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An Investigation into the Gliadin-Specific Immune Response in Schizophrenia

A thesis presented for the degree of Doctor of Philosophy at the University of Aberdeen

Ryan Thomas McLean (BSc hons, University of Keele)

2018

Declaration

This declaration certifies that I, Ryan T. McLean, composed this thesis and that all work described herein is my own unless otherwise stated. This work has not been accepted in any previous application for a degree. All quotations have been distinguished by quotation marks and the sources of information specifically acknowledged.

Signed:

Ryan Thomas McLean

Date: 14/01/2019

Abstract

A link between gluten consumption and the development of schizophrenia has been described in the literature. Furthermore increased levels of circulating antibodies directed against the gluten component, known as gliadin, have been observed in patients with schizophrenia. All studies reported to date measured antibodies against a mixture of native gliadin protein molecules, whereas dietary gliadin is partially digested, resulting in relatively long, indigestible, peptide fragments. In this study, levels of plasma antibodies against indigestible gliadin fragments were measured using an Enzyme-linked Immunosorbent Assay using a total of 405 archived plasma samples collected from patients with schizophrenia (n=169, 132 males and 37 females, aged 42.0 ± 13.3 years) and control subjects (n = 236, 159 males and 77 females, aged 44.7 ± 12.5 years). Patients were recruited from the North of Scotland in the period between 2003 and 2008 by NHS Grampian under Professor David St. Clair. Based on a genome-wide association study, 4 single nucleotide polymorphisms (SNPs) of interest were genotyped and their association with schizophrenia determined. Previously genotyped Human Leukocyte Antigen-DQ (HLA-DQ) variant data were also used to analyse HLA-DQ associations with plasma anti-gliadin antibody levels (AGA). Two cell-line based models were used to examine the effects of gliadin peptide incubation on cell maturation and/or differentiation of antigen-presenting cells.

The main finding of this thesis is that levels of plasma IgG against a γ-gliadin fragment, designated AAQ6C, are elevated in serum samples from patients with schizophrenia. Furthermore the gliadin-derived peptides were able to induce the maturation of a dendritic cell-like model, albeit with a reduced pro-inflammatory phenotype. A link between HLA-DQ variants and AGA remains elusive, despite the associations described in this thesis.

This thesis concludes that an immune response against AAQ6C is associated with schizophrenia. Although the mechanistic implications of this finding are unclear, it suggests that pathological contributions are unlikely to be mediated by cross-reactivity to proteins in the

central nervous system. The ability of the peptide antigens to induce the maturation of dendritic cells demonstrated that these peptides have immunogenic activity. The broader implications of this could be assessed with studies of the immunological response to gliadin peptides in the context of schizophrenia through the use of primary cell models.

Publications

Concepts and data within this thesis have been published previously as:

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Abbreviations

5-HT	5-Hydroxytryptamine (Serotonin)
AGA	Anti-gliadin Antibody
AMPA	α-amino-3-hydroxy-5methyl-4-isoxazolpropionic Acid
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
AUC	Area under the Curve
BBB	Blood Brain Barrier
BCR	B-cell Receptor
BG	Basal Ganglia
Breg	Regulatory B-Cell
C3	Complement Component 3
CCK8	Cell Counting Kit 8
CD	Coeliac Disease
CLP	Common Lymphocyte Precursor
CMV	Cytomegalovirus
CNS	Central Nervous System
CSF	Cerebral-Spinal Fluid
CV%	Coefficient of Variance
DA	Dopamine
DC	Dendritic Cell
DLPFC	Dorsolateral Pre-frontal Cortex
DMSO	Dimethylsulfoxide
DMT	N,N-dimethyltryptamine
DRD2	Dopamine Receptor 2
DSM	Diagnostic and Statistical Manual of Mental Disorders

ECACC	European Collection of Authenticated Cell Cultures
EEG	Electro-Encephalogram
ELISA	Enzyme-linked Immunosorbent Assay
FACs	Fluorescence Activated Cell Sorting
FES	First-Episode Schizophrenia
FITC	Fluorescein isothiocyanate
GABA	γ-Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GDP	Gross Domestic Product
GFD	Gluten-free Diet
GWA	Genome-wide Analysis
HLA	Human Leukocyte Antigen
Hz	Frequency (Herz)
IBS	Irritable Bowel Syndrome
ICD	International Classification of Diseases
iDC	Immature Dendritic Cell
Ig	Immunoglobulin
IL	Interleukin
KYNA	Kynurenic Acid
LP	Lamina Propria
LPS	Lipopolysaccharide
LR	Lower-Right
mAb	Monoclonal Antibody
MDD	Major Depressive Disorder
MEG	Magneto-Encephalogram
MHC	Major Histocompatibility Complex
MIA	Maternal Immune Activation

MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
NAc	Nucleus Accumbens
NC	Negative Control
NK	Natural Killer Cell
NMDAR	N-Methyl-D-aspartate Receptor
OD	Optical Density
OR	Odds Ratio
PANSS	Positive and Negative Syndrome Scale
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFC	Pre-frontal Cortex
PI	Propidium Iodide
Poly(IC)	Polyinosinic-polycytidylic
PP	Peyer's Patches
PQ	Proline and Glutamine
PSD	Post-synaptic Density
QA	Quinolinic Acid
QC	Quality Control
ROC	Receiver Operating Characteristic
SBI	Specific Binding Index
SNP	Single Nucleotide Polymorphism
TCR	T-Cell Receptor
Th	T-helper Cell
TLR	Toll-like Receptor
TRANK	Tetratricopeptide Repeat and Ankyrin Repeat Containing

Treg	T-Regulatory Cell
tTG	Tissue Transglutaminase
UR	Upper-Right
USA	United States of America
VC	Vehicle Control
VTA	Ventral Tegmental Area
WHO	World Health Organisation

1. Introduction

Schizophrenia is a relatively common but poorly understood form of mental illness. The pathology of schizophrenia was first characterised as *Dementia praecox* by Emil Kraeplin in the 19th Century (Kraepelin, 1919). As early onset dementia was not a common symptom of the condition, it was renamed Schizophrenia by Eugene Bleuler in 1911 (Kyziridis, 2005). One of the marked features of schizophrenia is the heterogeneity of clinical presentation, a feature that was noted by both Bleuler and Kraeplin, the former of whom subdivided schizophrenia into symptom-based categories. Additionally, psychiatrist Kurt Schnieder later assigned symptoms into either a first or second rank, with those in the first rank considered the most specific to schizophrenia. These classifications have persisted and remain relevant to the current diagnostic criteria, which have been set-out by the fifth edition of Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (Soares-Weiser et al., 2015).

Schizophrenia has no hallmark symptom and its clinical phenotype is a combination of so-called positive symptoms - delusions, hallucinations, speech disorders – and negative symptoms, including alogia, avolition anhedonia and affective flattening of the voice (Soares-Weiser et al., 2015). In addition to these two categories, a majority of patients also experience cognitive symptoms, which include poor memory and thought disorders (O'Carroll, 2000). To date, there is no objective molecular or genetic test available for the diagnosis of schizophrenia. Under the criteria of the third edition for the Diagnostic and Statistical Manual of Mental Disorders (DSM-III) criteria, the diagnosis of schizophrenia was broad, requiring only one of a core of symptoms to be present for a period of six months whereas the DSM-IV criteria stipulated an acute phase of one month to be required for diagnosis, as well as the presence of at least two of the core symptoms (Soares-Weiser et al., 2015). The diagnosis of schizophrenia is made exclusively by observation of a patient's adherence to the diagnostic criteria as

stipulated either by the DSM-V system or the International Classification of Diseases (ICD) system. The full criteria for diagnosis under DSM-V are listed (*Figure 1.1*).

Figure 1.1 The Criteria for the Diagnosis of Schizophrenia according to the DSM-V

Diagnostic Criteria for Schizophrenia (DSM-V)

A. Two or more of the following present during a 1-month period, with at least one being (1), (2) or (3):
    1. Delusions
    2. Hallucinations
    3. Disorganised speech
    4. Grossly disorganised or catatonic behaviour
    5. Negative symptoms (diminished emotional expression or avolition)
B. For a significant amount of time since onset, level of functioning in areas such as work, interpersonal relations or self-care is markedly below levels prior to onset
C. Continuous signs of disturbances for 6-months, including one month of symptoms (A), and may include periods of prodromal or residual symptoms.
D. Schizoaffective and bipolar disorder are excluded due to 1) No major depressive or manic episodes during the active phase or 2) mood episodes that occurred during the active phase were present for a minority of the duration.
E. Disturbances were not a physiological response to a substance, or another medical condition
F. If there is a history of autism or a communication disorder of childhood onset, the diagnosis of schizophrenia can only be made if delusions or hallucinations are present over the duration of 1-month.

The DSM-V criteria for the diagnosis of schizophrenia are reproduced from the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (American Psychiatric Association, 2013). Diagnosis of schizophrenia is made exclusively by adherence to the diagnostic criteria and there are currently no objective clinical measures available for the diagnosis of schizophrenia.

Both DSM-III and DSM-IV systems were broadly in agreement and differed primarily in the number of symptoms required for diagnosis. DSM-IV also introduced the five sub-groups of schizophrenia, based upon the pervading symptoms experienced by an affected individual, including: paranoia, disorganisation and undifferentiated and residual catatonia. The DSM-V system retains the five core symptoms but no longer privileges ‘bizarre delusions’ and requires

that two of the core symptoms must be delusions, hallucinations or disorganised speech (Soares-Weiser et al., 2015). Additionally, DSM-V has removed the subgroups introduced in the previous editions. As the heterogeneity of schizophrenia affects not only clinical presentation, but also biomedical measurements and the mechanism of susceptibility, it seems counterintuitive that the most recent edition of the DSM system has removed these subgroups; however, studies show that the subgroups of schizophrenia have not been useful either in clinical application or in research (Braff et al., 2013; Tandon et al., 2013).

Current clinical interventions for schizophrenia take the form of antipsychotic medication, which can be divided into first and second generation therapies. Despite this classification, there is no significant difference in the clinical efficacy between the two generations of antipsychotic drugs and both rely on blockade of the D_2 dopamine receptor as the primary mechanism of pharmacological action (Kapur et al., 2000; Lally and MacCabe, 2015). Patients on medication demonstrate a high heterogeneity in treatment response and approximately 40% of patients do not have a good response to any antipsychotic medication (Solanki et al., 2009). Clozapine is the only second-generation antipsychotic drug that has shown efficacy in treatment-resistant schizophrenia; approximately half of patients who did not respond to other antipsychotics responded to clozapine, possibly due to partial antagonism at the serotonin (5HT) receptor $5HT_{2A}$ receptor and the D_4 dopamine receptor (Lally and MacCabe, 2015; Meltzer, 1994).

Schizophrenia is a highly heterogeneous disorder in aetiology and clinical presentation and it may be the case that new subgroups based on common pathological mechanisms will dominate the future of schizophrenia research. Initial diagnostic guidelines specifically excluded ‘organic mental disorder’ as a criterion for schizophrenia diagnosis and it remains to be seen, as mechanisms of schizophrenia are elucidated, if these become new clinical entities in their own right or new subgroups of schizophrenia. Some clinicians have gone even further, suggesting that the term schizophrenia, implying a discrete disorder that is, in practice, applied

to a spectrum of psychiatric disorders, should be abandoned in the ICD-11 system and replaced with psychosis susceptibility syndromes (Os, 2016a).

1.1 Epidemiology of schizophrenia

Schizophrenia has mistakenly been termed as a disease of modernity; historical studies have identified remarkably 'schizophrenic-like' diseases that have been present throughout human history, from ancient Egypt and India to Europe in the Middle-Ages, suggesting a robust history as some form of clinical condition (Kyziridis, 2005). A previous, widely-cited study for the World Health Organisation (WHO), found that the incidence of schizophrenia was broadly constant, affecting ~1% of the population, and claimed that schizophrenia was 'ubiquitous', with similar clinical features across cultures (Jablensky et al., 1992). In contrast to these findings, however, several of the established risk factors for schizophrenia are geographically specific.

Urbanicity is one potential risk factor for schizophrenia (Freeman, 1994), and a meta-analysis showed an increase in the prevalence of schizophrenia in urban areas compared to rural areas (McGrath et al., 2004). A study of a Swedish cohort including 4.4 million participants suggested that the most densely populated centres had a 68-77% increase in the risk of schizophrenia when compared to the least densely populated areas, though substance abuse was not considered in this analysis (Sundquist et al., 2004). Despite this, substance abuse is a common factor which is often controlled in studies examining schizophrenia and urbanicity therefore unlikely to fully explain the link between schizophrenia and the urban environment (Esposito et al., 2002; Javitt, 1987; Os, 2004). It has been hypothesised that urban environments may confer a general increase in susceptibility that only manifests as schizophrenia in already susceptible individuals (Os, 2004).

A meta-analysis seeking to more closely examine the link between a nation's economic development and schizophrenia incidence confirmed a previously reported decrease in the prevalence of schizophrenia in developing countries, when compared to developed countries ($p= 0.04$) (Hopper and Wanderling, 2000; Jablensky et al., 1992; Saha et al., 2005). When discussing the differences in 'developed' versus 'developing' countries, it is important to note the inadequacy of these two terms. Firstly, there is much variability in socio-economic status within the countries of both categories. Secondly, what constitutes a "developed" or "developing" country is difficult to define. Finally, a comparison between developed and developing countries is difficult to make, since factors such as general access to healthcare, medication, social support and even attitudes towards mental illness may be highly variable, not only between groups but also within them. The comparisons between developing and developed countries have been rightly criticised even within studies examining these effects (Hopper and Wanderling, 2000). With these caveats, however, there appears to be a sustained decrease in prevalence of schizophrenia in less economically developed countries. A number of theories have been posited for this association, including increase in social care, increase in familial support, differing cultural attitudes towards schizophrenia and, controversially, the lack of pharmacological treatment (Cohen et al., 2008). Although the observed trend is robust, the explanation of possible mechanisms appears to be less so (Kulhara et al., 2009). A systematic review by Cohen et al. (2008) suggested that unmedicated patients in developing countries had clinical outcomes that were at least as poor as those in some developed countries, with patients in rural China having particularly poor outcomes (Ran et al., 2003). Furthermore the idea that the 'traditional' familial structures in the developing world aid in the care for patients with schizophrenia is at least overgeneralised (Cohen et al., 2008). Although the benefits of a supportive family environment are obvious, and substantiated by the literature (Pitschel-Walz et al., 2004), the evidence of familial support in developing countries is highly variable (Cohen et al., 2008). Confusingly, when gross domestic product (GDP) was used as a measure of economic development, no significant difference in the prevalence of schizophrenia was

observed between countries with ‘low’, ‘middle’ and ‘high’ GDP (Saha et al., 2006). The literature in its entirety suggests that there is a difference in the prevalence of schizophrenia between developed/developing countries, although a satisfactory explanation of this association remains elusive. It is likely that the prevalence of schizophrenia has no clear relationship with GDP, the primary measurement of the level of development of an economy.

The level of a country’s development as a risk factor for schizophrenia is further confounded by an apparent culture or ethnicity effect on the prevalence of schizophrenia. Studies examining the impact of culture on the risk of schizophrenia also suffer from the same over-generalisations as those examining the role of economic development. One study on a Korean-American population (n= 40) demonstrated no differences in schizophrenia symptoms between the Korean-American group and other ethnic groups (n= 183), though lower satisfaction with life was reported when compared to other groups (Bae and Brekke, 2002). Another study used a tri-ethnic design to examine cultural effects on schizophrenia symptoms, using the scores of empathy and social competence, and their relationship with schizophrenia symptoms (Brekke and Barrio, 1997). They found that white participants with schizophrenia had worse symptoms than African-American or Latino participants. Regression analysis of ethnicity, social competence and empathy showed that generally, social competence and empathy were the two largest predictors of symptom scores; since these scores were found to be increased in the African-American and Latino groups, the difference in schizophrenia symptoms was attributed to cultural differences between groups (Brekke and Barrio, 1997). The main flaw of this study is that both the empathy and social competence measures are likely to be confounded by heterogeneity of clinical presentation, and the differences observed in the scores between the groups may reflect distinct symptoms of schizophrenia amongst ethnic groups, rather than cultural differences, as concluded by the authors of the study (Brekke and Barrio, 1997). A study examining the effects of ethnic background on baseline symptoms of schizophrenia between Chinese, Malaysian and Indian participants suggested that Malaysians

had higher positive and negative scores than the other two groups (Lim et al., 2011). Additionally, patients in Sarawak on Borneo displayed an increase in the prevalence of auditory hallucinations with a shorter prodromal period when compared to Australian and Indian patients (McLean et al., 2015).

It has long been noted that individuals born in the winter months had an increased risk of developing schizophrenia, on account of the fact that individuals born within these months were over-represented in schizophrenia cohorts (Barry and Bary, 1961). The birth seasonality of schizophrenia has also been reported in both the northern and southern hemispheres. The effect is apparently clearer in the northern hemisphere, but this might be biased by the relative lack of studies from the south (Mendonça et al., 2009; Parker and Neilson, 1976). A large-scale study in Taiwan, at low latitude, demonstrated an increase of winter births in female patients with schizophrenia, but not in males (Cheng et al., 2013). A study of 9655 cases conducted in Singapore, which lies close to the equator, failed to demonstrate a difference in seasonal distributions of births of patients with schizophrenia (Parker et al., 2000). The main hypothesis put forward to explain the increase in winter births of schizophrenia patients is that, in winter, a number of factors associated with schizophrenia may accumulate *in utero*, including an increase in infections and lack of vitamin D due to lower levels of sunlight. A study in Spain further reinforced the link between schizophrenia and winter births; this study of 321 cases and 294 controls demonstrated that the effect was compounded by a time of hardship, namely the post-Civil War period, suggesting that increased risk of schizophrenia is conferred by a range of factors in the maternal environment (Martínez-Ortega et al., 2011).

While the risk of developing schizophrenia may be ~1% in the global population as suggested by Jablensky et al. (1992), a number of risk factors for schizophrenia have been proposed, such as urbanicity, seasonality, cultural and ethnicity. These factors are geographically specific and should therefore lead to variation in prevalence of the disease across countries, regions and environments. A review of the literature demonstrated a large variation

in the life-time prevalence of schizophrenia, ranging from 2.7/1000 to 8.3/1000 and an incidence of 0.11/1000/year – 0.7/1000/year (Messias et al., 2007). The initial WHO study aimed to provide a framework that would enable the study of schizophrenia across different geographical regions. It is therefore possible that the emphasis on reproducibility of schizophrenia diagnosis might have affected the results of these studies (Bhugra, 2005). Messias et al., (2007) concluded that there was a large variation in the prevalence and risks of schizophrenia across geographical regions. Furthermore, this variation may be indicative of a range of environmental and genetic risk factors, supporting the notion of multiple aetiologies of schizophrenia.

1.2 Pathophysiology of schizophrenia

In terms of pathophysiology, schizophrenia can be considered as a disorder of functional connectivity and communication in the central nervous system (CNS), particularly within the cortex and neocortex, resulting in a clinical presentation that forms the symptoms of schizophrenia.

1.2.1 Genetics and Onset

It is uncontroversial to state that schizophrenia has a large heritable component, although the precise delineation of the contribution of genetics to schizophrenia is unclear. It has been reported that, in comparison to the general population, first-degree relatives of patients with schizophrenia have a higher risk of developing schizophrenia, have higher PANSS scores, and are also more likely to develop schizophrenia-related psychiatric disorders such as schizoaffective or bipolar disorder (Hembram et al., 2014; Kendler et al., 1985). These observations suggest that a genetic component predisposes individuals to schizophrenia, in addition to potential environmental risk factors and triggers. There is debate over the level of

the contribution of an overall genetic burden to the development of schizophrenia, and to what degree the condition is heritable; however, twin studies suggest that the heritability of the disease is up to 80% (Cardno and Gottesman, 2000).

As alluded to in Section 1.1, a range of factors during foetal development can contribute to the risk of developing schizophrenia (Martínez-Ortega et al., 2011), suggesting that schizophrenia is a neurodevelopmental disorder. This is also seen in studies demonstrating an over-representation of individuals with neurodevelopmental disorders in families of patients with schizophrenia, and *vice versa* (Daniels et al., 2008; Greenwood et al., 2004). The current iteration of the neurodevelopmental hypothesis of schizophrenia states that subtle alterations in early neurodevelopment result in a brain-phenotype that either results in schizophrenia or predisposes an individual to develop schizophrenia in response to an environmental trigger (Nasrallah, 1986; Weinberger, 2017).

It is difficult to directly examine neurodevelopmental processes in patients with schizophrenia. One of the main reasons for this is related to one of the marked features of the condition; onset of the condition typically occurs around late adolescence, with the index admission occurring in the early 20s (Häfner et al., 1994). There is a slight gender difference, with the onset of schizophrenia in males generally occurring a few years earlier than in females (Häfner et al., 1994). This is somewhat later than the initial stages of neurodevelopment, though a longitudinal imaging study of high risk individuals during and after puberty showed progressive enlargement of the ventricles and a reduction in the grey matter of the prefrontal cortex, resembling an exaggerated variation of a typical neurodevelopmental process that occurs in adolescence (Gogtay et al., 2011; Rapoport et al., 1999).

Genetic studies of schizophrenia support the current notion that schizophrenia results from an accumulation of common genetic variants each individually conferring negligible risk, or from a few rare high risk variants (Birnbaum and Weinberger, 2017). Indeed, in the former case Weinberger wrote “schizophrenia may not reflect a discrete event or illness process at all,

but rather one end of the developmental spectrum that for genetic and/or other reasons 0.5% of the population will fall into" (Nasrallah, 1986). Genome-wide association (GWA) studies have proven a powerful genetic tool, relying on high-throughput DNA microarrays that are able to detect millions of single nucleotide polymorphisms (SNPs) across the human genome. Using large case-control cohorts, GWA studies can detect associations between genetic loci and a disease. This method is particularly useful for the genetic analysis of complex disorders with a polygenetic risk, like schizophrenia. Due to multiple testing of up to a million SNPs, the false discovery rate, the type-I error, from a GWA study is extremely high. As a result, a strict statistical significance level of a genome-wide cut-off ($p=5x10^{-8}$) has been applied to claim genetic association for a DNA marker at the genome-wide significance threshold (Bergen and Petryshen, 2012). Due to their high statistical power and consequent ability to amplify statistical noise, it is crucially important that GWA studies employ well-defined and appropriately matched case-control samples. Furthermore, in a phenotype-first approach, it is desirable that a well-defined phenotype is used to distinguish cases and controls.

The most recent large scale schizophrenia GWA identified 108 genetic loci with some 320 proximal genes that were associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). A number of rarer copy number variations (CNVs) have also been identified including at 1q21.1, 2p16.3, 3q29, 7q11.2, 15q13.3, 16p11.2, and 22q11.2 (Marshall et al., 2017). Pathway analysis suggested that these loci were associated with a loss of function in neuronal synapses, neuronal projection and nervous system development. Of these functions, only synaptic function remained significant when the relevant loci were removed from the analysis (Marshall et al., 2017). It is worth noting that these CNVs were carried by 1.4% of cases in this sample set, while combined SNP and CNVs associated with schizophrenia account for 5% of the heritable risk of schizophrenia (Marshall et al., 2017; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Additionally, a duplication at Xq28 conferred schizophrenia risk in both males and

females, though the risk was much higher in males (OR = ∞), while previous studies have demonstrated that duplications/deletions at this locus often result in intellectual disability (El-Hattab et al., 2015).

An unsurprising feature of genetic studies in schizophrenia, and a theme running through this introduction, is the level of heterogeneity of findings coupled with failed attempts at reproducing many of the genetic associations in schizophrenia. Very few candidate genes for schizophrenia have survived replication. One study examining 14 SNPs in candidate genes for schizophrenia that had previously been associated with schizophrenia found no single variant met genome-wide significance in schizophrenia (Sanders et al., 2008). A meta-analysis examining 25 historic candidate genes from the SZGene database found very few were replicated by large scale GWAS (Farrell et al., 2015). A selection of these findings are summarised (*Table 1.1*).

Table 1.1 Summary of Candidate Genes for Schizophrenia and their replication in a large scale GWA

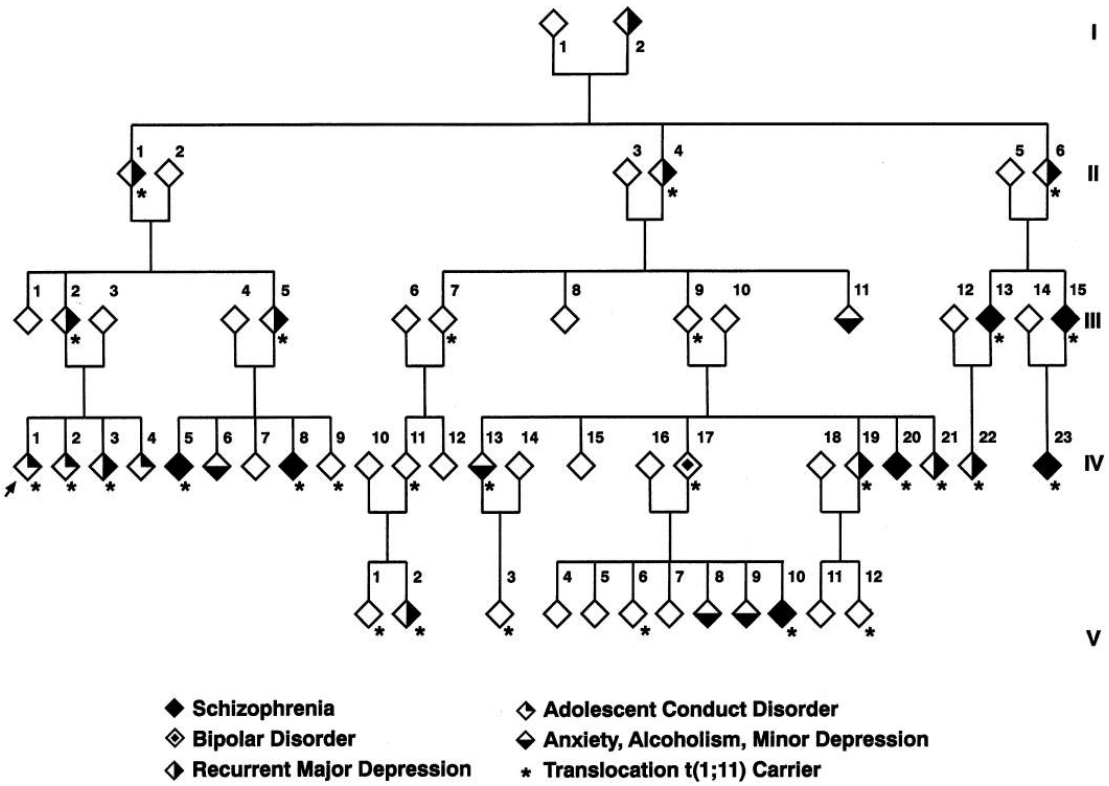
Gene	**Marker**	**SZGene OR (95% CI)**	**SZGene *P***	**108 OR (95% CI)**	**108 *P***
BDNF	270C/T	0.68 (0.52–0.87)	0.0028	1.01 (0.97–1.06) †	0.55
	rs6265	0.95 (0.87–1.04)	0.29	0.95 (0.92–0.97)	8.0×10^{-5}
CHRNA7	rs28531779	0.97 (0.72–1.30)	0.82	1.01 (0.96–1.05)	0.79
DISC1	rs999710	1.07 (1.00–1.14)	0.045	1.01 (0.99–1.03)	0.29
DRD2	rs1801028	0.85 (0.71–1.03)	0.1	0.95 (0.89–1.03)	0.22
DRD3	rs6280	1.03 (0.97–1.08)	0.33	0.99 (0.97–1.01)	0.31
DRD4	rs4646983	1.13 (0.76–1.67)	0.56	No data	No data
DTNBP1	rs3213207	1.10 (1.02–1.19)	0.015	1.04 (1.01–1.08)	0.012
HTR2A	rs6311	1.14 (1.06–1.23)	0.0005	1.01 (0.99–1.04)	0.18
KCNN3	1333T/C	1.12 (0.33–3.76)	0.86	0.95 (0.93–0.98) †	3.3×10^{-5}
MTHFR	rs1801133	1.09 (1.01–1.17)	0.026	1.01 (0.98–1.03)	0.55
NOTCH4	rs367398	1.00 (0.87–1.15)	0.99	No data	No data
NRG1	rs62510682	0.94 (0.88–1.01)	0.074	0.97 (0.95–1.00)	0.024
PPP3CC	rs7837713	0.99 (0.81–1.21)	0.91	1.01 (0.97–1.06)	0.62
RGS4	rs2661319	0.93 (0.88–0.99)	0.013	1.01 (0.99–1.03)	0.47
SLC6A4	5-HTTVNTR	1.11 (1.01–1.21)	0.024	0.91 (0.86–0.96) †	4.2×10^{-4}
	5-HTTLPR	1.01 (0.94–1.09)	0.75	1.03 (1.00–1.07) †	0.058
TNF	rs1800629	1.00 (0.86–1.17)	0.98	0.91 (0.89–0.94)	5.6×10^{-10}
ZDHHC8	rs175174	1.00 (0.90–1.11)	0.96	0.98 (0.96–1.01)	0.17

A selection of historic schizophrenia candidate genes and the strength of their schizophrenia association as identified by the Schizophrenia Working Group of the Psychiatric Genetics Consortium. HTR2A, MTHFR and RGS4 were not significantly associated with schizophrenia in this cohort. DISC-1 was also not significantly associated with schizophrenia in the Psychiatric Genetics Consortium cohort (Farrell et al., 2015).

As mentioned, the genetic risk of schizophrenia is conferred either by the accumulation of common low-risk variants or by a few rare high risk variants. One of the earliest examples of the latter is a translocation at Disrupted In Schizophrenia 1 (DISC-1) that was first identified in a Scottish pedigree (Blackwood et al., 2001; Millar et al., 2000). Of those included in the

pedigree that also carried the translocation at the DISC locus 7 had schizophrenia, 10 had major depressive disorder (MDD), 3 had Adolescent conduct disorder and one individual had bipolar disorder (Millar et al., 2000). 8 individuals had no psychiatric pathology despite carrying the translocation (Millar et al., 2000) (*Figure 1.2*).

Figure 1.2 DISC-Translocation in a Scottish Pedigree

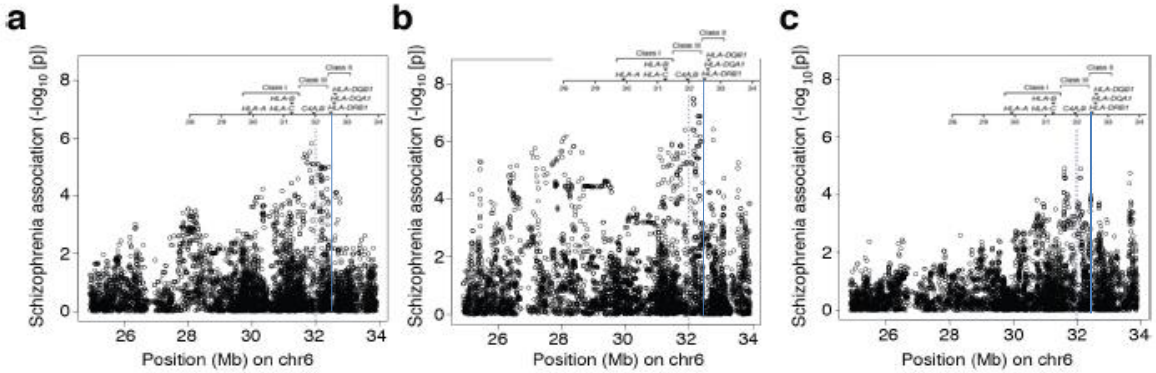


58 family members from the large Scottish family in which the DISC-1 translocation was discovered for whom karyotype data and psychiatric evaluations were available. The translocation segregates significantly with schizophrenia, Major Depressive Disorder and Adolescent Conduct Disorder. Figure from Millar et al., 2000.

Another hypothesis in the aetiology of schizophrenia, for which there is genetic evidence, is the immunological hypothesis. In the most recent large-scale GWA the SNP that showed the strongest association with schizophrenia was rs1233578, located proximal to the Human Leukocyte Antigen (HLA)-megalocus on the sort arm of chromosome 6 (28,303,247-28,712,247) (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The structure and function of the HLA locus is fully discussed in section 1.3.1. Briefly, the

HLA locus is best known for harbouring the genes required for antigen presentation to the adaptive arm of the immune system (MHC-I and MHC-II molecules). A further study suggested that SNVs in linkage with the C4 locus were responsible for the signal observed at the HLA-locus, rather than the conventional antigen presenting genes (Sekar et al., 2016). However, the authors of this study also noted considerable heterogeneity of HLA-locus variants in different schizophrenia cohorts, with some high signal SNPs located within the DRB and DQB loci (see *Figure 1.3*) (Sekar et al., 2016). In addition to the HLA-association observed, the GWAS also demonstrated a number of other immune system related loci were associated with schizophrenia (for further discussion see section 1.5.4). As many of the SNPs identified through the GWA reside in noncoding regions of the genome, there are two theories around potential associated mechanisms. It is possible that SNPs associated with schizophrenia may aid in the regulation of gene expression of proximal genes, or that loci identified may be in linkage disequilibrium with causal genetic variants, such as CNVs, deletions or duplications.

Figure 1.3 Manhattan Plots showing the strength of association between SNVs and Schizophrenia in different cohorts that make up the GWAS by The Schizophrenia Working Group of the Psychiatric Genetics Consortium



The three plots above (a, b, c) showing the level of association between SNVs and schizophrenia at the HLA-locus in a selection of schizophrenia cohorts that make up the GWAS performed by the Schizophrenia Working Group of the Psychiatric Genomics Consortium, (2014), overlaid with a map detailing gene-loci of interest. The dotted line denotes the C4 locus, while the solid blue line denotes the HLA-DRB1 locus. As demonstrated by these cohorts, schizophrenia risk SNPs extend into the HLA-II region. Figure taken from Sekar et al., (2016), with the HLA-locus map and solid blue line added by the candidate.

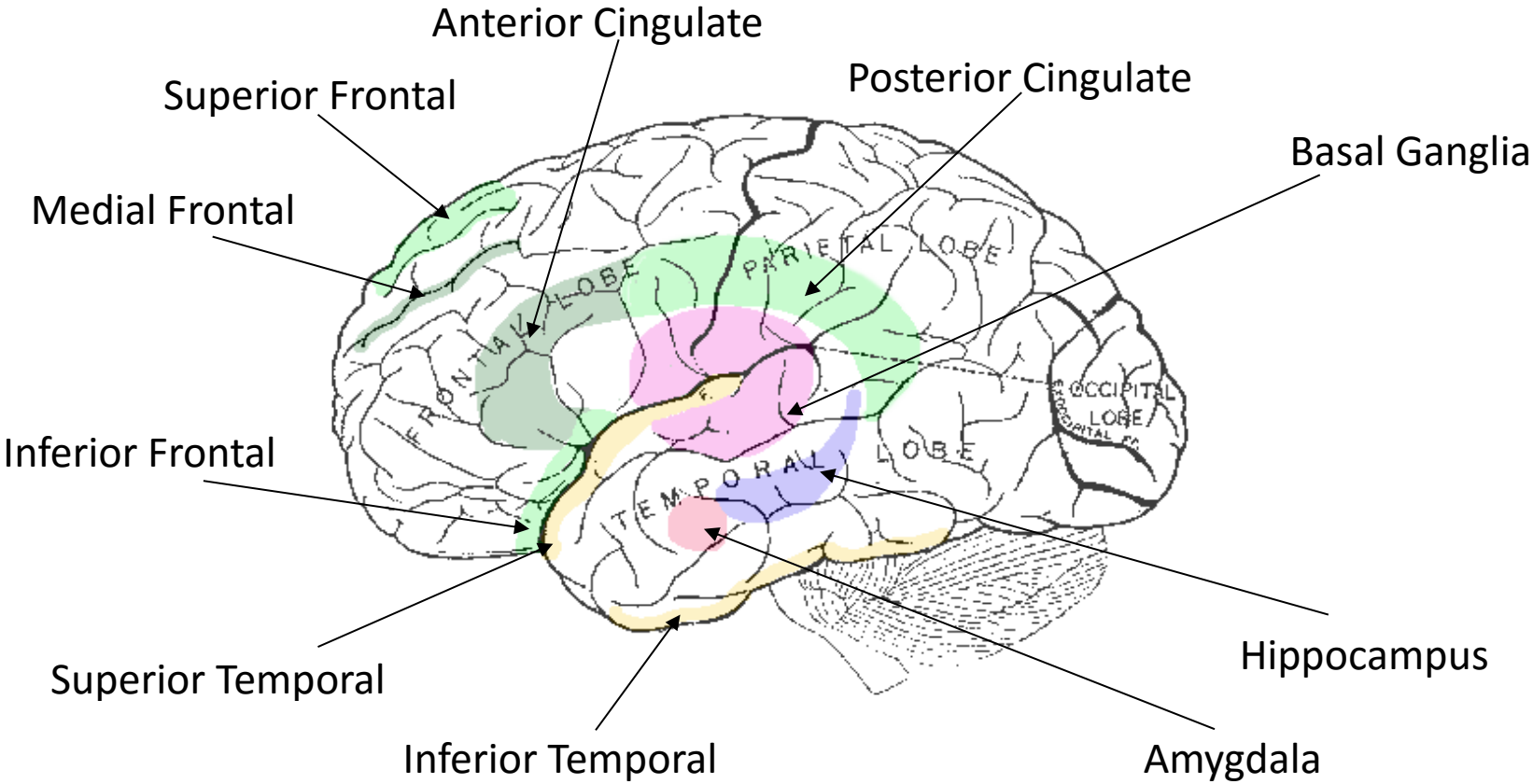
A feature of the genetic risk for schizophrenia is its overlap for genetic risk of other conditions. As mentioned in the previous paragraph and as can be seen from *Figure 1.2*, the DISC translocation also confers risk of MDD, while the duplication at Xq28 often results in intellectual disability (El-Hattab et al., 2015; Millar et al., 2000). Whole exome sequencing of 4,264 schizophrenia cases, 9,343 controls and 1,077 trios revealed loss-of function variants in the SETD1A gene were significantly associated with schizophrenia, while 7/10 individuals carrying loss-of function variants also displayed intellectual disability and strengthens the neurodevelopmental hypothesis (Singh et al., 2016).

An additional line of evidence that the phenotype of schizophrenia might result from abnormalities of neurodevelopment are imaging studies showing structural alterations of the brain in patients with schizophrenia (Owen et al., 2011).

1.2.2 Structural alterations of the brain

Schizophrenia was not initially categorised as an organic brain disease, although there are a number of structural alterations in brain areas associated with the condition. The literature surrounding structural brain alterations is comprehensive, albeit confounded by a number of factors, including age and gender as well as neuroleptic medication. In light of studies suggesting that antipsychotics may alter the structure of brain regions in schizophrenia, the most useful studies have examined antipsychotic naïve or first-episode schizophrenia (FES) patients (Guo et al., 2015; Shepherd et al., 2012). Some areas of the brain, in which structural alterations are most commonly found are summarised in *Figure 1.4*.

Figure 1.4 Brain regions where structural alterations are observed in patients with schizophrenia



Brain regions most consistently associated with schizophrenia highlighted by the PhD Candidate. Expansion of the ventricles generally suggests a loss of tissue in the brain, and this is observed in most regions of interest although the basal ganglia are enlarged in patients with chronic schizophrenia. This illustration is adapted from Gray, (1988). Int= Internal structure.

The most reproducible brain alteration in schizophrenia is ventricle expansion, particularly the lateral and third ventricles (Shepherd et al., 2012). Expansion of the ventricles is typically caused by either a loss of integrity of CNS tissue and/or an increase in cerebrospinal fluid (CSF) pressure, indicating morphological alterations of the brain. The most reproducible changes in schizophrenic brain are found to occur in the limbic and executive areas, namely the temporal lobe, the frontal lobe and the areas involved in the communication between the two regions, such as the basal ganglia and cingulate cortices (Chan et al., 2011; Leung et al., 2011; Shepherd et al., 2012).

The frontal lobe, including the anterior cingulate cortex, is crucial for higher cognitive function in humans, such as emotional processing, executive function and understanding language. In the frontal and prefrontal cortex, bilateral reductions in the grey matter of the medial frontal and inferior frontal gyri, along with the anterior cingulate cortex were observed in patients with schizophrenia (Shepherd et al., 2012). This change was demonstrated in both neuroleptic naïve and FES patients as well as individuals deemed at high risk of developing the disorder (Chan et al., 2011; Leung et al., 2011).

In humans, the temporal lobe, which contains the hippocampus, amygdala, Wernicke's area and the auditory cortex, performs a variety of functions related to memory, spatial awareness, emotional responses to internal and external stimuli, and language. The volumes of both the superior and inferior temporal gyri were smaller in neuroleptic naïve patients than healthy controls, while another study demonstrated a significant hemisphere effect in FES patients, with a reduction in the volume of the left temporal lobe (Chan et al., 2011; Leung et al., 2011). A meta-analysis of available literature, totalling 23,171 cases, collated evidence that the volume of the hippocampus in both hemispheres was moderately reduced in FES patients (Shepherd et al., 2012). Furthermore, the amygdala of individuals at risk of schizophrenia was

found to be significantly reduced compared to control subjects, a reduction that is also lateralised to the left hemisphere (Chan et al., 2011).

The basal ganglia (BG), located between the frontal and limbic regions, contain many of the midbrain nuclei including the ventral tegmental area (VTA) and substantia nigra, where a large number of dopaminergic neurons are located. In addition, the striatum, including the nucleus accumbens (NAc), is rich in dopamine (DA) and γ-aminobutyric acid (GABA) neurons. This area is also central to decision making, motivation, locomotion and emotional responses, and is a target for the glutamatergic and 5HT neuronal systems (Leung et al., 2011; Shepherd et al., 2012). The abundance of GABAergic interneurons and dopaminergic projections suggests that the BG plays a modulatory role on other brain regions, particularly at the pre-frontal cortex (PFC) and NAc regions (Carr and Sesack, 2000). Imaging studies demonstrated that the BG are significantly reduced in FES patients when compared to healthy controls, while the BG were found to be significantly larger in patients with a chronic form of the condition (Shepherd et al., 2012).

The cingulate cortex can be divided into anterior and posterior regions. The anterior cingulate cortex is a component of the PFC and plays a role in autonomic motor activity, as well as attention, motivation and reward anticipation. The posterior cingulate cortex is believed to play a role in the storage of memory and the interaction of emotion and memory. Bilateral reductions in the anterior cingulate cortex in FES patients can be observed, whereas reductions to the posterior cingulate appear to be lateralised to the left hemisphere of the brain (Leung et al., 2011; Shepherd et al., 2012). The reduced volume of the anterior cingulate cortex was also observed in individuals at high risk of developing schizophrenia, although this was not the case for the posterior cingulate cortex (Chan et al., 2011).

In addition to the above alterations in grey matter, which contains neuronal cell soma, significant reductions in the brain white matter of FES patients have also been documented (Lei et al., 2015).White matter is composed of the myelinated axon fibres of neurons and plays an

important role in intra-brain communication over relatively long distances, functionally connecting diverse areas of the brain. A meta-analysis demonstrated reproducible reductions in the volume of white-matter at the border of the frontal and parietal lobes, particularly in patients whose negative symptoms dominated the clinical presentation (Lei et al., 2015). Furthermore, significant reductions in white-matter in the insular lobe in unaffected first-degree relatives of schizophrenia patients were also observed (Lei et al., 2015). Another finding, with regards to white-matter changes in schizophrenia, is the level of lateralisation of deficits. Similar to the alterations in grey-matter, the white-matter of the left posterior cingulate was significantly reduced in patients with schizophrenia, while in the right hemisphere, significant reductions in the volume of the superior temporal region were identified (Federspiel et al., 2006). Alterations in the white-matter volume in the frontal areas were not lateralised, though these areas were significantly reduced when compared to healthy controls (Federspiel et al., 2006).

1.2.3 Dysconnectivity in Schizophrenia

The aggregate activity of neurons in the brain produces oscillating electrical signals that can be measured using an electroencephalogram (EEG). Neural oscillations measured by EEG correspond to the activity of specific cortical areas and are altered in a number of pathological conditions, most notably epilepsy (Buzsaki, 2006). The propagation of this oscillatory activity is the mechanism by which neuronal activity is coordinated across widely-distributed regions of the brain, allowing for the emergence of complex behaviours in response to external, or internal stimuli (Uhlhaas, 2013). Behaviours and activity of an organism are both likely to arise from the interactions of multiple oscillations at a range of frequencies (Hz) (Buzsaki, 2006).

One oscillation that is believed to be important for the integration of a number of different temporal regions in cognitive tasks and neural plasticity is the high frequency gamma-oscillation (30-100 Hz) (Buzsaki, 2006; Haenschel et al., 2009). Gamma-oscillations are

ubiquitous throughout the cortex of many organisms and increased during tasks that require activity from disparate regions of the brain. Reductions in both gamma-oscillations and neural synchrony are associated with late-adolescence and are followed by an increase in neural synchrony, possibly reflecting the reorganisation of cortical networks during this time (Uhlhaas et al., 2009). Due to this and the fact that gamma-oscillations are largely driven by GABAergic interneuron activity, alterations in gamma-band activity have been found to be associated with schizophrenia (Buzsaki, 2006; Uhlhaas, 2013).

In a manner similar to imaging studies, the EEG test has been reported in a number of studies among patients with schizophrenia, though the issues are often confounded by medication and chronicity of the patients. One study demonstrated a reduction in gamma-oscillations in the frontal cortex of FES patients (n= 53), when compared to healthy controls (n=29), during cognition-intensive behavioural tasks (Minzenberg et al., 2010). An additional study utilising the magneto-encephalogram (MEG) demonstrated an increase in gamma-oscillations in the left frontal and fronto-temporal areas in healthy volunteers during a mental arithmetic task, which is linked to the recall of previous answers (Kissler et al., 2000). In patients with schizophrenia, this effect was abolished, or activity was lateralised to the right hemisphere (Kissler et al., 2000). During resting states, MEG showed that patients with schizophrenia displayed a decrease in gamma-oscillations in the fronto-temporal and parieto-temporal areas (Kissler et al., 2000). Furthermore, deficits in the encoding of new information were associated with alterations in gamma-oscillations in patients with schizophrenia (Haenschel et al., 2009). Synchrony in the phase of gamma-oscillations was also found to be reduced between right and left auditory cortices in response to auditory stimuli, and decreased synchrony was correlated to auditory hallucination scores (Mulert et al., 2011).

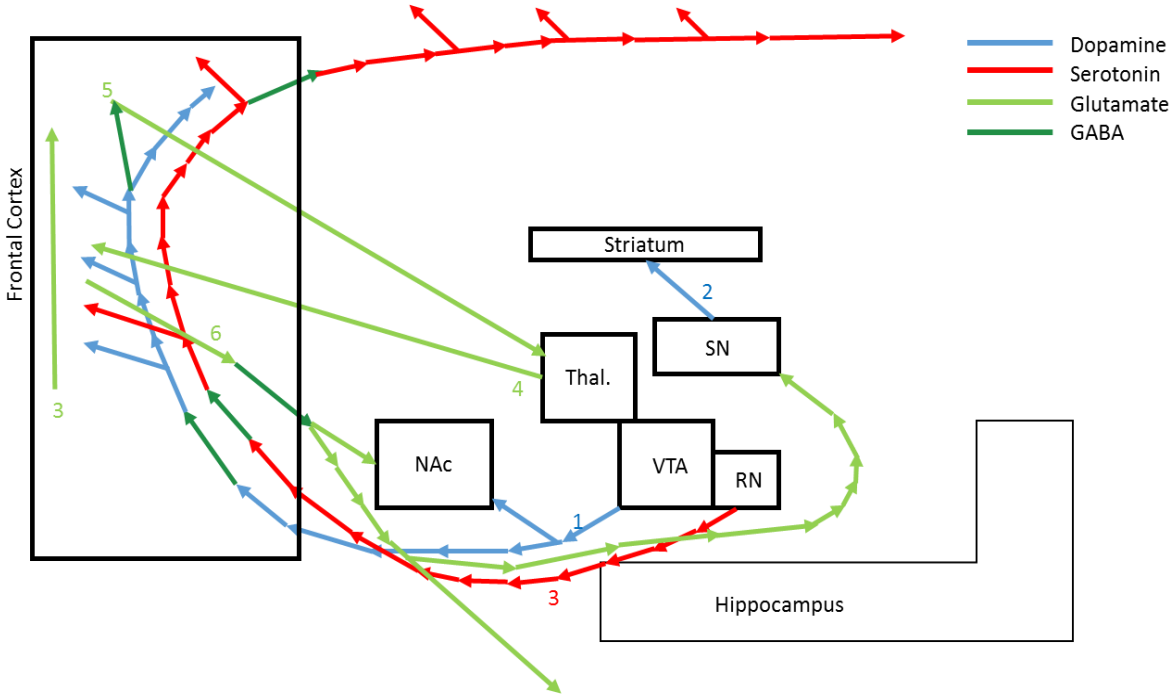
A response to a startling stimulus can be attenuated if paired with a weaker ‘prepulse’ given a short-time prior (Buckland et al., 1969). The mechanism behind prepulse inhibition (PPI) relies on the inhibition of inputs into startle network in the brain and distributed

postsynaptic inhibition of the startle network and therefore can be a correlate of the function of neuronal circuits (Frost et al., 2003). Deficits in PPI have long been observed in patients with schizophrenia, and one longitudinal study of 13 patients found that the observed decrease in PPI extends to the chronic form of the condition (Braff et al., 1978; Mena et al., 2016).

Alterations in gamma-oscillations as well as other phenotypes associated with schizophrenia support the hypothesis that schizophrenia may be a condition of altered neuronal connectivity both at rest or during the performance of tasks (Maran et al., 2016). Decreased phase synchrony in alpha-oscillations in the frontal lobe have been observed in schizophrenia patients at rest, while decreased phase synchrony in theta-oscillations were associated with impairments in working memory in schizophrenia (Maran et al., 2016). As previously mentioned, gamma-oscillations are generated primarily by the activity of GABAergic interneurons and the neurotransmitter systems play a central role in neural communications.

1.2.4 Alterations of neurotransmission

Figure 1.5 Neurotransmitter pathways of the brain



Simplified illustration of the neurotransmitter networks most commonly involved in schizophrenia with emphasis on the major pathways and the interconnected, reciprocal nature of the pathways. 1) Mesocorticolimbic pathway involved in higher order function, which plays roles in reward, cognition and motivation; 2) Nigrostriatal pathway involved in autonomic movement; 3) Rostral 5HT projections involved in higher cognitive function; 4) Thalamocortical pathway; 5) Corticothalamic pathway, and 6) Cortical-brainstem projection that mediates neurotransmitter release in the raphe nucleus (RN), substantia nigra (SN) and the nucleus accumbens (NAc). Glutamatergic pathways display excitatory activity at target regions. GABAergic interneurons are dispersed throughout the cortex and modulate the activity of other pathways.

The dopaminergic system in the context of the brain comprises the receptors and neurons targeted by DA neurotransmitters. Unlike other neurotransmitter systems, the dopaminergic system is highly organised into four main pathways, the striatonigral, the mesocortical, the mesolimbic pathways (*Figure 1.5*) (Björklund and Dunnett, 2007). Over half a century of evidence has suggested that the DA system is heavily involved in the regulation of behaviours, playing crucial roles in motivation, learning, conscious movement, decision making and memory. It is increasingly recognised that the four DA pathways may be oversimplified as recent studies suggest a large degree of complexity, with dopaminergic projections rigidly

organised in terms of inputs and outputs even within pathways (Beier et al., 2015; Lerner et al., 2015). Additional complexity arises at the receptor level, with five different DA receptors (D_1, D_2, D_3, D_4 and D_5), which have distinct functions and have either excitatory or inhibitory activities. In terms of functional segregation, the pathways in the midbrain are particularly important for behavioural aspects; for example, the mesocorticolimbic pathway is mainly responsible for motivation, memory and decision making, while the striatonigral pathway regulates conscious movement (Rossi et al., 2013).

Due to the role of the midbrain dopaminergic pathways in motivation and behaviour, it is generally thought that these pathways are likely to be involved in the pathophysiology of schizophrenia. The first evidence came from the discovery that antipsychotic drugs could block D_2 receptors (Carlsson, 1978), and further evidence showed that DA receptor blockade not only predicted clinical response but also caused extrapyramidal side effects, such as dystonia, tardive dyskinesia parkinsonism (Divac et al., 2014; Kapur et al., 2000). Furthermore, brain imaging studies with fluorophore-tagged DA revealed that patients with schizophrenia had an increase in striatal DA response to amphetamine and that this effect was reproducible in first-episode psychosis and drug-naïve patients with schizophrenia, but not those in remission (Abi-Dargham et al., 1998; Laruelle et al., 1999). This observation led to the hypothesis that schizophrenia is a disorder related to increased mesolimbic DA activity, accounting for the positive symptoms of schizophrenia; meanwhile, the negative symptoms may result from a decrease in extrastriatal DA activity, particularly in the mesocortical pathway (Davis et al., 1991).

The association of extrastriatal DA activity with schizophrenia is, however, controversial. A meta-analysis of 23 studies, including 278 patients and 265 controls, demonstrated no significant differences in extrastriatal DA activity between schizophrenia patients and healthy controls ($p= 0.074$), although this meta-analysis revealed heterogeneity amongst the studies (Kambeitz et al., 2014). Studies examining the role of striatal DA in schizophrenia have also failed to show uniform effects, as 8 out of 13 patients did not

demonstrate the typical increased DA response to amphetamine (Abi-Dargham et al., 1998). Additionally, studies in patients with treatment-resistant schizophrenia showed that while treatment-resistant patients had similar patterns of D_2 receptor blockade to treatment responders, treatment was nonetheless ineffective and the levels of mesolimbic pathway activation appeared to be similar to control subjects (Demjaha et al., 2012; Kapur et al., 2000; Wolkin et al., 1989). Finally, a gene enrichment analysis of the 11 genes most strongly associated with DA signalling showed no association with schizophrenia, suggesting that DA dysfunction may not be regulated at the genetic level in schizophrenia (Edwards et al., 2016). Therefore, it is possible that DA dysfunction represents a common, but not crucial process in the development of schizophrenia.

The 5-HT pathways are not as well defined as the dopaminergic pathways, likely owing to a comparative lack of organisation and segregation into discrete pathways. The raphe nuclei serve as the main source of 5-HT neurons in the CNS and have been divided into two classes, the rostral group and the caudal group, which project to almost all areas of the brain (Hornung, 2003). The areas with the greatest densities of 5-HT, previously known as serotonin, receptors are the hippocampus, the temporal lobe and the anterior cingulate cortex (Takano et al., 2011). Functionally, the 5-HT pathways aid in the regulation of a number of cognitive processes including aggression, arousal, cognition and memory as well as regulate activities of the autonomic nervous system (Niederkofler et al., 2015). 5-HT is able to differentially regulate (either excite or inhibit) target neurons, dependent upon which subtype of the 5-HT receptor is present (Celada et al., 2013). 5-HT signalling via the $5\text{-}HT_{1A}$ receptor decreases the probability of action potential firing, while the $5\text{-}HT_{2A}$ receptor allows 5-HT to act as an excitatory neurotransmitter (Santana et al., 2004; Sprouse and Aghajanian, 1987).

When N,N-dimethytryptamine (DMT), a $5\text{-}HT_{2A}$ receptor agonist, was administered to healthy volunteers, they developed transient psychiatric phenomena representative of the positive symptoms of schizophrenia (Gouzoulis-Mayfrank et al., 2005). A meta-analysis of

post-mortem and live *in vivo* imaging studies concluded, though caveated by a heterogeneity of findings, that binding affinity of DMT to the 5-HT_{2A} receptor was decreased in patients with schizophrenia (Selvaraj et al., 2014). Although the studies of post-mortem tissue included, by definition, chronic and long-term medicated patients, they demonstrated a decrease in 5-HT_{2A} receptor binding affinity, while another study in drug-naïve patients failed to demonstrate any significant change, suggesting that this may be a consequence of treatment rather than a consequence of schizophrenia (Hurlemann et al., 2008; Lewis et al., 1999). Furthermore, a post-mortem study found that 5-HT uptake in the anterior cingulate and prefrontal cortex was significantly lower in patients with schizophrenia than healthy controls, while the densities of both 5-HT_{1A} and 5-HT_2 receptors were significantly elevated (Joyce et al., 1993). In addition, this study demonstrated that increased 5-HT uptake in the caudate putamen and nucleus accumbens was associated with schizophrenia. 5-HT uptake and its receptor density were also increased in the temporal cortex of patients with schizophrenia (Joyce et al., 1993).

The activity of neuronal circuits is determined by both excitatory glutamatergic and inhibitory GABAergic neurons. As a result, these neurotransmitters and their receptors are considered as regulators of neuronal activity, other neurotransmitter pathways and even the aggregate activity of whole cortical networks. This is demonstrated by the reciprocal regulation between cortical and striatal activities, which is primarily mediated by glutamatergic and GABAergic neurons projecting to dopaminergic neurons and *vice versa* (Carr and Sesack, 2000). GABAergic interneurons are classified by their morphology and expression of certain markers. Parvalbumin-containing interneurons are fast spiking, and are required for the induction of high frequency gamma-oscillations (Buzsaki, 2006; Petilla Interneuron Nomenclature Group et al., 2008).

Disruption of the GABAergic and glutamatergic systems and the subsequent imbalance between excitation and inhibition of neural activity are the most widespread and reproducible findings in schizophrenia (Gonzalez-Burgos et al., 2010). Transcranial magnetic stimulation

revealed a reduction in cortical inhibition in patients with schizophrenia (Wobrock et al., 2008). There is evidence suggesting that due to their fast-spiking firing profile and the high metabolic costs of maintaining this, parvalbumin-containing interneurons may be particularly vulnerable to oxidative stress and cell death (Carter and Bean, 2009). A decrease in the expression of parvalbumin, GABA transporter and glutamic-acid decarboxylase (GAD), was observed in post-mortem schizophrenia brain samples (Gonzalez-Burgos et al., 2010). A recent magnetic resonance imaging (MRI) study directly measured GABA and glutamate in the cortex of 21 patients with schizophrenia and 24 healthy controls; the results showed that the levels of both neurotransmitters were significantly decreased in the patient group (Thakkar et al., 2016). However, studies of parvalbumin cell density in the cortex of schizophrenia patients failed to confirm a significant decrease in parvalbumin-containing cells; a recent study demonstrated that schizophrenia might instead be associated with a decrease in parvalbumin levels rather than parvalbumin-positive interneurons (Benes et al., 2001; Enwright et al., 2016). The decreased expression of parvalbumin, GABA transporter and GAD have been found throughout the post-mortem brains of patients with schizophrenia, including the dorsolateral prefrontal and anterior cingulate cortices (Hashimoto et al., 2008). In situ hybridisation of post-mortem tissues from patients with schizophrenia revealed that the density of GAD-containing neurons was decreased in cortical layers II and IV, and the density of GAD-expressing neurons that also expressed the N-methyl-D-aspartate receptors (NMDARs) were decreased by up to 73% in cortical layer II of patients with schizophrenia (Woo et al., 2004).

NMDARs are a type of glutamate receptor expressed in GABAergic interneurons, and therefore play a central role in eliciting the inhibitory activity of interneurons. However, despite evidence for behavioural changes induced by antagonism of NMDARs in humans, evidence of NMDAR alterations in schizophrenia is mixed at both the transcriptional and protein levels (Hu et al., 2015). Similarly, reports on altered expression, generally a reduction in the expression, of other glutamate receptors in schizophrenia are also mixed, with inconclusive evidence for

abnormal expression of both α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic (AMPA) receptors and kainate receptors (Hu et al., 2015). Despite inconsistency within the literature, some evidence supports the region-specific decreases of glutamate receptor expression. Levels of NMDAR subunit mRNA appear to be decreased in the frontal cortex, the temporal cortex and the hippocampus, while mRNA levels of AMPA receptor subunits have been found to be decreased in the frontal cortex along with the hippocampus; kainate receptors were mainly decreased in the frontal cortex (Hu et al., 2015; Weickert et al., 2013). Studies measuring protein expression of glutamate receptors have demonstrated an even more nuanced picture. In the anterior cingulate cortex, NMDAR expression was found to be increased, while no significant difference in NMDAR expression was found in the dorsolateral prefrontal cortex (DLPFC) (Kristiansen et al., 2006). However, a more recent study of 37 cases and controls demonstrated a decrease in the final protein products of NMDARs in the DLPFC (Weickert et al., 2013). Moreover, an increase in cortical kynurenic acid (KYNA), an NMDAR antagonist, has also been observed in schizophrenia, further reinforcing the link between NMDAR hypofunction and schizophrenia (Schwarcz et al., 2001). Whether or not these factors operate independently, to reflect differing aetiologies for NMDAR hypofunction in schizophrenia, remains unknown.

Although alterations in GABAergic/glutamatergic signalling have been observed in patients with schizophrenia, evidence for their behavioural alterations has primarily come from animal studies. The selective ablation of the NR1 subunit of the NMDAR in 40-50% of cortical interneurons resulted in the development of a ‘schizophrenia-like’ phenotype, including negative and cognitive symptoms in a murine model (Belforte et al., 2010). This phenotype was only apparent with a post-natal ablation of NMDARs, and post-adolescent ablation did not result in ‘schizophrenia-like’ symptoms (Belforte et al., 2010). Furthermore, a reduction in parvalbumin interneurons resulted in an inhibition of gamma-band activity, which was associated with cognitive and social deficits in rodents (Lodge et al., 2009). In humans, the only

ethical investigation into the behavioural effects of NMDAR hypofunction is the administration of NMDAR antagonists. Several studies found that subanaesthetic doses of ketamine were able to induce some symptoms of schizophrenia in healthy volunteers and triggered psychotic episodes in patients with schizophrenia (Lahti et al., 1995; Morgan et al., 2004). However, since NMDAR antagonism only selectively blocks NMDARs but does not duplicate dysfunctions of glutamaterigic or GABAergic signalling, the use of pharmacological models can only hint at behavioural consequences of excitatory or inhibitory alterations in schizophrenia (Steeds et al., 2015).

1.3 Antigen presentation and humoural immunity

Thematic similarities can be observed between the central nervous system and the immune system. Both are large networks requiring a complex system of communication, the responses of both are altered by exposure to exogenous factors and both have mechanisms for remembering information. Recent years have bought renewed interest in the functional links between these two systems, previously believed to be distinct.

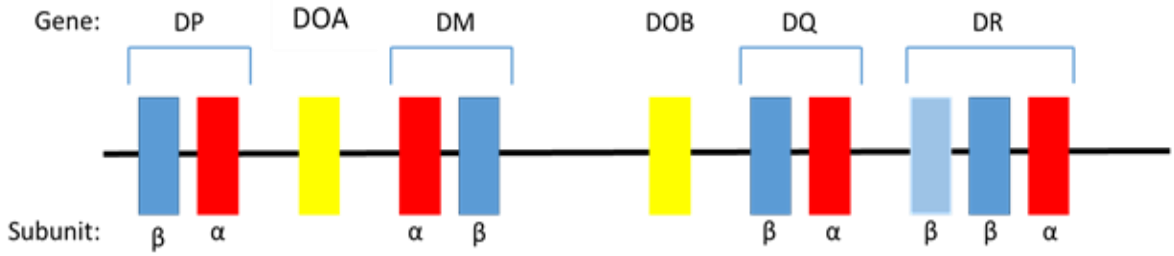
The immune system is traditionally divided between the innate and adaptive arms, the latter of which can be further divided into humoural and tissue immunity. It is important to note communication between all these systems are required for efficient functioning of the immune system. The innate immune system is often considered as the body's first line of defence. It broadly recognises pathogens and its response doesn't change over time, nor is it tailored to any specific pathogen. In contrast, the adaptive arm of the immune response is tailored to a specific pathogen, becoming even more specific over the course of an infection and is able to rapidly recognise previous encountered infectious agents through the use of memory-lymphocytes. As this thesis primarily focusses on an adaptive immune response in schizophrenia the following section will largely detail adaptive immune system processes.

1.3.1 The Human Leukocyte Antigen System

As mentioned, one of the functions of the innate immune system is to activate the adaptive immune system. The innate immune system is able to modulate the adaptive immune system, as well as its self, through the secretion of immune-factors such as cytokines. Additionally, specialised innate immune cells are able to phagocytose pathogens and present pathogen-derived antigens on their cell surface via the major histocompatibility complex (MHC). In humans, the MHC is known as the human leukocyte antigen (HLA).

There are two classes of antigen-presenting MHC proteins, MHC-I and MHC-II. MHC-I proteins are present on the surface of all nucleated cells and present antigen derived from the cytosolic environment, which may include self-antigens. In the case of infection non self-antigens will be presented by MHC-I and the cell will be killed by cytotoxic T-cells. MHC-II proteins, by contrast, are only expressed on a handful of specialised antigen presenting cells including macrophages, microglia, dendritic cells and B-cells.

Figure 1.6 Schematic Diagram of the HLA-II Locus



This simplified schematic diagram shows the genes in the MHC-II locus directly involved in antigen presentation. HLA-DR and DQ are the most highly expressed HLA-II genes involved in antigen presentation, while HLA-DM is primarily involved in catalysing the loading of peptide antigen to HLA-DR, DQ and DP. HLA-DR, DR, DM, DO and DP are comprised of an alpha (red) and a beta (blue) subunit, though some individuals carry two beta HLA-DR subunits (light blue). This figure was adapted from (Murphy and Weaver, 2016).

Genetically, MHC is under the control of the MHC genes, of which there are three classes, MHC class I, class II and class III. The MHC genes are on the short arm of chromosome 6. The class I and II genes code for the antigen presenting molecules of MHC class I and class II, respectively, while the class III genes encode non-antigen presenting genes, including the complement proteins. The HLA locus has been termed a mega locus, containing over 200 genes in 7 million base pairs (Murphy and Weaver, 2016). In humans MHC-I antigen presenting proteins are three alpha subunits coded for by three genes, HLA-A, HLA-B and HLA-C.

Antigen binding by MHC-II genes is determined by the HLA-DR, HLA-DQ and HLA-DP clusters of genes (*Figure 1.6*). Each cluster is made up of a pair of genes, each coding for an alpha or beta subunit, which forms the structure of a HLA-II molecule. The antigen binding site is a pocket formed by the dimerization of the alpha and beta subunits. A significant number of individuals carry a second beta-subunit at the HLA-DR cluster, which can also form an additional HLA-DR molecule with the alpha subunit resulting in four different antigen-recognising MHC-II peptides arising from three clusters of genes (Murphy and Weaver, 2016).

Table 1.2 Number of MHC-II polymorphisms for each antigen presenting gene

Gene	***DRA***	***DRB***	***DQA1***	***DQB1***	***DPA1***	***DPA2***	***DPB1***	***DPB2***	***DMA***	***DMB***	***DOA***	***DOB***
Alleles	7	2,593	95	1,257	67	5	1,014	6	7	13	12	13
Proteins	2	1,878	35	838	29	2	692	3	4	7	3	5

The polymorphic natures of the HLA-II antigen-presenting molecules are driven primarily by the large number of available beta-subunit alleles, a total of 3423 beta subunits have been identified so far, while the alpha subunits are less polymorphic and a total of 75 submits have been identified. This information in this table is based on the latest information from HLA database at https://www.ebi.ac.uk/ipd/imgt/hla/stats.html (Information accurate as of 18/10/18).

The HLA genes are highly polymorphic, with the HLA-megalocus considered as one of the most polymorphic regions in the human genome. Since an individual will inherit two of each parental MHC-II gene cluster, the inheritance of MHC-II alleles from two unrelated parents further increases the diversity of antigen recognition (Howell et al., 2010). The most polymorphic of these, the HLA-DRB genes have 2593 alleles identified, encoding 1878

proteins. At the HLA-DR locus polygenecity is largely driven by the HLA-DRB subunit, with only two functional variants identified at the HLA-DRA locus (*Table 1.2*).

1.3.2 MHC-class II antigen processing and presentation

APCs internalise pathogens, or protein complexes via phagocytosis or receptor mediated endocytosis. Once internalised into endosomes, pH dependent proteases digest the internalised proteins into peptide fragments. The endosome will fuse to vesicles containing MHC-II molecules being trafficked to the cell surface. If the peptide has a complementary structure to the MHC-II molecule's binding site, the two proteins will bind and the protein antigen will be presented, with the MHC-II molecule on the APC's cell surface.

Once an antigen is presented bound to MHC-II on the cell surface, it can be recognised by T-helper cells, the receptor of which must recognise both MHC-II and the bound antigen. Recognition of a bound antigen by the TCR results in the activation of that T-Cell, including the upregulation of costimulatory receptors and factors required for B-Cell activation. B-cells are also able to take up antigen, process and present it on MHC-II molecules. When an activated Th cell recognises antigen bound to MHC-II on a B-cell it will induce that B-cell to undergo rapid clonal expansion, antibody production class switching and somatic hypermutation.

Certain combinations of HLA genes can carry increased risk for the development of autoimmune diseases. One example of HLA-mediated autoimmune disease is Coeliac Disease (CD). The development of Coeliac Disease is largely dependent upon the presence of *DQB1*0201* and *DQB1*0202* HLA-alleles, which code for the HLA-DQ2.5 serotype. HLA-DQ2.5 recognises the 33-mer peptide derived from α2-gliadin responsible for triggering symptoms of CD, is present in >90% of CD patients (Green and Jabri, 2003; Qiao et al., 2005; Shan et al., 2002a). Up to 5% of CD patients are negative for HLA-DQ2.5 but positive for HLA-DQ8 and HLA-DQ2.2. It has been suggested that these HLA molecules recognise peptide

fragments derived from γ-gliadin, rather than the CD-immuno-dominant α-gliadin (Vartdal et al., 1996). While up to 95% of CD patients carry HLA-DQ2, these variants are relatively common in Caucasian populations with up to 30% of western European individuals carrying HLA-DQ2; therefore, some factors other than HLA-binding affinity are likely to play a role in the development of an immune response against gliadin in CD patients (Cummins and Roberts-Thomson, 2009).

1.3.3 B-cell development

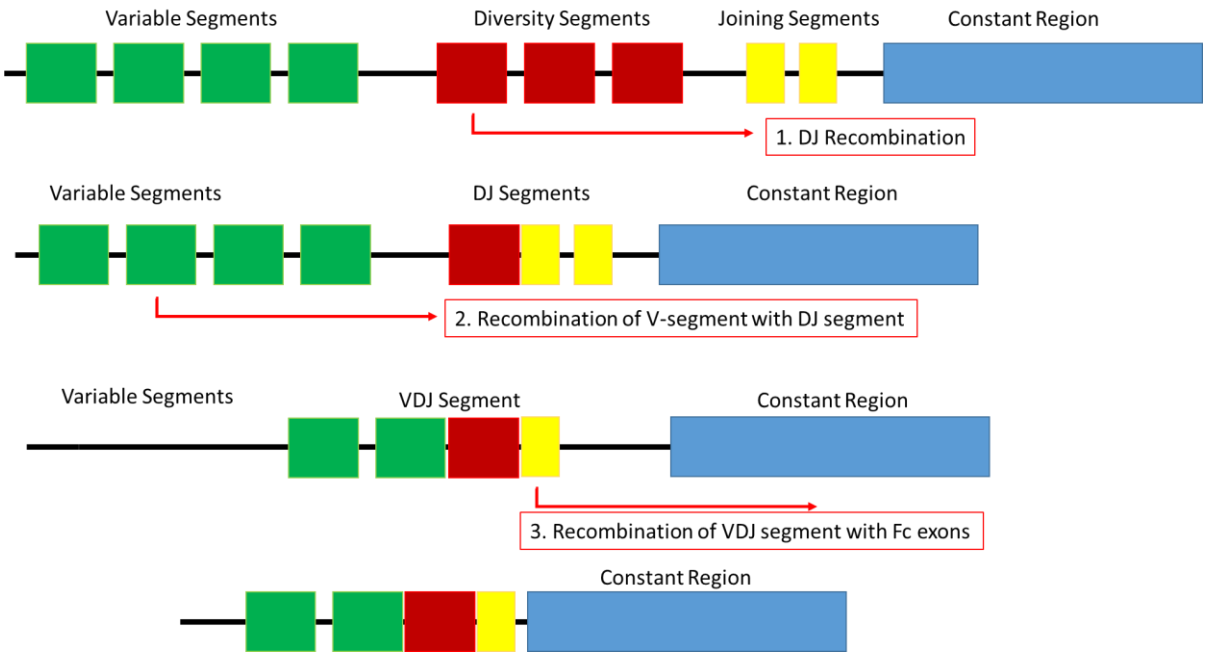
All lymphocytes find their origin in the bone marrow. T-cell precursors migrate from the bone marrow to the thymus in order to complete their development. All lymphocytes are derived from the common lymphocyte precursor (CLP) in the bone marrow. Commitment to the B-cell lineage is determined by CLP adhesion to cell surface receptors on stromal cells, which induce differentiation of CLPs into Pro-B cells. Maintaining contact with stromal cells, pro B-cells differentiate into pre-B cells and finally becoming an immature B cell.

One of the key stages in B-cell development is the formation of the specific membrane bound form of antibody, unique to that B-cell, which forms the B-cell Receptor (BCR). It can be said that the B-cell repertoire is potentially infinite, therefore, in order to avoid an impossibly high genetic burden of a gene coding for each individual antibody specificity, B-cells employ V(D)J recombination. Antibody heavy and light chains are encoded for by Variable (V)and Joining (J) genes, while Diverse (D) genes are used to encode only the heavy chain. The heavy chain locus contains 51 V-segments, 27 D-segments and 6 J-segments, while the light chain locus is comprised of 40 V-segments and 5 J segments (Alberts et al., 2002). In early pro-B cell development DNA between one of the D segments and one of the J-segments at the heavy chain locus is deleted by enzymes encoded by Recombinant Activating Genes (RAG-1 and RAG-2), resulting in one D-segment joined to one J-segment. During the pre-B cell development stage, recombination between the V-segment and the newly formed DJ segment occurs. Furthermore, the VDJ segment is then recombined with the Constant domain, of which there are 9,

corresponding to each of the 9 classes of antibody. At this stage in B cell developments the Cμ-segment is expressed, which is the constant fragment (F_c) of IgM (*Figure 1.7*).

During the immature stage of B cell development, a light chain V-segment and a light chain J-segment undergo recombination, leading to the expression of a fully operational IgM-class antibody. The combinational diversity of the rearrangement of DNA in this manner generates considerable antibody diversity, with one estimate being 2.5×10^{14} when the error-prone nature of the RAG enzymes is taken into account (Alberts et al., 2002). This potential diversity of BCR is over an order of magnitude higher than the total number of B-cells in the human body, 1×10^{13} (Alberts et al., 2002).

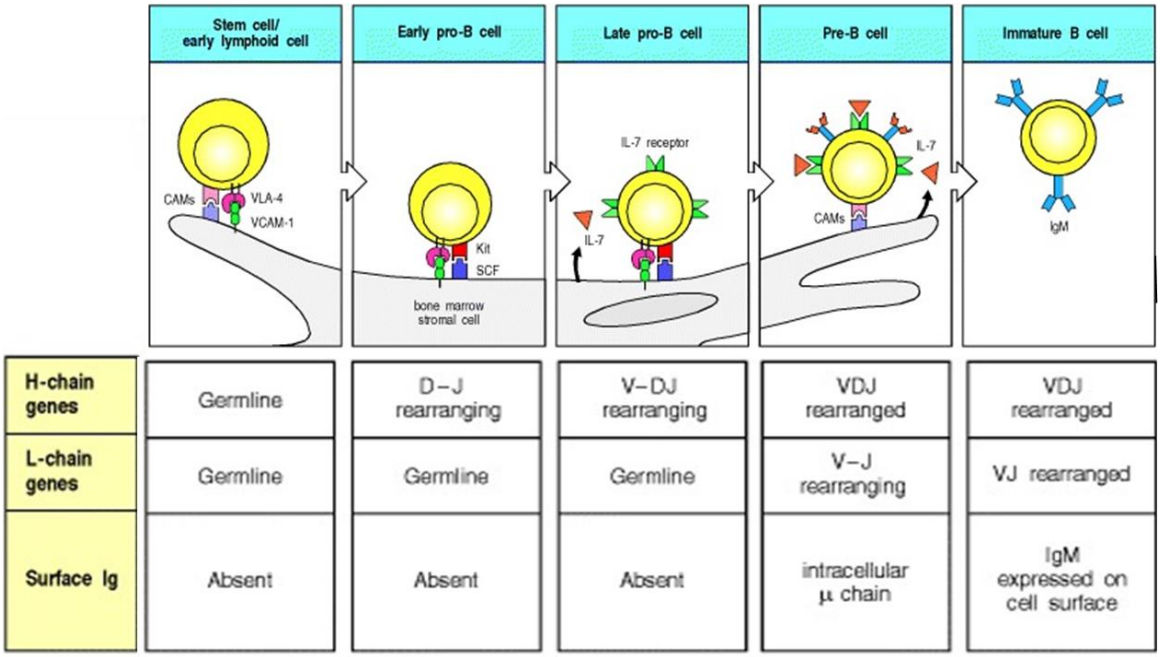
Figure 1.7 Summary Diagram of VDJ Recombination of the heavy chain



VDJ recombination of the heavy chain occurs in three stages. In Pro-B cell development RAG1 and RAG2 enzymes delete DNA between a D and a J segment and re-join the two segments in DJ Recombination. During pre B cell development these enzymes again delete DNA between a V segment and the newly formed DJ segment to form the VDJ segment in V-DJ recombination. Finally, the Fcμ is then joined to the VDJ segment. During the immature B cell phase light chain rearrangement occurs (not shown) and a fully formed cell surface IgM is expressed. Figure adapted from (Murphy and Weaver, 2016).

Prior to B cell egress from the bone marrow the BCR is tested for functionality by dimerization with other BCRs on the same cell. Successful cell signalling then inhibits further heavy-chain VDJ recombination, while those cells that fail to produce functional BCRs are excluded by apoptosis. Finally, the BCR is tested for self-reactivity against antigens in the cellular environment. Cells that bind strongly to self-antigens undergo either apoptosis, anergy or receptor editing, while BCRs that bind soluble self-antigens at low affinity are able to enter the peripheral immune organs along with cells that show no self-reactivity (Murphy and Weaver, 2016).

Figure 1.8 Overview of B cell maturation in the bone marrow



B cells arise from the common lymphocyte precursor. Signals from stromal cells drive much of the development of the B cell lineage. VDJ recombination is an important process in the development of a B cell, leading to the expression of the BCR, which is tested for functionality through cross-linking of neighbouring BCRs, and self-reactivity. Figure adapted from (Murphy and Weaver, 2016).

The diversity of antigens available for sampling in the bone marrow is limited and tolerance in the peripheral immune organs must also be maintained. This is achieved through sampling of self-antigens by the BCR in the lymph nodes, where tissue and organ associated

antigens are readily available. Antigen sampling in localised lymph nodes may also underlie tolerance to immune privileged sites such as the CNS or the testis (DeLuca et al., 2009; Harris et al., 2014; Tostanoski et al., 2016; Wolvers et al., 1999). One aspect of peripheral tolerance is that although it can be induced in a site-specific manner, tolerance is a systemic quality (Wolvers et al., 1999). This is discussed in more detail in section 1.5.3. Mature B cells that demonstrate high-affinity, self-reactivity to multivalent antigens undergo apoptosis, while B cells that demonstrate low affinity reactivity against soluble antigen remain alive but are said to be clonally ignorant (Murphy and Weaver, 2016). The surviving cells are considered to be fully mature B cells and are $CD19^+$, $CD20^+$, $CD27^-$, $CD86^+$ expressing functional HLA-DQ and HLA-DR.

1.3.4 B cell Activation

Mature inactive B cells are found in the peripheral immune sites, the lymph node and spleen, as well as in circulation. B cell activation can broadly be categorised by two mechanisms related to the involvement of T cells; Thymus-dependent (TD) or Thymus-independent (TI).

TI, or T-independent antigens, are primarily derived from common pathogen-associated molecular patterns (PAMPs) and can be further categorised as class 1 (TI-1) and class 2 (TI-2). TI-1 antigens, such as polysaccharides on bacterial cell walls, are Toll-Like Receptor (TLR) agonists. TLRs are specialised at recognising PAMPs, and their ligation results in a non-specific IgM antibody response. A more specific antibody response can be produced from a TI-1 antigens direct ligation to a complementary BCR. This results in upregulation of TLRs allowing specific TI-1 B cells to respond to much lower levels of TI-1 antigens. TI-2 antigens are also made up of repeated molecular patterns but elicit immune responses by crosslinking with many BCRs, resulting in B cell differentiation into plasmablasts, short-lived IgM producing cells. The antibody response to TI-2 antigens, unlike that from TI-1 antigens, can further be shaped by the

secretion of cytokines from either innate immune cells or T cells to induce isotype class-switching (Murphy and Weaver, 2016).

The humoural immune response can be split into two stages. In the first stage, as previously mentioned, requires B cell co-stimulation by T-helper cells and recognition of antigen by the BCR. Antigens circulate via the blood to the spleen and via lymphatic vessels to the lymph nodes. When the B cell encounters its BCR-complimentary antigen the BCR-antigen complex is internalised, bound to MHC-II proteins and presented on the B cell surface membrane. Recognition of the antigen-MHC complex by the TCR, in conjunction with other co-stimulatory factors such as CD40L prolong the survival of B cells, while antigen-stimulated B cells that fail to be activated by T cells will die. After stimulation by Th cells, some B-cells migrate to a region of the lymph node near the efferent ducts known as the primary focus to proliferate and differentiate into short-lived plasmablasts and leave the lymph node, while others migrate to the lymph node follicle and form the germinal centre for the second stage of the adaptive humoural response.

The germinal centre is made up of proliferating B cells in the centre (dark zone) and T helper cells on the periphery (light zone). Cells in the germinal centre undergo somatic hypermutation, the process by which the BCR undergoes additional mutations to the Ig Variable domains. Briefly, the enzyme activation induced cytidine deaminase (AID) deaminates cytosine into uracil during DNA transcription. This produces a mismatched base with guanine and induces error-prone DNA repair mechanisms, whereby mismatched DNA is excised and replaced with random amino acid bases. The majority of these changes result in a loss of function of the BCR to recognise and bind its complimentary antigen. Some mutations, however, will result in a BCR with increased affinity for its antigen. B cells that are able to take up more antigen as a result of increased affinity are selected by Th cells for survival, while those that take up less antigen, or none at all (as a result of a lower affinity BCR), will not receive survival signals from Th cells and undergo apoptosis. By this method of hypermutation,

rapid proliferation and selection, over a number of cellular generations as B cells migrate out of and then back in to the germinal centre, high affinity antibodies are selected for and released into circulation (Murphy and Weaver, 2016).

In addition to somatic hypermutation, B cells also receive signals from Th cells to undergo class switching, which is the process by which IgM secreting B cells change antibody class. This usually takes the form of class switching from IgM to IgG or IgA. This process is also under the enzymatic control of AID and is determined by repetitive switch regions of DNA between C genes and J-segments at the heavy chain locus. AID, and other enzymes, introduce double stranded breaks in DNA at the switch region associated with the IgM constant domain and the switch region associated with the constant domain of another antibody class. DNA repair enzymes excise the intervening sequence and join the two switch regions. Determination of the c-region selected occurs via cytokines released by Th cells in the light zone, though cytokines from other immune cells can also influence this. For example, IgG class switching is promoted by IL-4, IFN-γ and IL-21, while IgA class switching is induced by IL-21, TGF-β and IL-5 (Murphy and Weaver, 2016).

The final aspect of adaptive immunity discussed in this section is its capacity for memory. Immunologic memory is a property that emerges from the persistence of long-lived memory cells. Memory B cells are long-lived plasma cells left over from a primary immune response, and express low levels of membrane bound IgM and IgD. Furthermore, memory B-cells preferentially secrete high affinity IgG and IgA in response to a repeat infection. During a secondary infection, high affinity IgG from the initial infection is available to opsonise and neutralise pathogens while any excess antigen can be circulated through the lymphatic system to peripheral lymph organs. Much like in the initial immune response B cells are activated by Th cells, proliferate and enter circulation, while some form germinal centres and undergo affinity maturation. This leads to sequentially quicker and more efficient production of antigen-specific immune responses with repeated immunisation.

1.4 The role of the immune system in schizophrenia

The beginning of the 'immune-hypothesis' of schizophrenia can be retroactively traced back to Kraeplin, who believed that schizophrenia may be caused by endotoxins resulting from infection (Noll, 2004). However, it appears that, with the then recent discovery of the germ theory of disease, this was not an uncommon hypothesis applied to a number of conditions at the turn of the 20th Century (Noll, 2004). A literature search on NCBI PubMed using the terms 'immune' or 'autoimmune' and 'schizophrenia' reveals that the earliest synthesis of the immune hypothesis has its roots in the mid-20th Century via studies examining the relationship between autoimmune conditions and schizophrenia, notably systemic lupus erythematosus and, later, rheumatoid arthritis (Fessel, 1961; Taylor, 1978). As early as 1954 a study noted 'Histamine tolerance in schizophrenia', although the full text of the article is sadly unavailable (Lucy, 1954). Finally, there appears to be a wealth of unavailable studies ranging from the 1950s to the 1970s, mainly in Russian, suggesting 'modifications of allergic manifestations following electroshock therapy' or examining the effect of schizophrenia patient serum on protein turnover in the brain (Hyvert and Fagard, 1951; Us and Bozhko, 1971).

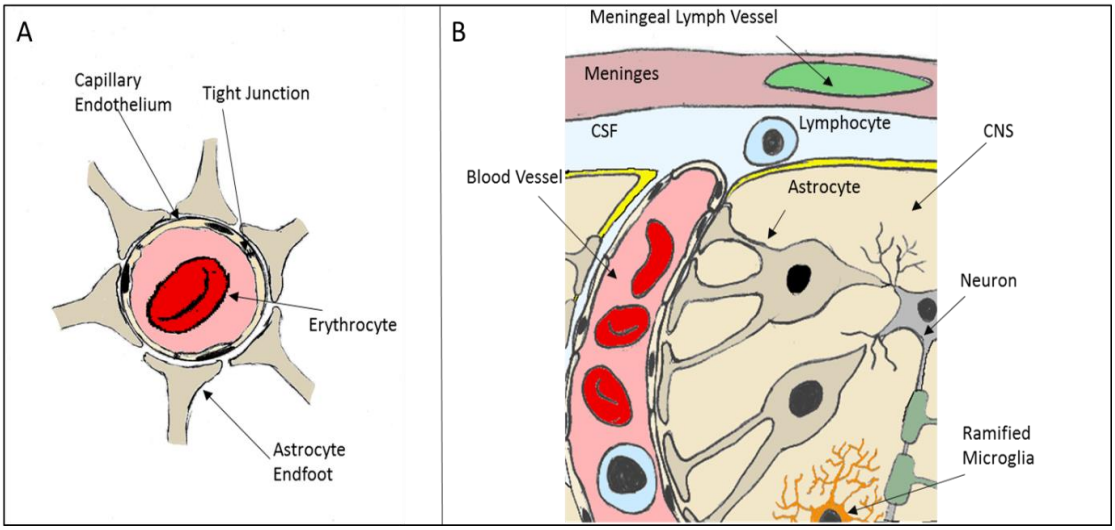
More recently, new and rediscovered links between the CNS and the peripheral immune system have led to a greater appreciation of the potential for the immune system to alter CNS activity, behaviour and to contribute to the development of psychiatric illness.

1.4.1 Immune alterations in the CNS

Previously, the brain was considered an immuno-privileged organ whereby the cells and proteins associated with the immune system were excluded from the brain parenchyma, which itself maintained an immunosuppressive environment; immune cells of the adaptive immune system were said to be ignorant rather than tolerant of CNS tissues. Moreover, the resident immune cells in the CNS, the microglia, are tightly regulated even when activated, increasing

the bias towards an inhibitory immune response in the brain, where damage cannot be so easily repaired.

Figure 1.9 Illustration of physiological interactions between the CNS and peripheral immune system



A. Illustrative diagram of the main components for the blood-brain barrier (BBB) under steady state conditions. Tight junctions of the endothelium exclude molecules based on polarity and size. Astrocyte end-feet are another barrier. There is evidence that the BBB may act as an interface between the CNS and peripheral immune system.
B. Illustrative diagram showing the main components of the CNS immune system under steady state conditions. Ramified microglia are stationary and anti-inflammatory, while lymphocytes in the CSF associated with the meninges may be able to alter the activity of neurons and are drained by meningeal lymph vessel. Recreated and adapted from literature (Louveau et al., 2015).

In fact, this immune privilege is relative rather than absolute. The recent discovery of functional lymph vessels in the meninges of the brain demonstrates that the CNS is integrated into the immune system and that immune tolerance in the CNS may be maintained by antigen sampling rather than simply by immune exclusion (Harris et al., 2014; Louveau et al., 2015; Mohammad et al., 2014). Additional studies demonstrated that peripheral immunological factors, such as TNF-α and IFN-γ, influenced sociability and performance in cognitive tasks in animal models (Brynskikh et al., 2008; Filiano et al., 2016). Under steady state conditions, immune cells of the peripheral immune system are generally excluded from the brain parenchyma, instead exerting effects via secreted soluble factors at the *glia limitans* of the meninges (Filiano et al., 2016). However, under inflammatory conditions, microglia are able to

recruit peripheral monocytes and interleukin (IL)-8 may promote B-cell migration across the BBB (Alter et al., 2003; D'Mello et al., 2009). Finally, there is evidence that immune-associated molecules are synthesised in the brain and play specialised roles within the CNS. A number of pro-inflammatory cytokines are expressed in the rat brain in a time-dependent manner, correlating to critical time points of neurodevelopment (Stolp, 2013). Finally, complement proteins regulate synaptogenesis by tagging synapses for engulfment by microglia (Mastellos, 2014; Perez-Alcazar et al., 2014). The immunological architecture of the CNS is summarized in *Figure 1.9*.

An increase in activated microglia has been observed in both first episode of schizophrenia (FES) patients and those at high risk of developing schizophrenia (Bloomfield et al., 2015). Activated microglia are the only cells within the CNS to produce quinolinic acid (QA), an endogenous NMDAR agonist that can induce excitotoxic injury via the selective death of interneurons; as such QA could potentially play a role in the development of schizophrenia. With regards to the role of QA in schizophrenia, however, the literature is sparse. A study reported an increased QA concentration in the cerebellum, CSF and the frontal lobes of 13 patients with schizophrenia when compared to 12 healthy controls, although another study demonstrated decreased QA in microglia of 17 patients with schizophrenia, when compared to 23 control individuals (Gos et al., 2014; Torrey et al., 1998). Kynurenic Acid (KYNA) has drawn greater attention in the study of schizophrenia, which has consistently shown an increase in the activity of kynurenine pathway and an alteration of KYNA-associated metabolites in the brains of patients with schizophrenia (Kegel et al., 2014; Schwarcz et al., 2001; Schwieler et al., 2015). Furthermore, patients with increased KYNA levels appeared to display a decrease in QA levels, suggesting an upregulation of the kynurenine branch of the kynurenine pathway at the expense of the QA branch (Kegel et al., 2014). Tryptophan metabolism in the brain via the kynurenine pathway appears to be segregated between microglia and astrocytes, with the latter being responsible for KYNA production and the former for QA (Campbell et al., 2014); yet

evidence of increased astrocyte activation in schizophrenia has been sparse. A meta-analysis of the evidence for post-mortem inflammation in schizophrenia suggested either a decrease or no change in astrocyte-activating markers associated with schizophrenia, though there was a significant association between brain inflammation and astrogliosis (Catts et al., 2014; Trépanier et al., 2016).

Another role of the immune system in schizophrenia is the involvement of the complement system. In the periphery, the complement system is a signalling cascade made up of the complement proteins that either tag pathogens for destruction via phagocytosis or directly lyse them via the formation of the membrane attack complex (Lydyard et al., 2011a). All types of cells in the CNS express receptors for complement proteins, though the role of endogenous complements in the CNS was initially believed to be conventionally immunological, allowing the CNS environment to directly control internal inflammatory processes (Mastellos, 2014). It has since been discovered that complement 3 (C3) and complement complex C1q regulates microglial clearance of synapses via their synthesis by neurons in an activity-dependent manner (Mastellos, 2014; Perez-Alcazar et al., 2014). Since there appears to be very little cell death in schizophrenia but reductions in neuronal density and region-specific brain volume have been observed, it is possible that these changes are attributable to an increase in synaptic pruning in schizophrenia (Faludi and Mirnics, 2011). Studies have further revealed region-specific reductions in presynaptic markers such as synapsin I as well as post-synaptic density (PSD) proteins (Faludi and Mirnics, 2011). A recent study demonstrated that the C4 alleles were associated with increased risk of developing schizophrenia, and this increase in risk was positively correlated with copy number length of the C4 gene and increased levels of C4 expression in the brains of schizophrenia patients (Sekar et al., 2016). Further examination of a C4 knockout demonstrated a decrease in synaptic pruning. Although the effects of C4 overexpression on synaptic pruning were not examined in this model, this study suggested that

some cases of schizophrenia might involve aberrant complement-mediated synaptic pruning (Sekar et al., 2016).

An increase in lymphocyte infiltration has also been observed in the CNS of patients with schizophrenia, including a trend towards increased $CD3^+$ T cells associated with paranoid schizophrenia, while the mild residual form of schizophrenia has been found to be associated with a significant increase in $CD20^+$ B cells (Busse et al., 2012a). However, the sample size of this study was small, so replication is required to confirm the finding of CNS lymphocyte infiltration in schizophrenia. As emphasised by Busse et al. (2012a), lymphocyte infiltration into the cerebrospinal fluid is not necessarily correlated to CNS lymphocyte infiltration, but an increase in activated T-cells was detected in the CSF of patients with schizophrenia (McAllister et al., 1991). Another study demonstrated an increase in activated lymphoid cells, with an atypical morphology in the CSF of patients with schizophrenia (Nikkilä et al., 2001).

1.4.2 Dysfunction of the blood brain barrier

As previously mentioned, the BBB is no longer considered as an absolute barrier between the CNS and the peripheral immune system, but the functional role of the BBB cannot be ignored. Indeed, BBB dysfunction may be a necessary prerequisite for antibody action in autoantibody-mediated encephalitis, which could result in psychosis and schizophrenia-like symptoms (Hammer et al., 2014). BBB function in schizophrenia has not been well characterised, likely due to the difficulty in measuring subtle changes in BBB integrity, particularly in psychiatric diseases where CNS samples are rare and *ex-vivo* samples are not available. Studies in animals suggested that an increase in peripheral and central inflammation could increase BBB permeability via activity of IL-6 (Cohen et al., 2013). Furthermore, IL-8 mediated signalling is important for the upregulation of the adhesion molecule VLA-4, whose blockade can inhibit migration across the BBB of an *in vitro* model (Alter et al., 2003).

Therefore, inflammatory processes, which are upregulated in schizophrenia, may contribute to increased permeability of the BBB. S100 calcium-binding protein B (s100B) is present in the processes of astrocytes that coat the brain capillary endothelium and its elevated levels in the circulation may represent a putative marker of BBB dysfunction due to the breakdown of astrocyte end-feet (Blyth et al., 2009). A number of studies examined s100B levels in the serum of patients with schizophrenia and demonstrated increased levels of serum s100B, suggesting that potential BBB disruption is involved in schizophrenia, particularly in patients with cognitive dysfunction (Chen et al., 2016). Additionally, elevated levels of serum s100B have been found to be correlated to the levels of IL-23, an important pro-inflammatory cytokine associated with the development of the experimental autoimmune encephalitis in a model of multiple sclerosis (MS) (Hong et al., 2016). Serum s100B levels were also positively correlated with the anti-inflammatory IL-10 and TGF-β1 levels, but negatively with scored positive and negative syndrome scales (PANSS) (Hong et al., 2016). Direct examination of capillary endothelium in the brain is limited in patients with schizophrenia, though a recent laser-capture microdissection in 24 matched cases and controls demonstrated a downregulation of the genes associated with ion transport, cell proliferation and adhesion, suggesting an impairment of the BBB (Harris et al., 2008); this study also demonstrated a decreased expression of the genes associated with the inflammatory response in the vascular endothelium in schizophrenia. It is therefore possible that an increase in inflammatory responses may not result in BBB dysfunction (Harris et al., 2008). In other words, inflammatory processes are unlikely to be involved in or irrelevant for, BBB dysfunction in schizophrenia. Even if BBB dysfunction is independent of inflammatory processes, a combination of these two factors is likely to be required, at least transiently, for potential involvement of the immune system in schizophrenia. For example, the increased levels in serum S100B suggest that BBB dysfunction in schizophrenia may be mediated at least in part by a breakdown of astrocyte end-feet; further studies are required to examine the role of inflammation and BBB dysfunction in schizophrenia.

1.5.3 Peripheral immune alterations

In addition to abnormal immune processes in the CNS, there is a large body of evidence suggesting the alteration of peripheral immune responses in schizophrenia, although caution must be exercised in the interpretation of many of these findings as it is known that antipsychotic medication can alter the populations of circulating immune cells (Alvir et al., 1993; Atkin et al., 1996). A flow cytometry study examined peripheral immune cell populations in schizophrenia patients undergoing treatment with clozapine and demonstrated a decrease in the number of dendritic cells (DCs), although this effect was not dependent upon serum concentrations of clozapine, the authors rightly state that measuring serum concentrations are subject to metabolic variation between individuals and so a medication effect could not be fully assessed (Fernandez-Egea et al., 2016). Furthermore, a shift from antigen-exposed T-cells and regulatory T (Treg) cells towards naïve T-cells was observed in schizophrenia, along with increases in the population of natural killer (NK) cells and B-cells (Fernandez-Egea et al., 2016). A pilot study examined acutely ill patients in an unmedicated subgroup and demonstrated a decrease in T-helper (Th) cells, consistent with the report by Fernandez-Egea et al. (2016) and with the observation of increased B-cell counts and a possible shift from cellular to humoral immunity (Steiner et al., 2010). An additional study of endothelial adhesion molecule receptors on peripheral T-cell populations in a medicated schizophrenia cohort demonstrated an increase in VLA4 expression associated with $CD4^+$ and $CD8^+$ T-cells, suggesting an increased potential of T-cells to cross the BBB in schizophrenia. This study also supports the idea that BBB permeability in schizophrenia is not dependent upon inflammatory processes within the BBB endothelium (Harris et al., 2008; Müller et al., 1999).

Paraneoplastic syndrome is a complication of some cancers, leading to the production of autoantibodies. If these antibodies are bound to proteins associated with neuronal

communication like NMDARs, schizophrenia-like symptoms may occur. Antibodies binding to NMDARs would explain a number of observations regarding schizophrenia, including the diversity of symptoms. Several studies of anti-NMDAR antibodies in schizophrenia using a variety of techniques, have resulted in a diversity of outcomes, with some suggesting an increase in prevalence of these antibodies, while others failed to show a significant difference in anti-NMDAR antibody levels between patients with schizophrenia and healthy controls (Ando et al., 2016; Masopust et al., 2015; Pathmanandavel et al., 2015; Steiner et al., 2013). A systematic review and meta-analysis of 3387 individuals suggested that a minority of patients with schizophrenia were positive for antibodies against NMDARs (OR, 3.10; 95% CI, 1.04-9.27; P=.043) (Pearlman and Najjar, 2014; Pollak et al., 2014). Due to the overlap of symptoms between anti-NMADR encephalitis and schizophrenia, it is likely that a number of patients with anti-NMDAR encephalitis may have been misdiagnosed with schizophrenia; for example, two patients in one study were initially diagnosed as having schizophrenia, accounting for ~1% of all cases in this study cohort (n= 121) (Steiner et al., 2013). Other autoantibodies against neuronal proteins have also been reported in schizophrenia, including antibodies against the D2 receptor, M1 subunit of the muscarinic acetylcholine receptor and α-7 subunit of the nicotinic receptor (Chandley et al., 2009; Jones et al., 2014; Pathmanandavel et al., 2015). However, a study examined 24 antigens derived from neuronal proteins in both rat hippocampus cell-based assay and HeLa-cell based assay, and showed no neuronal autoantibodies in plasma samples (van Mierlo et al., 2015). Other studies have attempted to measure serum reactivity to CNS tissue, rather than individual proteins. One case-control study showed seropositivity to rat CNS in 53.3% of patients with schizophrenia, compared to 5.1% of controls, demonstrating that sera from patients with schizophrenia was more likely to stain CNS tissue than sera from control individuals (Margari et al., 2013). A follow-up study measuring serum-reactivity to the human hippocampus and cerebellum demonstrated an increase in anti-CNS antibodies associated with schizophrenia and corroborated the early finding that sera from patients with schizophrenia was more likely to stain CNS tissue than sera from control individuals (Margari et al., 2015). A

recent study examining autoantibody targets selected from a GWA that identified 108 schizophrenia associated genetic loci (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), demonstrated that 20.7% of patients with schizophrenia carried plasma immunoglobulin (Ig) G against tetratricopeptide repeat and ankyrin repeat containing 1 (TRANK1), compared to 4.8% of healthy controls (p= <0.001) (Whelan et al., 2018). While the function of TRANK1 protein has not been established, it has been studied as lupus brain antigen 1 and found to be associated with systemic lupus erythematosus-like pathology in murine models (Moore et al., 1998).

The potential effect of infectious agents on psychiatric disease had been posited even before schizophrenia was characterised (Noll, 2007). Increased levels of antibodies against a number of infectious agents have been found to be associated with schizophrenia, including *Toxoplasma gondii*, herpes simplex viruses, and Epstein Barr Virus (Leweke et al., 2004). The diversity of infectious agents involved and evidence of genetic association with these antibodies in patients with schizophrenia suggest that the immune response to pathogens, rather than direct action by pathogenic agents, is more likely to play a role in the development of schizophrenia (Avramopoulos et al., 2015).

It has been suggested that the association between winter births and schizophrenia may also be due to prenatal, maternal infection (Martínez-Ortega et al., 2011). Offspring from rodents immunised with lipopolysaccharide (LPS) demonstrated age-specific behavioural deficits in response to startle stimuli, while an increase in microglial activation was observed in the offspring of PolyI:C treated mice in an age-specific manner (Basta-Kaim et al., 2012; Manitz et al., 2016). Furthermore, cytokines are expressed in an age- and region-dependent manner in the developing brain, suggesting a role as neurotrophic or neurodevelopmental factors, with this pattern of cytokine expression altered in a maternal immune activation (MIA) model, including an increase in pro-inflammatory cytokines in frontal lobes (Garay et al., 2013). A recent study showed that coherence between the medial-prefrontal cortex and the

hippocampus was reduced in low-frequency gamma-band and theta-band signals in the PolyI:C treated MIA model (D. D. Dickerson et al., 2010a). This finding strengthened the validity of the model as a correlate of schizophrenia, suggesting that MIA is able to induce changes in communication between distal brain regions (D. D. Dickerson et al., 2010b).

1.5.4 Immunogenetics of schizophrenia

As mentioned in section 1.2.1 a genome wide association (GWA)-based meta-analysis confirmed 108 genetic loci that were strongly associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The strongest association signal was identified in the short arm of chromosome 6 that harbours the MHC or HLA genes, consistent with previous GWA studies reporting the association between the HLA locus and schizophrenia (International Schizophrenia Consortium et al., 2009; Shi et al., 2009). The structure and function of HLA molecules are discussed elsewhere (section 1.3.1). Although no specific HLA-II variant has been found to be associated with schizophrenia, a number of associations have arisen between HLA genotypes, infection exposure and schizophrenia (Bamne et al., 2012; Fellerhoff et al., 2007). A GWA study also highlighted a number of other loci associated with genes that have a dual enrichment in brain and immune tissues, however, many of which were in noncoding regions. Accordingly, the current hypothesis has been proposed to illustrate that these genetic variations may be responsible for the regulation of gene expression and that expression changes in a number of combined genes may represent accumulated risk of developing schizophrenia (Roussos et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Indeed, a genetic association between the C4 gene and schizophrenia appears to be conferred by the third most common genotype, C4AL-C4AL, and therefore, other genes or environmental factors, are likely to contribute to the burden of risk (Sekar et al., 2016). Furthermore, this study suggested that the signal

observed by the Schizophrenia Working Group of the Psychiatric Genomics Consortium, (2014) at the HLA-region is explained by linkage with C4 alleles.

A gene-enrichment study to compare schizophrenia with five autoimmune conditions demonstrated limited enrichment outside the MHC region for classical immune-related genes in schizophrenia when compared to autoimmune diseases (Pouget et al., 2016). Along with more canonical autoimmune diseases six candidate genes with dual immune brain associations were also enriched in schizophrenia, *DPP4*, *HSPD1*, *EGRI1*, *CLU*, *ESAM* and *NFATC3* with functions including immune system activation, cell-cell adhesion, myelination, synaptic plasticity and blood brain barrier permeability (Pouget et al., 2016). This suggests that the main immune pathology in schizophrenia is derived from immune proteins with a dual role in the peripheral immune system and neurodevelopment (Pouget et al., 2016). This study, therefore, does not suggest that immune alterations have no role in the pathogenesis of schizophrenia but rather, that schizophrenia did not display the genetic characteristics of a typical autoimmune disease.

Concordance for schizophrenia in monozygotic twins is up to 50%, and an estimated heritability ranges from 30% to 80% (Gejman et al., 2010; Light et al., 2014); the offspring of unaffected twins also carry an increased risk of schizophrenia. These observations further emphasise the heritable nature of the disorder and support the theory that a variety of genetic alterations may collectively contribute to an overall burden of risk for the development of schizophrenia (Gejman et al., 2010). Some autoimmune conditions may be over-represented in patients with schizophrenia or their families, while others autoimmune conditions are less prevalent. For example, data from patient interviews suggested that 40% of their first-degree relatives were more likely to have a risk of developing an autoimmune disease such as type-1 diabetes (Wright et al., 1996). A large Danish study found an overrepresentation of coeliac disease (CD) in the histories of patients with schizophrenia (Eaton et al., 2004). A systematic review of the literature suggested that individuals with a non-neurological autoimmune disease

had an increased risk of schizophrenia diagnosis (OR = 1.51) (Cullen et al., 2017). This analysis also sustained a negative association between rheumatoid arthritis and schizophrenia (OR 0.75) as well as positive associations between schizophrenia and autoimmune anaemia (OR 1.87), type I diabetes (OR= 1.40) and coeliac disease (CD) (OR= 8.40) (Cullen et al., 2017).

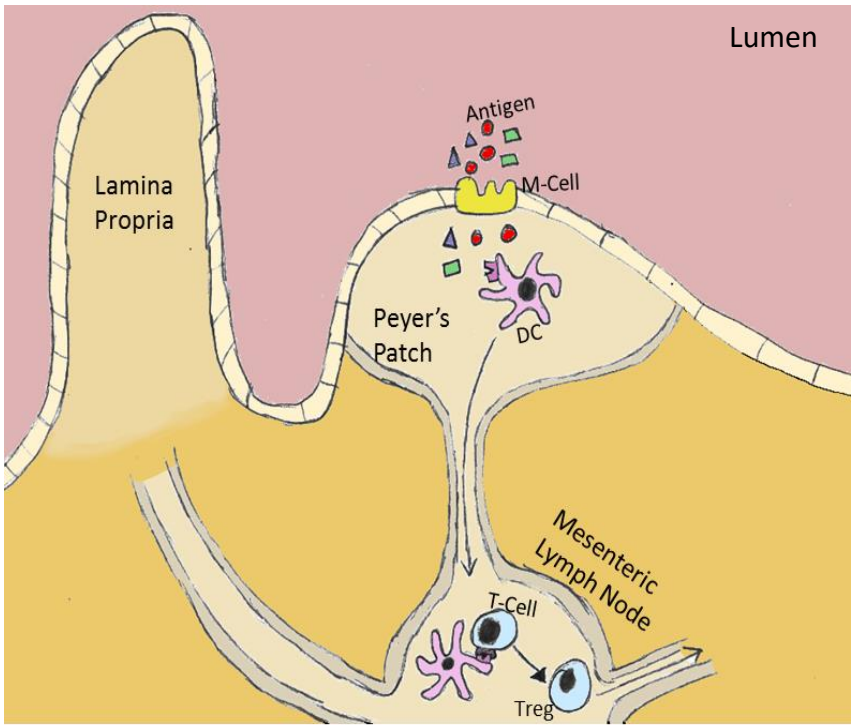
1.6 The anti-gliadin immune response in schizophrenia

1.6.1 Oral tolerance

DCs are the main professional APCs and are crucial as a bridge between the adaptive and innate arms of the immune system. They present antigens directly to T-cells that in turn interact with B-cells and modulate B-cell responses to exogenous antigens, making them key regulators of both humoral immunity and cellular immunity. DCs represent a heterogeneous type of immune cells with monocyte and bone marrow derived lineages, and a variety of phenotypes and functions. In response to inflammatory conditions, DCs mature and present antigens to T cells, inducing T-helper (Th) cells and/or cytotoxic T-cells, leading to an increase in inflammatory conditions, the up-regulation of the humoral immune response and further recruitment of the innate immune cells (Lydyard et al., 2011a). Immature DCs (iDCs), however, secrete anti-inflammatory factors, such as IL-10 (Esterházy et al., 2016).

Despite their nomenclature, iDCs are also capable of processing and presenting antigens, but under anti-inflammatory or steady-state conditions. iDCs promote immune tolerance to an antigen rather than initiating an immune response (Mahnke et al., 2002). The precise mechanism by which iDCs induce peripheral tolerance is not fully understood. The expression of both HLA-II molecules and TCR co-stimulatory molecules is lower in iDCs than mature DCs, leading to a decrease in TCR signalling and potentially death or anergy (Mahnke et al., 2002). Furthermore, iDCs contribute to their own tolerogenic environment via IL-10 secretion and inhibit the production of pro-inflammatory factors.

Figure 1.10 Diagram of proposed mechanism of the induction of oral tolerance



The gut is an important immunological tissue. Peyer's Patches are the inductor sites for the mucosal immune response. Under steady state conditions, M-cells, specialised epithelial cells, transport antigens from the lumen into peyer's patches where iDCs take up antigens and present them to naïve T-Cells in the mesenteric lymph nodes to induce maturation of Treg cells. It is unclear how oral tolerance becomes systemic but it is believed to involve the dissemination of tolerised immune cells. Under inflammatory conditions, mature DCs (MDCs) induce naïve T-cells into Th-cells, which in turn activate B-cells that then migrate to the lamina propria and secrete dimeric IgA into the lumen.

Due to constant exposure to antigens either from the microbiome or dietary proteins, the mucosal surfaces require a strong tolerogenic environment but also needs to be able to respond effectively to pathogens. Much like peripheral systemic tolerance, immune tolerance in the gut requires the presentation of antigen under steady-state conditions and the subsequent induction of T-reg cells. Antigen sampling from the lumen of the intestine is performed either by macrophages via phagocytosis or by antigen transcytosis, and the antigens are subsequently taken up by DCs at the lamina propria (LP) or by Peyer's patches (PP), respectively (Pabst and Mowat, 2012). Although there is debate surrounding the precise importance of the LP or PPs in the induction of immune tolerance to dietary proteins, it is clear that the presentation of antigens to naïve T-cells by DCs in the mesenteric lymph nodes is a crucial step in this process (Pabst and Mowat, 2012). It is worth noting that oral tolerance is not the same as systemic tolerance, however an important facet of oral tolerance is that it may induce systemic tolerance

although this process requires additional steps. Diverting blood flow from the liver via shunting through the hepatic portal vein, can prevent the induction of systemic immune tolerance, suggesting that the dissemination of either antigens or regulatory immune cells may be required to induce the systemic component of oral tolerance as summarised in *Figure 1.10* (Pabst and Mowat, 2012).

No study has yet examined the status of oral tolerance in patients with schizophrenia, although some have attempted to examine gut comorbidities. One study examined the prevalence of irritable bowel syndrome (IBS) in patients with schizophrenia and found that 9/47 patients in the case group had IBS compared to 1/40 patients in the control group that was seeking medical treatment for an unrelated illness (Gupta et al., 1997). The major limitation of this study, however, was the small sample size and the lack of subsequent repetition. Therefore, the link between the two conditions remains to be conclusively established. There is also disparate evidence of intestinal inflammation in some patients with schizophrenia, which could lead to a decrease in tolerogenic processes at the gut mucosa. It was previously demonstrated that anti-*Saccharomyces cerevisiae* IgG, a marker of gastrointestinal inflammation and a serological indicator for Crohn's disease, was increased in patients with schizophrenia and correlated to the levels of antibodies against dietary proteins (Severance et al., 2012a). However, despite some evidence suggesting gut inflammation in schizophrenia and gut responses to psychological stress, a relationship between gut pathology and schizophrenia has not been conclusively established.

1.6.2 Gliadin proteins

Gluten is the primary storage protein of grains, including wheat, rye and barley. It has been a component of human diets consumed around the Mediterranean basin and western Eurasia since the dawn of agriculture. Although gluten proteins are not essential for survival, wheat is a good source of essential amino-acids while the ease of harvest, adaptability and versatile utility have led to the widespread use of wheat across the world and it is almost ubiquitous in 'Western'

diets (Shewry, 2009). In addition to wheat-based products, gluten is often added to other foods due to its texture and viscoelastic properties (Shewry, 2009). Wheat gluten is a complex of proteins mainly composed of gliadin and glutenin. Gliadin has been thought to be the major component for health conditions such as CD.

There are 42 active genes that encode for gliadin proteins in *Triticum aestivum,* the dominant domesticated wheat species, with gliadins being subtyped according to molecular weight and primary structure differences (Wang et al., 2017). There are three subfamilies of gliadin proteins, α-gliadin (25 genes), γ-gliadin (11 genes) and ω-gliadin (5 genes) (Wang et al., 2017). Gliadin molecules contain many long motifs of proline and glutamine (PQ) residues, and as such, are only partially digestible by the enzymes present in the human gut (Janssen et al., 2015; Palová-Jelínková et al., 2005). This results in indigestible gliadin peptides, some of which are 20-100 amino acids in length and carry HLA-II restricted epitopes; therefore, these gliadin-derived peptides show immunogenic potential in the presence of certain types of HLA variants (O'Brien et al., 2008).

1.6.3 The link between gluten consumption and schizophrenia

Diet has long been suspected to play a role in the development of autoimmune diseases.

A link between schizophrenia and gluten consumption was observed initially by Dohan (1966) who reported that schizophrenia admissions to civilian hospitals, were correlated with national wheat consumption, as measured by grain imports, during the Second World War. It is obvious that such a study was confounded by a number of factors, though this trend was independent of increase or decrease in imports of grains. Furthermore, grain imports were examined across a number of different countries, some under occupation, some at war but not occupied and neutral countries. The United States of America (USA) was a notable exception to many of the conditions that may have affected schizophrenia diagnosis in the other countries surveyed. The

USA was at war but not occupied and no fighting took place within the continental states; grain imports were increased during this period and hospital admissions for schizophrenia also went up, opposite to the other countries included in this analysis. A follow-up study applied a more robust test to examine the hypothesis that gluten consumption was linked to schizophrenia by examining the effects of a gluten-free diet (GFD) on hospitalization of schizophrenia patients, based on the time spent in a maximum security ward as a correlate of the clinical condition (Dohan et al., 1969). This study showed that patients who received GFD were released into the general wards significantly earlier than patients who did not receive GFD.

A number of subsequent smaller studies since then have attempted to examine whether GFD has a beneficial effect in patients with schizophrenia, but the outcomes were mixed (Kalaydjian et al., 2006). One study demonstrated that patients with schizophrenia had no alleviation of symptoms with gluten withdrawal, nor worsening of symptoms with gluten challenge (Potkin et al., 1981). Another study attempted to more closely examine Dohan's original findings in twenty-six patients in a secure ward with either gluten-free or gluten-containing cookies, but they found no worsening of symptoms with a gluten challenge (Storms et al., 1982). Meanwhile another study on a secure ward identified 2/24 patients whose symptoms improved on a GFD and relapsed upon double-blind gluten challenge (Vlissides et al., 1986). Several case studies reported that individuals without CD could develop gluten-induced psychosis, at least one of whom was diagnosed as having schizophrenia (Eaton et al., 2015a; Jackson et al., 2012; Lionetti et al., 2015). Kalaydjian et al. (2006) suggested that a number of studies examining GFD in schizophrenia suffered from small sample sizes; a power calculation by Potkin et al. (1981) assumed that a gluten-sensitive subgroup made up 3% of total schizophrenia cases and suggested that a sample size of eight would have a 75% likelihood of failure to detect such a subgroup. Collectively, these studies, including the original observations by Dohan, suggest that GFD may be beneficial to a small subgroup of patients with schizophrenia. A recent study employed a double-blind design to test the whether a larger

GFD trial in patients with schizophrenia was feasible (Kelly et al., 2018). Fourteen patients in total were assigned either a GFD or a non-GFD. Patients in the GFD group reported lower scores for negative symptoms, with a moderate effect size of 0.53 compared to the non-GFD group (Kelly et al., 2018). Additionally, a 35% reduction in levels of anti-gliadin IgG was observed in the GFD group, while levels of anti-gliadin IgG were reduced 17% in the non-GFD group (Kelly et al., 2018). However, an adequately controlled double-blind trial of GFD with a large sample size is still required in order to establish the extent of this subgroup in schizophrenia.

1.6.4 Schizophrenia and CD

Coeliac disease (CD) is an autoimmune gasteroenteropathy characterised by gastric symptoms caused by an immune response to the dietary gluten proteins. In addition to the gastric symptoms, patients with CD may also experience ataxia or other neurological symptoms including delusions and hallucinations (Gujral et al., 2012). It has been reported in the literature that CD and schizophrenia have a similar prevalence, though several large studies suggest that CD diagnosis increases the risk for schizophrenia up to 3-fold and that schizophrenia is more prevalent in patients with CD than in control subjects (Cascella et al., 2011; Kalaydjian et al., 2006; Porcelli et al., 2014). These observations are, however, balanced by genetic and molecular studies that demonstrated a decrease in the prevalence of HLA-DQ 2.5 alleles in patients with schizophrenia and a decrease in serum CD markers, such as IgG against tissue transglutamase (tTG) (F. Dickerson et al., 2010; International Schizophrenia Consortium et al., 2009; Samaroo et al., 2010). Additionally, a large UK-based study with the general practice research database, found no difference in the prevalence of schizophrenia in patients with CD, Crohn’s disease or ulcerative colitis, and decreases in the prevalence of schizophrenia in individuals with these conditions when compared to controls (West et al., 2006). In addition, of those patients with both schizophrenia and enteropathy, diagnosis of the former condition

preceded diagnosis of the latter in the majority of cases, in line with another study showing that up to 3.1% of patients with schizophrenia developed an autoimmune condition (Benros et al., 2012; West et al., 2006). Together this suggests that Crohn's and colitis may in fact be protective against schizophrenia.

In conclusion, the link between schizophrenia and CD is unclear with both compelling and conflicting evidence in the scientific literature. The finding of no positive association between schizophrenia and CD is supported by the studies indicating a decrease in genetic and serological markers for CD in patients with schizophrenia. Therefore, it is unlikely that the link between gluten consumption and schizophrenia is a result of an over-representation of patients with CD in schizophrenia cohorts.

1.6.5 Anti-gliadin antibodies and schizophrenia

Although the levels of some serological markers diagnostically relevant for CD are decreased in patients with schizophrenia, both conditions share an increase in the levels of circulating antibodies against gliadin (F. Dickerson et al., 2010; Samaroo et al., 2010). However, anti-native gliadin antibodies (AGAs) are directed against the γ-gliadin fraction in schizophrenia (Samaroo et al., 2010), while those found in CD patients are mainly directed against the α2-gliadin fraction (Samaroo et al., 2010; Shan et al., 2002b).

Studies of AGAs in schizophrenia have demonstrated increases in both IgG and IgA classes of antibodies against native gliadins, but there has been considerable variability between studies, with consistent replication of an increase in circulating AGA IgA levels (Cascella et al., 2011; Dickerson et al., 2015; F. Dickerson et al., 2010; Jackson et al., 2014; Okusaga et al., 2013; Reichelt and Landmark, 1995; Samaroo et al., 2010). An increase in AGA IgA levels, but not IgG levels, was also observed in patients with schizophrenia in a Chinese population (Jin et al., 2012). Finally, an increase in maternal AGA IgG levels was found to be associated

with an increased risk of non-affective psychosis in their offspring (odds ratio (OR) 1.7, 95%CI 1.1-2.8) (Karlsson et al., 2012). A meta-analysis of AGA in patients with schizophrenia calculated that the OR of AGA IgG in schizophrenia was 2.31 (95%CI 1.16-4.58), while the OR of AGA IgA was 2.57 (95%CI 1.15-5.82) (Lachance and McKenzie, 2014). For a summary of results see *Table 1.3*.

Table 1.3 Summary of findings from studies examining levels of anti-gliadin antibodies in schizophrenia

Study	Assay	Outcome				Comment
		Group (n)	Mean/Median/Pos. (SD)	p	OR (95% CI)	
Jackson et.al 2014	Anti-Gliadin ELISA IgG	Control (100)	1.3 (1.3)	0.0475		
		Case (100)	2.9 (7.7)			
	Anti-Gliadin ELISA IgA	Control (100)	1.9 (0.9)	0.3861		
		Case (100)	2.0 (1.5)			
Okusaga et.al 2013	Anti-Gliadin ELISA IgG	Control (1000)	0.52 (0.56)	<0.0001	2.13 (1.57-2.91)	
		Case (950)	0.81 (0.79)			
Reichelt and Landmark (1995)	Anti-Gliadin ELISA IgG	Control (13)	0.42 (range 0.01 - 1.11)	>0.05		
		Case (48)	0.33 (range 0.01-0.82)			
	Anti-Gliadin ELISA IgA	Control (13)	0/13 patients	0.01		
		Case (48)	9/48 patients			
Jin et.al 2012	Anti-Gliadin ELISA IgG	Control (478)	0.836 (0.552)	0.269	1.32 (0.92-1.88)	Chinese Population
		Case (419)	0.878 (0.587)			
	Anti-Gliadin ELISA IgA	Control (461)	0.951 (0.505)	0.001	1.72 (1.25-2.35)	
		Case(473)	1.066 (0.528)			
Karlsson et.al 2012	Anti-Gliadin ELISPOT IgG	Control (553)	27/553 patients		95th%ile: 2.55 (1.4-4.7)	Maternal AGAIgG
		Case (211)	23/211 patients			
Dickerson et.al 2010	Anti-Gliadin Immunoassay IgG	Control (151)	1.0 (0.61) Reference	Reference		Control subjects were used as a reference group, with levels in case groups normalised to the control group
		Schizophrenia (191)	1.40 (1.03)	<0.0001		
		Recent-Onset (129)	2.17 (1.77)	<0.0001		
	Anti-Gliadin Immunoassay IgA	Control (151)	1.0 (0.64) Reference	Reference		
		Schizophrenia (191)	1.38 (1.21)	0.056		
		Recent-Onset (129)	1.48 (1.20)	<0.0001		
Cascella et.al 2011	Anti-Gliadin ELISA IgG	Control (900)	3.3% +	>0.05	Not given	
		Case (1401)	1.4% +			
	Anti-Gliadin ELISA IgA	Control (900)	3.1% +	<0.001	9.2 (5.8-14.8)	
		Case (1401)	23.1% +			
Lachance and McKenzie 2014	AGA-IgG Meta-analysis	Control (2427)			2.31 (1.16-4.58)	Meta-analysis of 5 studies examining AGA
		Case (2888)				
	AGA-IgA Meta-analysis	Control (1415)			2.57 (1.13-5.82)	
		Case (1969)				

Summary of the outcomes of previous studies measuring levels of anti-gliadin antibodies in serum from patients with schizophrenia and control subjects. Of note is the large study from Cascella et al., (2011), showing an OR of 9.2 for AGA-IgA positivity and schizophrenia. All studies, with the exception of (F. Dickerson et al., 2010) showed an increase in AGA-IgA in patients with schizophrenia when compared to control individuals, though this particular study did show that levels were increased in recent-onset patients. Findings of increased levels of AGA-IgG are more varied, though the large study by Okusaga et al., (2013) shows an OR of 2.13 for increased AGA-IgG and schizophrenia. The meta-analysis by Lachance and McKenzie, (2014) suggested that increased levels of AGA-IgG and AGA-IgA had a collective OR of 2.31 and 2.57, respectively. (pos= positivity, referring for either % or frequency of individuals positive for AGA)

Functionally, the role of AGAs in the development of schizophrenia is unclear and remains to be demonstrated. In patients with CD, cross-reactivity between AGAs and synapsin I, a regulator of neurotransmitter release has been observed (Alaedini et al., 2007). Because the immune response to wheat gluten in schizophrenia appears to be distinct from CD, it may be possible that the putative role of AGAs in schizophrenia is not related to cross-reactivity, and therefore it is important to focus on investigating a possible role of anti-gliadin IgG as an activator of the complement system in developing schizophrenia. A study of rats infected with *T. Gondii* demonstrated a significant increase in anti-gliadin IgG and complexes with C1q (Severance et al., 2012c). Maternal AGA IgG and C1q levels were also significantly correlated in mothers whose offspring developed psychosis later in life; a further study demonstrated that *T. Gondii* infection was able to upregulate cortical C1q in rats (Severance et al., 2014; Xiao et al., 2016). Furthermore, cortical expression of C1q was found to be co-localised with astrocytes, the cell type responsible for KYNA production in the CNS, and serum C1q levels were significantly higher in schizophrenia patients positive for AGAs than those negative for AGAs (Catts et al., 2014; Okusaga et al., 2016; Severance et al., 2014).

It is important to note that AGAs are not specific to schizophrenia; in addition to their presence in 80% of CD patients, a significant number of healthy individuals also display increased levels of circulating AGAs (Jin et al., 2012; McLean et al., 2017). The distinguishing feature between AGAs in patients with schizophrenia and healthy subjects is unclear, but as with anti-NMDAR encephalitis, CNS access may be an important factor (Hammer et al., 2014). Epitope specificity for AGAs in healthy controls is also unclear as AGAs from patients with CD directed against α2-gliadin and AGAs from patients with schizophrenia directed against γ-gliadin, although this study used AGA-negative healthy individuals as controls (Samaroo et al., 2010). Consequently, it is unknown whether antibody targets from gliadin-derived fragments are distinct between patients with schizophrenia and healthy controls.

1.7 Aims of this PhD study and outlook

The aim of this PhD study was to test the hypothesis that the gluten immune response observed in schizophrenia is antigen-specific and mediated by autoimmune processes. To this end, serum samples from schizophrenia patients and non-schizophrenia controls were employed in order to identify potential antibody targets from gliadin-derived fragments. The data collected were used to examine associations between genetic variants and circulating anti-gliadin antibodies in order to determine if altered levels of anti-gliadin antibodies are dependent upon HLA-type. Finally, cell culture models were developed with immune cell lines to test the hypothesis that selected gliadin-derived peptides differentially induce the maturation of antigen presenting cells.

2. General Methodology

2.1 Study samples

All the samples used for this study were collected through the University of Aberdeen and NHS Grampian under the leadership of Professor David St. Clair. A total of 467 patients with schizophrenia (mean aged 41± 13.3 years) and 490 healthy controls (aged 44± 12.5 years) were recruited from the North of Scotland in the period between 2003 and 2008. The case samples were identified through psychiatric hospitals or outpatient facilities and control subjects were volunteers screened for psychiatric disorders, or a family history of mental illness by a self-reporting questionnaire. All schizophrenia samples met the criteria for schizophrenia diagnosis under DSM-IV or ICD-10 and all participants were British Caucasian individuals. All participants gave written consent for taking their blood samples for genetic and serological analyses. The study was approved by local ethics committees (Research Ethics Council reference: 10/S0802/18) and conformed to the Declaration of Helsinki and its amendments.

2.1.1 Plasma samples

Whole blood samples were extracted from patients with schizophrenia and unrelated healthy controls in Aberdeen. Plasma was separated by centrifugation by Dr Colette Mustard at the University of the Highlands and Islands, using a routine protocol. The plasma was then aliquoted before storage at -80˚C until use. A further aliquot was prepared just prior to experiments and stored at -20˚C for less than a year.

2.1.2 Extraction of DNA samples

DNA samples were extracted from whole blood using DNeasy® Blood and Tissue Kits (Qiagen, Germany) according to the manufacturer's instructions. Extractions were performed by Dr Lorna Halley and Dr Colette Mustard at the University of the Highlands and Islands. A volume of 200µl whole blood was processed with 20µl of proteinase K, followed by 200µl of lysis buffer and 200µl of ethanol. The mixture was then vortexed for approximately 15-30

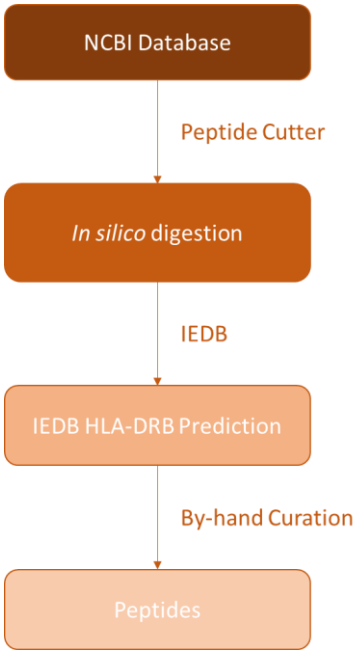
seconds and incubated at 56°C for 10 min. The mixture was then transferred to the collection column provided and spun in a mini centrifuge (Microstar 17, VWR) at 6000 x *g* for 1 min; 500µl of wash buffer 1 was added to the column and spun at 6000 x g for 1 min; 500µl of wash buffer 2 was added to the column and spun at 17,000 x *g* for 4 min. The collection tube was replaced before further centrifugation at 17,000 x *g* for 1 min. The collection tube was replaced and 150µl of elution buffer was added to the column before elusion was completed by centrifugation at 6000 x *g* for 1 min. The DNA samples were stored at -20°C prior to use for genotyping single nucleotide polymorphisms (SNPs) with a polymerase chain reaction (PCR)-based protocol.

2.2 Gliadin-derived Peptides

The peptides used in this study were designed by Professor Jun Wei. The α- and γ-gliadin sequences were retrieved from the NCBI protein database (http://www.ncbi.nlm.nih.gove/protein) and analysed *in silico* using PeptideCutter software; sequences >11 amino acids long that did not contain cutting sites for pepsin, trypsin and chymotrypsin underwent HLA-II prediction (Gasteiger et al., 2003). Target peptides were selected based on computational prediction for HLA-II restricted epitopes using SMM_align output from IEDB (Söllner et al., 2010; Wang et al., 2010). As it is the most polymorphic, HLA-DR was selected for the prediction of HLA-restriction. Twelve alleles were used for the prediction; DRB1*0101 , DRB1*0301, DRB1*0401, DRB1*0404, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301, DRB1*1302, DRB1*1401, DRB1*1501 and DRB1*1601, accounting for 88.2% of all HLA-DRB1 alleles identified in European-derived Caucasian populations (de Bakker et al., 2006). As per the IEDB, a score of <50nM suggests that a peptide will strongly bind to its predicted HLA-allele while <500nM suggests intermediate binding, consequently, for the purposes of this thesis a score of >500nM was considered as unlikely to bind. Identified HLA-restricted sequences were then digested *in silico* compared with pepsin,

trypsin and chymotrypsin using PeptideCutter software (https://web.expasy.org/peptide_cutter/). The final peptide list was hand curated by Professor Wei (see *Figure 2.1*). Detailed information about these peptide sequences, their NBCI accession number and position in native gliadin molecules are given in *Table 2.1*. A 29-mer peptide was also designed based on a maize protein (NCBI accession 1BFA_A) as the control antigen and its sequence of amino acids is as follows: H-HAQLEGRLHDLPGCPREVQRGFAATLVTN-OH. All peptides designed were then synthesised with solid-phase chemistry with a purity of >95% (Severn Biotech Ltd, Worcs, UK).

Figure 2.1 Flow diagram detailing the process of peptide design



Peptides were retrieved from the NCBI database and underwent *in silico* digestion with peptide cutter software. The SMM IC50 output scores were used to determine prediction of HLA-recognition. The final peptide selection was curated by hand. For outputs see Appendix 1.

Table 2.1 Sequence information of indigestible gliadin-derived fragments used in this study

NCBI Accession (antigen)	Sequence	Position (aa)	Native molecule	Predicted HLA Binding
CAB76957 (AL1G1)	KCSFQSSQQNPQAQGSVQPQQLPQ	205 - 226	α1-Gliadin	DRB1*04:01
CAB76964 (AL2G1)	CPFRPQQPYPQSQPQYSQPQQPISQK	88 - 111	α2-Gliadin	DRB1*01:01 DRB1*04:01
CAB76964 (AL2G2)	KNVYIPPYCTIAPVGIFGTNYR	270 - 290	α2-Gliadin	DRB1*01:01 DRB1*07:01 DRB1*15:01
AAQ6387 (AAQ6A)	CHFIQPQQPFPQQPQQSFPQQQPSLIK	59 - 72 110 - 119	γ-Gliadin	DRB1*01:01
AAQ6387 (AAQ6B)	CHSIIMQQEQQEQRQGVQILVPLSQK	185 - 208	γ-Gliadin	DRB1*01:01 DRB1*15:01 DRB1*11:01
AAQ6387 (AAQ6C)	HPKCSIMRAPFASIVAGIGGQYRD	253 - 274	γ-Gliadin	DRB1*01:01 DRB1*15:01 DRB1*07:01
ABO37962 (ABO3a)	KATTIATANMQVDPSGQVQWPQQQPFRC	13 - 38	γ5-Gliadin	DRB1*07:01 DRB1*13:02 DRB1*01:01
ABO37962 (ABO3b)	KYVRPDCSTINAPFASIVAGISGQH	263 - 285	γ5-Gliadin	DRB1*01:01 DRB1*07:01 DRB1*04:01 DRB1*15:01 DRB1*11:01

The designation, accession numbers, sequence, position, parent gliadin and predicted HLA-binding are displayed. Alleles for predicted HLA-binding are listed in order of the confidence of their binding prediction to each sequence.

2.3 Anti-gliadin antibody assay

Each synthetic peptide antigen was dissolved in 67% acetic acid to a concentration of 5mg/ml as stock solution and stored at -20°C prior to use. An enzyme-linked immunosorbent assay (ELISA) was developed in-house to detect two types of plasma antibodies, IgG and IgA, against indigestible MHC-II restricted gliadin-derived peptides (Section 2.2) in case-control plasma samples. A detailed protocol is given in Chapter 3 (Section 3.2.3).

Based on work by Dr Lorna Halley, which showed that the OD signal against all antigens tested remained stable at antigen concentrations >10µg/ml this concentration was selected for the development of in-house ELISAs (Halley, 2014). The 10µg/ml solution of peptide was made with coating buffer (phosphate-buffered saline, PBS, containing 0.1% azide, pH 7.4,) and 100µl added to each well on Nunc-Immuno Maxisorp 96-well plates (DIS-971-030J, Fisher Scientific). Each plate was coated with two gliadin-derived peptides and the control peptide for non-specific binding. After overnight incubation, the plate was washed three times with wash buffer (Tris-buffered saline with Tween®20, pH8.0) and 100µl of plasma sample was diluted 1:100 for IgA assay or 1:150 for IgG assay, in assay buffer (PBS containing 1.5% bovine serum albumin, BSA), then added to each well and incubated for 1.5 hours at room temperature. Negative control wells contained assay buffer only. The plate was washed in triplicate to remove excess antibodies in plasma before 100µl of the secondary antibodies, either goat anti-human IgG-peroxidase antibody (ab98624, Abcam) or goat anti-human IgA-peroxidase antibody (A0295, Sigma Aldrich) diluted in assay buffer, was added to each well. After additional washing steps, the colour reaction was initiated by adding 100µl of stabilised chromogen (TMB) and terminated after 20 minutes using 50µl of Stop Solution.

The intensity of the resulting colour change was measured as optical density (OD) at λ=450nm with a reference wavelength of λ=620nm on a Varioscan microplate reader. A specific binding index (SBI) was used to present the data in the levels of circulating antibodies against gliadin-derived peptide antigens. SBI was calculated according to the resulting OD from

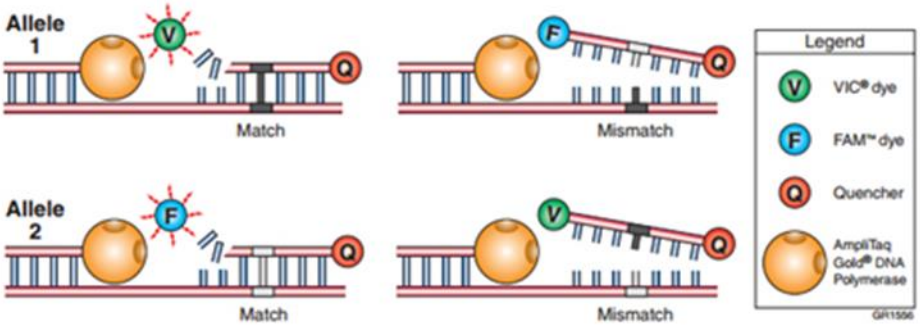
antibody-specific biding to gliadin-derived peptides relative to that from non-specific binding to the maize-derived control peptide. Expressed as: SBI = [OD gliadin – OD_{NC}] / [OD maize – OC_{NC}]

2.4 Genotyping of SNPs

Schizophrenia-associated SNPs confirmed by the Schizophrenia Working Group of the Psychiatric Genomics Consortium (2014) were selected for genotyping according to the following criteria: (1) the associated genes are highly expressed in B-lymphocytes (over 3-fold medians) based on the BioGPS gene expression database (Wu et al., 2009), (2) odds ratio (OR) from combined samples should be >1.05 or <0.95 based on the GWA study (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), (3) greater than mean expression in the CNS, and (4) TaqMan genotyping reagents are commercially available. This process is discussed in further detail in section 4.2.2. This thesis also used data from genotyped HLA allele tagging SNPs and this work was performed by Dr Lorna Halley, though the analysis of HLA-tagged SNPs and levels of antibodies against gliadins was performed by the candidate.

The presence of the selected SNPs in the schizophrenia cohort was tested by the real-time PCRTaqMan protocol. In brief, the PCR reaction was performed in a 10µl volume containing 1.5µl of DNA sample, 3.25µl of autoclaved water, 0.25µl of primers and fluorescent probes (VIC and FAM fluorophores) with a quencher as well as 5µl of master mix containing four deoxynucleoside triphosphates and TaqPolymerases (Thermofisher Scientific, USA). The negative control well contained autoclaved water instead of a DNA sample. To limit enzymatic activity, reagent and sample loading onto the plate was performed on a cooling block at -20 ˚C. The plate was then sealed to stop evaporation during the PCR reaction and centrifuged to ensure all regents were present in solution.

Figure 2.2 Schematic diagram of the principles of Taq-Man PCR



A substantial increase in...	Indicates...
VIC® dye fluorescence only	Homozygosity for allele 1
FAM™ dye fluorescence only	Homozygosity for allele 2
Both fluorescence signals	Heterozygosity for allele 1 and allele 2

Intact DNA is heated to 95°C to separate complementary DNA strands. At 60°C the DNA-probe containing the SNP of interest, conjugated with the reporter dye (FAM) and a quencher anneal to complementary bases. DNA polymerase then synthesises a new complementary DNA strand, cleaving FAM/VIC from the quencher as it does so. No longer in the presence of the quencher, the reporter dye is able to fluoresce, which can then be detected and quantified. Taq-Man Polymerase uses two probes, one for the minor allele and one for the major allele. If a probe cannot bind to a complementary strand then the quencher and dye remain in proximity and therefore that fluorescent signal is not amplified. Genotype calling is determined by the quantification of the fluorescent signal from each dye. Image taken from ("DNALINK," n.d.) Accessed on 19/10/2018.

A StepOne Plus® Real-Time PCR system was used for DNA amplification. The conditions used for PCR amplification are listed in *Table 2.2*. The plate underwent an initial activation step at 60˚C for 2 min, followed by 95˚C for 10 minutes for polymerase activation and then cycling rapidly between 95˚C and 60˚C for melting annealing and extension (*Table 2.2* and *Figure 2.2*).

Table 2.2 The conditions used for PCR-based TaqMan genotyping

Stage	Temperature	Time (mins)
Pre-PCR Read	60°C	0.5
	95°C	10
Cycling Stage (50 cycles)	95°C	0.25
	60°C	1
Post PCR Read	60°C	0.5

The PCR-plate undergoes rapid cycle of heating and cooling in order to denature and reanneal DNA and SNP probes.

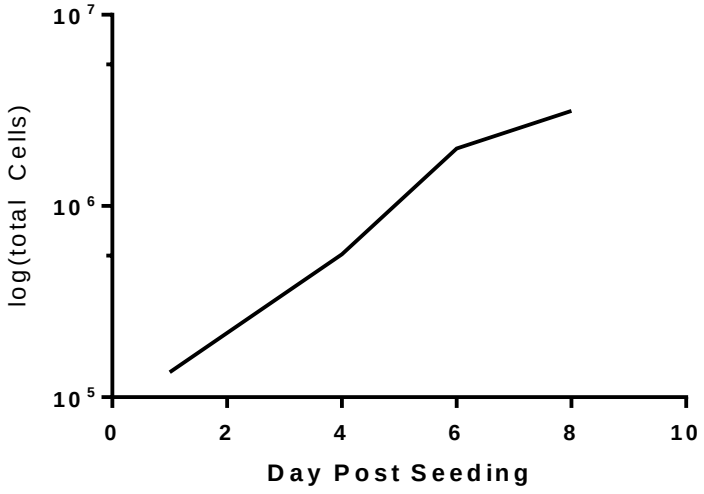
2.5 Cell culture

Two cell lines were used to develop models for investigating the interaction between gliadin-derived antigens and immune cells. These two cell lines included the WIL.2.NS cells, a B lymphoblast cell line derived from the spleen of a Caucasian male with spherocytic anaemia (Levy et al., 1968), and the THP-1 cell line, which was derived from an 1-year old male child with acute monocytic leukaemia. The cell culture methods are outlined in the following section.

2.5.1 Culture of WIL.2.NS cells

WIL.2.NS cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC), UK, and delivered in a frozen vial. The frozen cells were rapidly thawed at 36°C and cultured under standard conditions (37 °C and 5% CO_2) in complete RPMI1640 medium containing 10% FBS and 2mM L-glutamine (Sigma-Aldrich, USA), and maintained at a density of 0.5-2x10^6 cells/ml, with medium change every other day until cells were harvested for experiments. As shown in *Figure 2.3*, cells entered the growth phase from day 4 of post-seeding incubation. Further details are described in Chapter 5 (Section 5.2.2).

Figure 2.3 Growth curve of the WIL.2.NS B-cell line

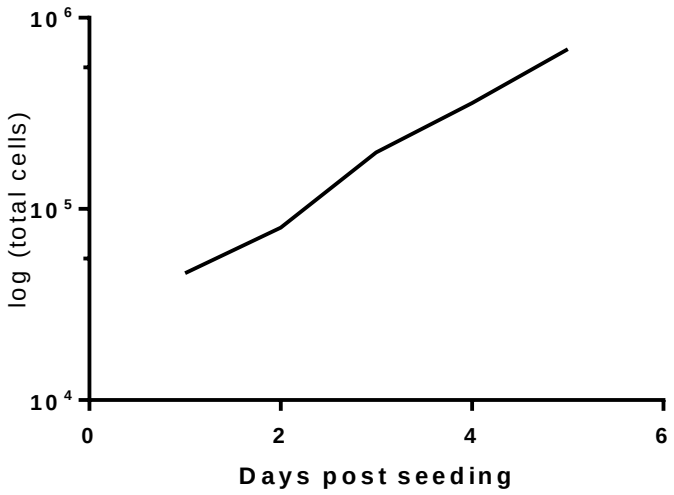


WIL.2.NS B cells were rapidly thawed and left to incubate for ~1 day. Cells were then counted every other day. The cells were in growth phase after 24 hours post-seeding.

2.5.2 Culture of THP1 cells

THP-1 cells were purchased from the ECACC and delivered in a frozen vial. The frozen cells were rapidly thawed at 36°C and resuspended at a density of $0.5x10^6$ cells/ml in complete RPMI-1640 medium containing 2mM L-glutamine and 10% FBS. Before experiments started, THP-1 cells were allowed to enter into the growth phase, at approximately three days of post-seeding incubation (*Figure 2.4*), and the density of cultured cells was maintained at $0.5\text{-}1x10^6$ cells/ml, with medium change every other day before culture under experimental conditions (Section 5.2.6)

Figure 2.4 Growth curve of the THP-1 monocyte cell-line



Monocyte-derived THP-1 cells were rapidly thawed and left to incubate for ~1 day. Cells were then counted every other day. The cells were in growth phase after 24 hours post-seeding.

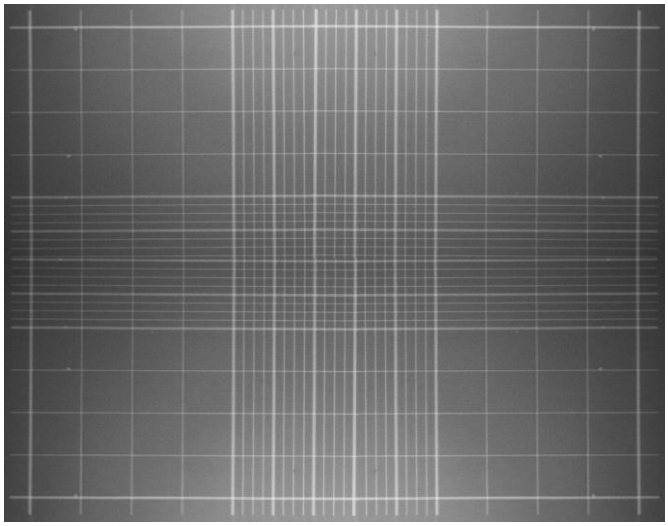
2.5.3 Cell counting

Cells were counted using a haemocytometer (*Figure 2.5*). An aliquot of cells was diluted 1:1 with 0.4% trypan blue, a cell surface membrane impermeable dye, and 10µl of the cell suspension was then placed on a covered haemocytometer. The microscopic gridlines were visualised on a microscope at 10x magnification and the cells were counted with a hand counter, which was reset after counting every viable cell in each of the four quadrants. The final cell density was calculated according to the total number of cells in all 4 quadrants using the following equation: Average cell number x 10,000 x trypan blue dilution factor

The final density was expressed as yx10^4 cells/ml, where y= cell number

.

Figure 2.5 Composite image of the Haemocytometer used for cell counting

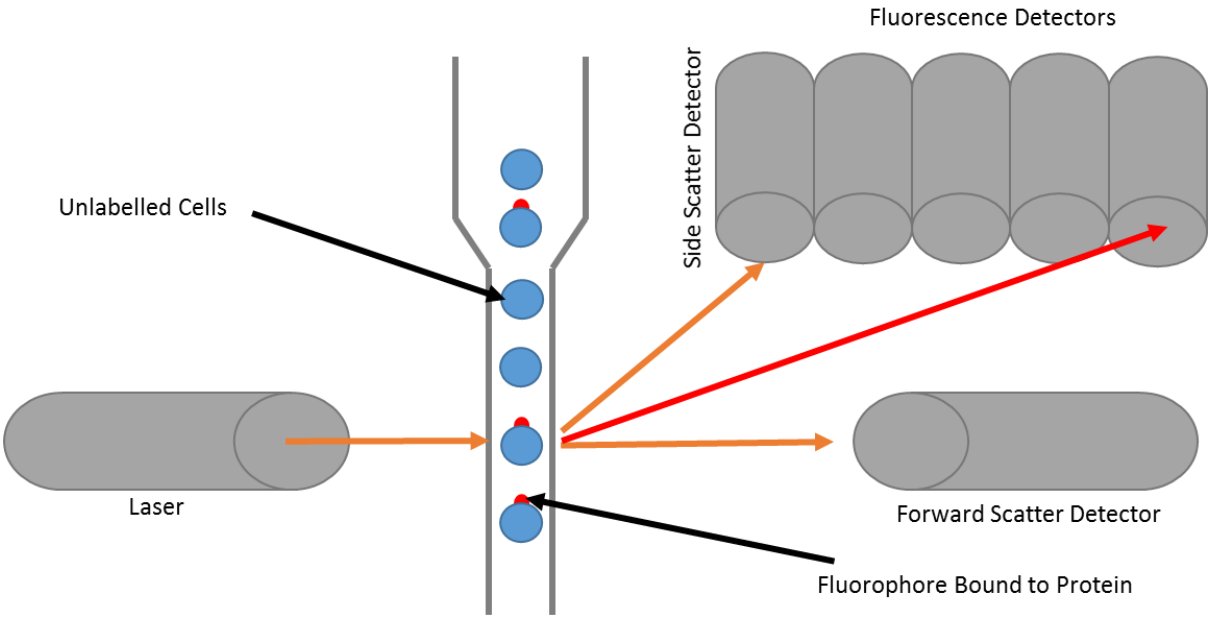


Haemocytometer for cell counting. Live cells, determined by the exclusion of trypan blue, in each of the four quadrants were counted and the average number of cells over the four quadrants was used to determine the final cell density.

2.5.4 Fluorescence activated cell sorting

Fluorescence-activated cell sorting (FACS) is a method by which the characteristics of a population of cells can be quantified. Cells are pumped through a flow system, which allows them to pass through a laser beam one at a time. Light-detectors are then able to measure the light-scattering properties of cells in order to determine cell size (forward scatter) or intracellular complexity (side scatter) (*Figure 2.6*). Furthermore, through the use of a fluorescent probes, often a monoclonal antibody, stained cells can be further characterised based on the expression of intracellular or extracellular proteins, although the detection of intracellular markers requires additional permeablising steps.

Figure 2.6 A Schematic diagram of the principle components of fluorescence activated cell sorting

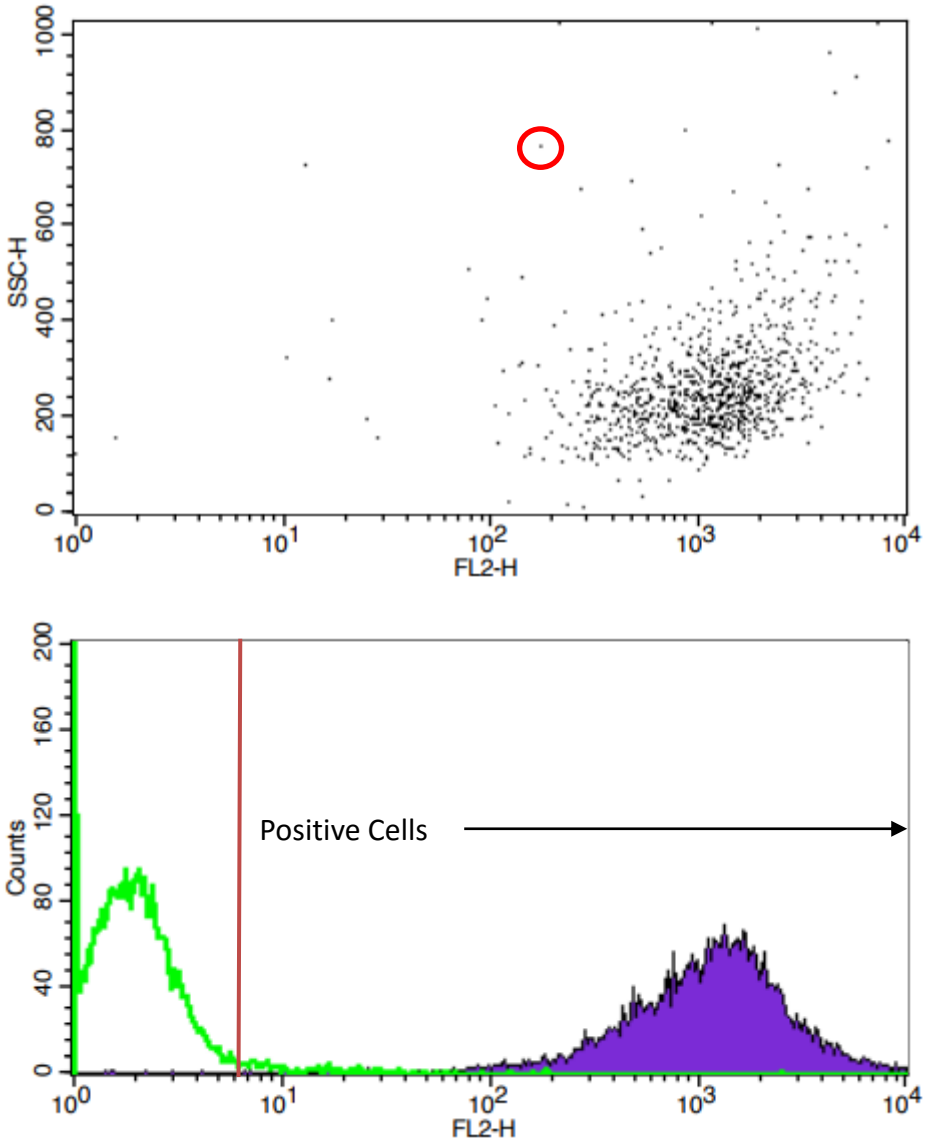


Cells pre-stained with fluorophores of interest are passed through a narrow column and through the path of a laser emitting the excitation frequency of the fluorophore bound to the protein of interest. If the fluorophore is present, it is excited and emits light, which can be filtered and detected. Each individual cell can therefore be quantified according the forward/side scatter and emission of fluorescent light.

As cells enter the laser beam, bound fluorophores emit a specific wavelength of light, which can then be detected by light-detectors for that colour. Since flow cytometers contain multiple detectors, different mAbs with different fluorophores can be used simultaneously to stain cells and measure multiple proteins. Since detectors are able to detect overlapping wavelengths of fluorescence, however, bleeding between detectors must be compensated by altering the optimal wavelength detection, particularly in the case of multiple fluorophores with overlapping emission spectra (for example, in the case of Fluorescein isothiocyanate (FITC) and propidium iodide (PI), as detailed in Section 2.5.4). It is therefore ideal to carefully compare the emission spectra of fluorophores and, if possible, to avoid overlapping spectra. Additionally, due to the presence of Ig-Fc receptors that recognise the constant region of mAbs, class controls must be employed in order to determine specific mAb staining. Class controls are matched to specific mAbs by immunoglobulin class, derived animal and conjugated fluorophore. For example, rabbit IgG-II anti-human CD86-FITC would require a class control

of rabbit IgG-II conjugated with FITC fluorophore. In the output, each cell in suspension can be visualised, typically in a dot-plot or histogram, according to its side/forward scatter properties of emission properties (*Figure 2.7*).

Figure 2.7 Example of flow cytometry output



Upper: Dot plot showing 10% of total events. An individual event, usually a cell, is highlighted with a red circle, displaying a relatively high level of intracellular complexity (SSCH) and emitting light detected in the FL-2 Channel. Lower: Histogram of fluorescent cells. Events to the right of the red line are considered positive, based on the level of fluorescence from the negative class-control, which is outlined in green. Florescence from the specific antibody is filled in purple

2.5.5 Detection of FITC-Annexin V as a marker of apoptosis

Apoptosis is a process of regulated physiological cell death (Kerr et al., 1972). Many physiological processes can drive apoptosis to help protect against harmful cell mutation, or as

part of the control mechanisms associated with embryonic development and tissue homeostasis. During the early stages of apoptosis, the membrane bound protein phosphotidyl serine is translocated from the inner to the outer plasma membrane. Annexin V is a Ca^{2+}-dependent phospholipid binding protein and can bind with a high affinity to phosphotidyl serine. Annexin V binding to phosphotidyl serine can therefore act as a marker of the early-stage of apoptosis, which can be detected with fluorophore-conjugated Annexin V. During the late stages of apoptosis, the plasma and nuclear membranes lose integrity, allowing the vital DNA-binding dye PI to gain access to DNA. This means that PI cannot discriminate between necrosis and apoptosis. However, dual staining of conjugated-Annexin V and PI is able to measure cell death (Annexin V+/PI+ cells) and apoptosis (Annexin V+/PI-).

Since necrotic processes may result in uncontrolled and unregulated cell death and lead to the loss of membrane integrity and cell lysis, necrotic cells can also be stained positive for Annexin V and PI. Therefore, this test cannot determine whether Annexin V+/PI+ cells are the result of apoptosis or necrosis, thus in the absence of time course experiments, only Annexin V+/PI- cells can be confidently considered to be apoptotic.

As the fluorescent spectra of FITC (conjugated to Annexin V) and PI have a high degree of overlap, a number of controls are required for flow cytometry analysis. Cells were incubated overnight with staurosporine, an inducer of apoptosis, as a positive control, while untreated cells were used as a negative control. Both treated and untreated cells with Annexin V/PI single staining were used to determine the cut-off for Annexin V positivity and PI positivity, while Annexin V/PI dual staining with treated and untreated cells was used to subsequently determine the level of compensation between the two fluorophores. This is done by manually altering the optimal detection wavelength of a detector to remove, or compensate for, spillover fluorescence being detected. PI is primarily detected by channel 1 (FL1), but spills over into FL2, which is the detector for FITC. Using the single stained controls, PI and FITC positive events were

placed into their respective quadrants using the compensation sliders in the CellQuest® Software. The PI/FITC dual stain was then used to further refine these settings.

2.5.6 Detection of cell viability

To examine cell adherence of cultured THP-1 cells following wash, Cell Counting Kit 8 (CCK-8, Sigma-Aldrich) was used to analyse cell viability as a proxy for cell adherence. As the differentiated THP-1 cells used in this assay were adherent, while undifferentiated cells were not, washing the wells removed all non-adherent cells and the CCK-8 assay could then be used to indirectly measure the relative amounts of remaining, differentiated adherent cells. CCK-8 is a colourimetric assay that relies on the activity of cell dehydrogenases to reduce water-soluble tetrazolium salt 8 (WST-8), producing an orange formazan product in the presence of viable cells. Although only viable cells will have dehydrogenase activity, one limitation of the assay is that stimuli or treatments that affect dehydrogenase activity could generate a false impression of viable cell number.

2.6 Data analysis

Appropriate statistical tests were applied to analyse experimental data collected in these studies. Statistical tests were performed using the following statistical software packages: UNPHASED version 3.0.12 (Dudbridge, 2008), IBM SPSS Statistic Version 23.0, SPSS SamplePower 2.0, GraphPad Prism 6 and Past3. Due to multiple testing, where appropriate the Bonferroni correction was applied. With each independent test within a hypothesis, the chance of a type I error increases by 5%, meaning that with two independent reported $p < 0.05$, the actual probability that differences observed are due to random chance is 10%, rather than the conventionally acceptable 5%. With three independent measures, this rises to 15%. With 10 independent tests, it becomes probable that at least one statistically significant result is due to

random chance, rather than the variable of interest. This can be corrected for by dividing the statistical cut-off (0.05) by the number of independent tests performed. Further details regarding the data analysis and justifications for the tests employed are described in individual Chapters of this thesis.

3. Differential Antibody Response Against Indigestible Gliadin Antigens in Patients with Schizophrenia

Results from this chapter have been published in *Translational Psychiatry* by the PhD Candidate (McLean et al., 2017)

3.1 Introduction

Schizophrenia is a complex psychiatric disorder, demonstrating heterogeneity in clinical presentation with a combination of positive, negative and cognitive symptoms (Tandon et al., 2013). The causes of schizophrenia remain unknown, but alterations of neuronal communication are believed to underlie the pathophysiology of the illness (Abi-Dargham et al., 1998; Maran et al., 2016; Perry et al., 1979; Wobrock et al., 2008). Due to the diversity of clinical presentation, differences in treatment response and variable epidemiology, it is likely that multifactorial mechanisms contribute to a spectrum of schizophrenic illnesses (Jablensky et al., 1992; Os, 2016b; Solanki et al., 2009).

A role for the consumption of gluten in the development of schizophrenia was initially proposed based on the observation of a positive correlation between national wheat imports and hospital admissions for schizophrenia (Dohan, 1966). Although the outcomes have been inconsistent, studies have attempted to examine the efficacy of gluten-free diets (GFD) in the treatment of schizophrenia, demonstrating improvement against clinical grading and earlier recovery in some patients treated with GFD (Dohan et al., 1969; Dohan and Grasberger, 1973; Jackson et al., 2014; Kalaydjian et al., 2006). Case studies in the literature have further demonstrated the induction of psychosis and schizophrenia-like symptoms in response to gluten challenge, as well as the resolution of these symptoms with GFD (Eaton et al., 2015b; Lionetti et al., 2015).

A mechanism by which gluten consumption may play a role in the development of schizophrenia has yet to be demonstrated. A number of immunological alterations have been found to be associated with schizophrenia, including an increase in pro-inflammatory cytokines and microglia activation (Bloomfield et al., 2015; Dickerson et al., 2015; Müller et al., 2015; Schwieler et al., 2015). Additionally, an increase in immunoglobulin (Ig) G and A classes against native gliadin, a major component of gluten, was previously observed in a proportion of patients with schizophrenia (Cascella et al., 2011; F. Dickerson et al., 2010; Jackson et al.,

2014; Jin et al., 2012; Lachance and McKenzie, 2014; Okusaga et al., 2013; Reichelt and Landmark, 1995) (Summarised in *Table 1.3*). The initiation of antibody production relies upon the recognition and presentation of antigens by the human leukocyte antigen class II (HLA-II) molecules and genome-wide association (GWA) studies revealed that the loci most strongly associated with schizophrenia reside in the HLA region (International Schizophrenia Consortium et al., 2009; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Shi et al., 2009).

The epitopes recognised by anti-native gliadin antibodies (AGAs) detected in schizophrenia may be different from those identified in coeliac disease. Of schizophrenia patients who were positive for AGA IgA, only 3.8% were positive for IgA against CD-specific gliadin-derived epitopes, compared to 12.2% of control subjects (Jin et al., 2012; Schwertz et al., 2004). Furthermore, patients pre-selected for high AGA levels did not display high levels of CD-specific serological markers, such as plasma antibodies against tissue transglutaminase (tTG) (Samaroo et al., 2010). Previous studies suggested that the pathogenic gluten fragments for CD were mainly derived from α-gliadin and γ5-gliadin (Shan et al., 2005, 2002), while immune reactivity to γ3-gliadin and its homologous sequence (NCBI accession AAQ6387) was associated with schizophrenia (Samaroo et al., 2010).

To date, all the tests for circulating AGAs in schizophrenia have been developed with mixtures of full-length native gliadins consisting of ~300 amino acid residues. Such a test would detect antibodies against not only linear epitopes, but also conformational epitopes that are unlikely to survive digestion in the gut. In this study, we measured plasma levels of IgG and IgA against indigestible peptide fragments derived from γ-gliadins and α-gliadins, which harbour HLA-II restricted epitopes, with an in-house enzyme-linked immunosorbent assay (ELISA) in individuals with schizophrenia and healthy controls. Circulating AGA levels in the case-control samples were also tested using commercially available ELISA kits. The

association between previously genotyped HLA-DR/DQ variants and levels of antibodies against gliadins was also examined.

3.2 Materials and methods

3.2.1 Subjects

A total of 405 archived plasma samples collected from patients with schizophrenia (n=169, 132 males and 37 females, aged 42.0 ± 13.3 years) and control subjects (n = 236, 159 males and 77 females, aged 44.7 ± 12.5 years), were used to examine the levels of circulating antibodies against gliadin-derived peptide antigens. These samples were collected through the University of Aberdeen in the period between 2003 and 2008, and had been stored long term at -80 °C without defrosting until they were aliquoted for antibody testing. All patients were diagnosed as having schizophrenia based on the DSM-IV criteria. Control subjects were recruited from a local population in the North of Scotland and screened for psychiatric disorders by a questionnaire and excluded (International Schizophrenia Consortium et al., 2008; 2009). No case samples were reported to have CD. Both case and control samples were collected in the same period and stored under the same conditions. Antipsychotic drugs used by schizophrenia patients at the time of sampling are listed in *Table 3.1*, with 128 patients taking a single antipsychotic drug, 14 taking more than one drug and 27 without medication details. All the subjects were classified as British Caucasian individuals and they all gave informed written consent to donate blood samples for research of the pathology of schizophrenia (available demographic information given in Chapter 2). This study (Research Ethics Council reference: 10/S0802/18) was approved by a local ethics committee and conformed to the provisions of the Declaration of Helsinki and its amendments.

Table 3.1 Medication information of patients with schizophrenia in this study cohort

Medication	**Patient (n)**	**Percentage (%)**	**Male (n)**	**Age** (mean ± SD)
Amisulpride	9	5.3	7	38.2 ± 15.4
Clozapine	39	22.8	30	37.4 ± 15.4
Flupenthixol	28	16.4	21	53.3 ± 9.7
Haloperidol	6	3.5	5	41.2 ± 11.5
Olanzapine	30	17.5	22	39.6 ± 11.9
Phenothiazine	17	9.9	16	45.5 ± 14.6
Quetiapine	9	5.3	8	48.8 ± 13.8
Risperidone	14	8.2	11	40.2 ± 12.7
Sulpiride	4	2.3	3	37.7 ± 22.8
Dual Medication	14	10.4	13	41.8 ± 8.2
Unknown	27	15.8	23	50.9±10.8

Available medication information of patients with schizophrenia, including patient number, percentage of the total number of patients taking a medication, number of males and mean age. Each medication group includes those individuals that were also taking another medication, therefore some patients were included twice.

3.2.2 Antigen selection

Based on previous literature suggesting immune responses to γ-gliadins in schizophrenia and α-gliadins in CD, the initial selection of gliadins was limited to these types (Samaroo et al., 2010; Shan et al., 2002a). All gliadin sequences of interest were retrieved from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) and analysed *in silico* to determine indigestible fragments using PeptideCutter software (Gasteiger et al., 2003). The linear peptide antigens used in this study were selected based upon the presence of computationally predicted HLA-II binding epitopes (Söllner et al., 2010; Wang et al., 2010). The resulting sequences were HLA-II restricted and did not contain cutting sites for pepsin, trypsin or chymotrypsin (*Table 2.1*). A 29-mer peptide (H-HAQLEGRLHDLPGCPREVQRGFAATLVTN-OH) derived from a maize protein (NCBI accession 1BFA_A) was used as control peptide for non-specific binding. All peptide antigens were synthesised by solid-phase chemistry with a purity of >95%

(Severn Biotech Ltd, Worcs, UK). A full list of peptides, sequence and parent molecule are given in Chapter 2 (Section 2.2)

3.2.3 In-house ELISA for plasma antibodies against anti-gliadin derived antigens

Each synthetic peptide was dissolved in 67% acetic acid to generate a 5 mg/ml stock solution and stored long-term at -20°C. The working solution was made by diluting the stock solution with phosphate-buffered saline (PBS)-based coating buffer (Sigma-Aldrich, Dorset, UK) to 10 µg/ml for both gliadin-derived antigens and the control antigen; 100 µl working solution was added to each well on Nunc-Immuno Maxisorp 96-well microtiter plates (Thermo Fisher Scientific, Loughborough, UK). Each plate was coated with two gliadin-derived antigens and the control peptide. After incubation at 4°C overnight, the plate was washed 3 times with wash buffer (Sigma-Aldrich, USA); 100 µl plasma samples were diluted 1:100 in assay buffer (PBS containing 1.5% BSA) for IgA assay and 1:150 for IgG assay, and then added to each sample well. The negative control (NC) wells contained 100 µl assay buffer only. Following incubation for 1.5 hours at room temperature, the plate underwent additional washing as described above, and was then incubated for 1.0 hour with 100 µl of peroxidase-conjugated goat antibodies raised against either human IgG (Abcam, Cambridge, UK) or human IgA (Sigma-Aldrich, USA) diluted 1:30000-50000 in assay buffer. The plate underwent additional washing steps; colour development was initiated by adding 100 µl Stabilized Chromogen (Life Technologies, Glasgow, UK) and terminated 20 min later with 50µl Stop Solution (Life Technologies, UK). The resulting colour change was measured as optical density (OD) at λ=450 nm with a reference wavelength of λ=620 nm on a microplate reader. An inter-assay deviation was estimated using quality control (QC) samples, which were pooled from 20-30 healthy control samples and tested on every 96-well plate; a coefficient of variation (CV%) was used to represent the reproducibility of the in-house ELISA. The CV% was expressed as the SD of the mean OD readings of the QC samples as a percentage of the mean OD readings of the QC samples.

Each sample was tested in duplicate. In order to reduce the effects of inter-plate variation, both case and control samples were tested in parallel on every plate. To reduce the interference from non-specific signals due to the passive absorption of various antibodies in plasma to 96-well microplates, a specific binding index (SBI) was introduced to express the relative levels of circulating antibodies against gliadin-derived fragments. SBI was calculated as follows: SBI= [OD gliadin – OD_{NC}] / [OD maize – OD_{NC}].

3.2.4 Testing of plasma antibodies against native gliadins

Plasma AGAs were assayed using commercially available kits for both IgG (Gliadin IgG ELISA Kit) and IgA (Gliadin IgA ELISA Kit) against the full-length native gliadin molecules (Omega Diagnostics, Cambridge, UK). All assays were performed according to manufacturer's instructions (http://www.omegadiagnostics.com/). In brief, plasma samples from healthy controls and patients with schizophrenia were diluted 1:100 in the provided sample diluent and 100 µl of the diluted sample, standards and negative controls were added to the ELISA plate, prior to 30-minute incubation at room temperature. The plate was washed in triplicate with the wash buffer provided (diluted 1:9 in distilled water) and 100 µl of peroxidase-conjugated antibody was dispensed into each well for 30 minutes at room temperature. Subsequently, the plate underwent four additional washes before incubation with 100 µl of TMB for 10 minutes, and the colour change reaction was halted with the addition of 100 µl of STOP solution. The OD was read at λ=450nm-620nm and the reading of each sample was normalised to the mean OD reading of four-well standards provided for qualitative analysis.

3.2.5 TaqMan® genotyping of HLA Variants

Index SNPs in strong linkage with HLA-alleles were genotyped by Dr Lorna Halley, according to the protocols outlined in section 2.4 and formed part of her doctoral thesis (Halley, 2014). The MHC-II tagging SNPs used in this study are detailed in *Table 3.2*.

Table 3.2 HLA-tagging SNPs used in this study

SNPs/HLA	Base change	Chromosomal Location	Haplotype tagged
rs6457614 DR1	G/T	6:32651900	DRB1*0101-DQA1*0101-DQB1*0501
rs3135388 DQ6.2	C/T	6:32413051	DRB1*1501-DQA1*01-DQB1*0602
rs2187668 DQ*2.5	A/G	6:32605884	DRB1*0301-DQA1*0501-DQB1*0201
rs7454108 DQ*8.1	G/T	6:32681483	DRB1*04-DQA1*0301-DQB1*0302

The details of HLA-tagging SNPs used in this study. Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley and the table is reproduced from Dr Halley's doctoral thesis (Halley, 2014).

3.2.6 Total IgA assay

Total IgA levels in plasma were measured using a commercially available kit, according to the manufacturer's instructions (Life Technologies). The ELISA plate was coated with a 100 µl solution of coating buffer containing the capture antibody, sealed and stored at 4°C overnight. Subsequently, the plate was washed twice using washing buffer (1 x PBS containing 0.05% Tween 20) and blocked for 2 hours at room temperature. Meanwhile, standards were prepared

by a 2-fold serial dilution to produce a standard curve at the following concentrations; 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.563 ng/mL and vehicle alone (blank). Plasma samples were serially diluted 10,000-fold with Assay Buffer A supplied by the manufacturer and 20 µl of each pre-diluted sample was added to each well after 80 µl Assay Buffer A supplied was added to each sample-well. Finally, 100 µl of each standard was added to the plate in duplicate and the plate was incubated at room temperature for 2 hours. After the plate was washed four times, 100 µl of detection antibody (diluted 1:250 in Assay Buffer B, provided) was added and incubated for 1 hour at room temperature. Following a final quadruple washing stage, the plate was incubated with 100 µl TMB at room temperature for 15 min before the colour change reaction was halted by adding 100 µl of STOP solution. The colour change reaction was visualised at λ=450-570 nm. The standard curve and sample concentrations were determined automatically by SkanIt Software Research Edition 4.1 (Thermo Fisher Scientific).

3.2.7 Data analysis

The Kolmogorov-Smirnov test failed to show a normal distribution of levels of antibodies against gliadin-derived fragments and AGAs in either the patient group or the control groups (*Table 3.3*), so the Mann-Whitney U test was applied to examine the differences in the levels of antibodies against gliadin-derived antigens and AGAs between the two groups. Due to multiple testing, the Bonferroni correction was applied to reduce the risk of type-I errors and $p<0.006$ was considered to be statistically significant, after accounting for Bonferroni correction.

Table 3.3. Distributions of anti-gliadin antibody levels in both case and control samples

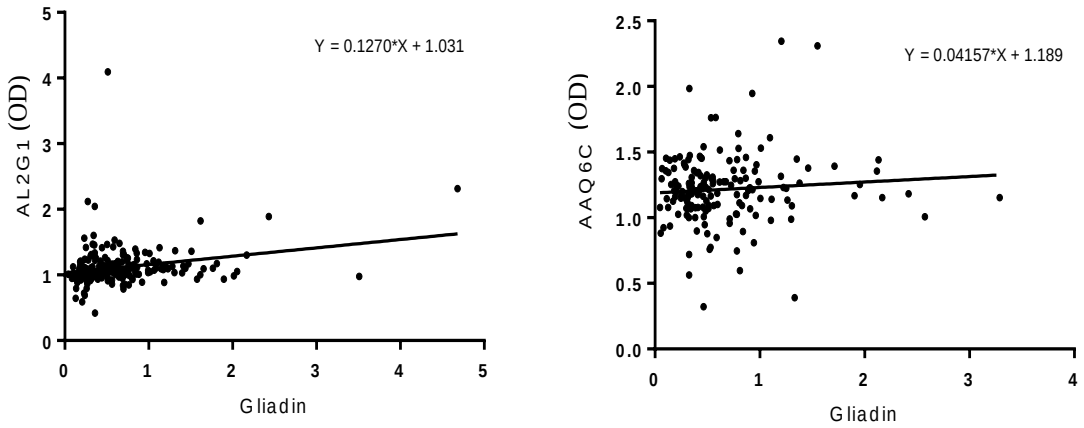
Antigen	IgG				IgA			
	Skewness	**Kurtosis**	**D Statistic**	**p**	**Skewness**	**Kurtosis**	**D Statistic**	**p**
Case								
AL1G1	0.05	6.9	0.13	**<0.001**	0.67	2.4	0.09	**<0.001**
AL2G1	5.37	48.1	0.19	**<0.001**	4.28	34.9	0.14	**<0.001**
AL2G2	7.50	89.1	0.20	**<0.001**	-0.24	0.6	0.04	**>0.100**
AAQ6A	4.15	24.0	0.23	**<0.001**	3.91	24.9	0.17	**<0.001**
AAQ6B	0.48	2.4	0.07	0.012	4.17	30.2	0.16	**<0.001**
AAQ6C	1.13	8.1	0.11	**<0.001**	0.24	1.0	0.05	>0.100
ABO3a	8.91	112.4	0.20	**<0.001**	6.42	53.0	0.25	**<0.001**
ABO3b	-1.88	9.9	0.09	**<0.001**	-0.28	1.1	0.05	>0.100
Gliadin	3.20	16.9	0.17	**<0.001**	1.62	3.8	0.14	**<0.001**
Control								
AL1G1	1.31	4.9	0.09	**0.001**	1.88	8.3	0.13	**<0.001**
AL2G1	0.83	4.4	0.13	**<0.001**	1.63	9.9	0.12	**<0.001**
AL2G2	1.11	16.8	0.15	**<0.001**	-2.32	14.8	0.12	**<0.001**
AAQ6A	4.40	21.9	0.29	**<0.001**	4.45	24.7	0.26	**<0.001**
AAQ6B	7.14	62.0	0.28	**<0.001**	3.90	22.4	0.19	**<0.001**
AAQ6C	0.59	4.6	0.10	**<0.001**	0.02	0.6	0.07	0.048
ABO3a	0.92	1.7	0.16	**<0.001**	2.13	10.9	0.12	**<0.001**
ABO3b	-1.32	7.2	0.09	**0.003**	0.17	4.7	0.06	>0.100
Gliadin	2.08	5.8	0.16	**<0.001**	3.37	15.1	0.18	**<0.001**

Kolmogorov-Smirnov test was applied to test the distribution of antibodies against gliadins in cases and controls. The majority of anti-gliadin antibodies showed a skewed distribution and so non-parametric tests were used to examine the differences in levels of antibodies against gliadins (p >0.006)

Receiver operating characteristic (ROC) curve analysis, which is a statistical method that can be employed to determine the validity of a medical test by plotting the true positive (condition positive and marker positive) against false positive (condition negative, marker positive). The output is given as a ROC curve, with the overall effectiveness represented by the area under the curve (AUC). The sensitivity (the true positive rate) can then be determined at a given specificity (false positive rate) (*Figure 3.4*). ROC analysis was applied to calculate the area under the curve (AUC) with calculation of the ELISA sensitivity against a specificity of ≥95%.

Linear regression was applied to examine which antipsychotic drugs might affect the secretion of circulating antibodies against gliadin-derived fragments and AGAs. Patients on medications were categorised into 9 subgroups based on antipsychotic drugs taken. In this analysis, the antibody levels were considered to be the dependent variable, and medication, age and sex were the independent variables. Based upon the assumption that a systemically administered drug would not alter the secretion of a single antibody, Fisher's combining probability test was applied to determine combined p-values based on nine drug-group tests for altered levels of plasma antibodies reacting with each antigen (Elston, 1991). Multivariate linear regression and the mann-whitney U test were applied to examine the correlations between the levels of AGAs and antibodies against gliadin-derived fragments in a combined schizophrenia-control group and cases and controls respectively. As some associations between AGA and antibodies against gliadin fragments were significant, albeit not particularly strong, examples of the two strongest associations are given and these are typical of the associations observed (*Figure 3.1*).

Figure 3.1 Examples of Regression Analysis between AGA and antibodies against indigestible gliadin fragments



Linear regression was used to examine the relationship between levels of antibodies against gliadin fragments and levels of antibodies against native gliadin. Examples of the correlation between AGA and levels of IgG against two gliadin-derived fragments, AL2G1 and AAQ6C.

The Mann-Whitney U test was used to examine the link between genotype and anti-gliadin antibodies. Due to the low frequency of the minor allele homozygotes (2/2) of SNPs

genotyped in this study, the given false variables were created with the major allele homozygote (1/1) denoted by 1, while the combination of heterozygotes (1/2) and minor allele homozygotes was denoted by 2. Bonferroni correction was applied to reduce the type-I errors due to multiple testing and $p < 0.006$ was determined to be statistically significant. In order to examine a genotype effect on the levels of AGAs, cases and controls were grouped together and then split by case and control in order to examine state-specific associations between genotype and AGAs.

In order to further evaluate the relationship between HLA-alleles and plasma anti-gliadin antibody levels, a robust regression analysis was performed with antibody levels as the dependent variable and genotypes as the independent variable, inputted as false variables based on the number of minor alleles present, i.e. 0, 1 or 2. As neither antibody levels nor residuals in the linear models showed a normal distribution, the robust regression was performed using bootstrapping (n=1999 tests). Robust regression analysis was performed using the statistical software Past3.

3.3 Results

3.3.1 Levels of circulating antibodies against indigestible gliadin-derived fragments

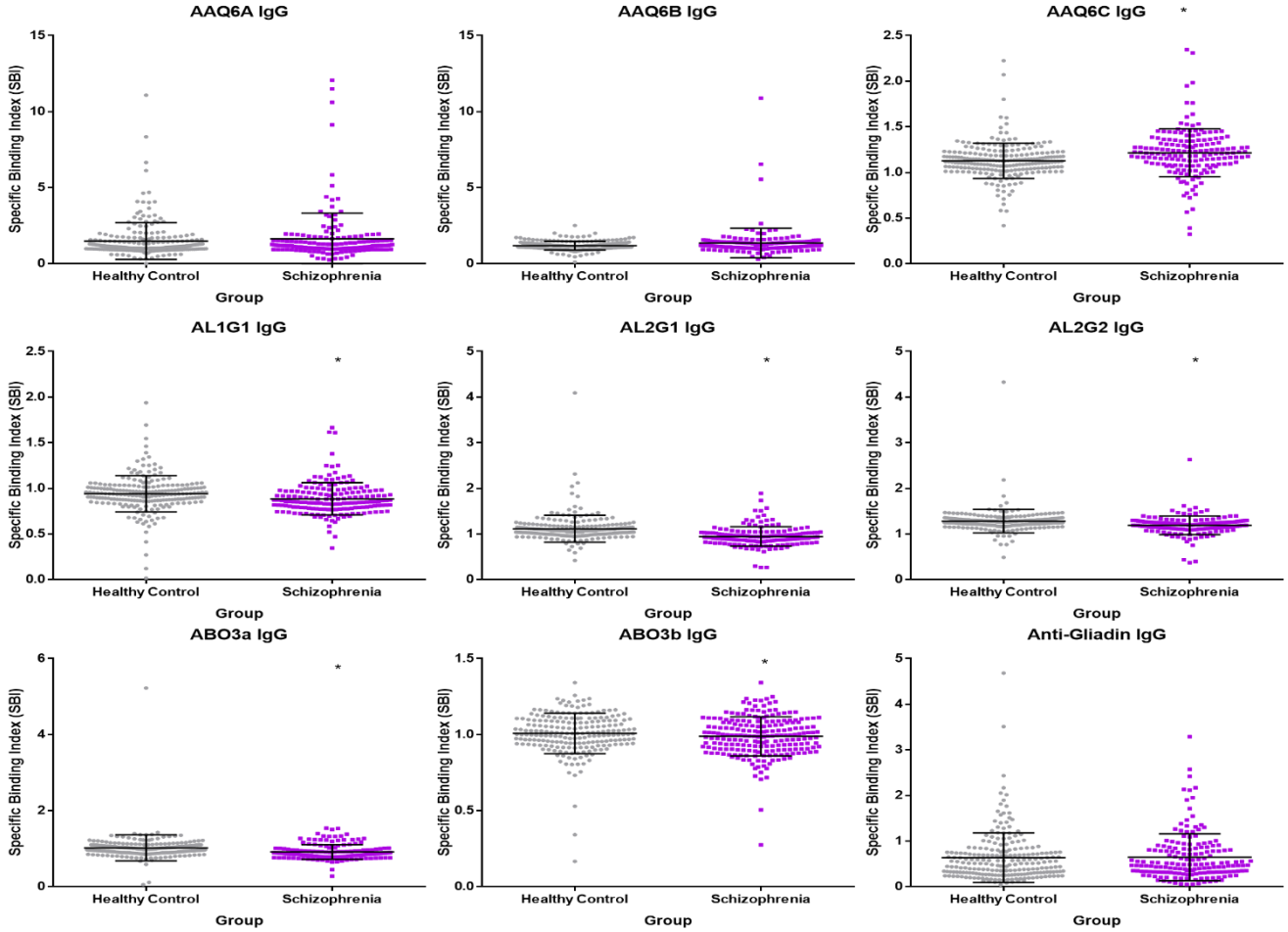
As shown in *Table 3.4* and *Figure 3.2*, patients with schizophrenia had significantly higher levels of plasma anti-AAQ6C IgG than control subjects ($Z= -4.65$, $p < 0.001$), but significantly lower levels of IgG antibodies against AL1G1 ($Z= -4.65$, $p < 0.001$) AL2G1 ($Z= -8.72$, $p < 0.001$), AL2G2 ($Z= -6.01$, $p < 0.001$), ABO3a ($Z= –6.37$, $p < 0.001$) and ABO3b ($Z= -5.32$, $p < 0.001$).

Table 3.4. The levels of plasma IgG against indigestible gliadin derived fragments

Antigen	Control			Case			Z	P
	n	Median	IQR	n	Median	IQR		
AL1G1	218	0.940	0.157	169	0.855	0.189	-4.65	**<0.001**
AL2G1	224	1.047	0.174	167	0.922	0.181	-8.72	**<0.001**
AL2G2	224	1.280	0.140	167	1.205	0.150	-6.01	**<0.001**
AAQ6A	224	1.100	0.674	167	1.192	0.548	-1.19	0.264
AAQ6B	222	1.110	0.353	167	1.202	0.377	-2.72	0.0066
AAQ6C	223	1.117	0.186	167	1.224	0.267	-4.65	**<0.001**
ABO3a	211	0.987	0.190	161	0.866	0.200	-6.37	**<0.001**
ABO3b	211	1.002	0.150	161	0.950	0.130	-5.32	**<0.001**

Mann-Whitney U test was applied in order to examine statistical differences in the levels of IgG against indigestible gliadin fragments between schizophrenia cases and control individuals. Levels of IgG against AL1G1, AL2G1 and AL2G2 were significantly lower in patients with schizophrenia when compared to controls (p= <0.001). Levels of IgG against AAQ6A and AAQ6B were not significantly different between the groups (=>0.006), while levels of IgG against AAQ6C were significantly elevated in patients with schizophrenia when compared to control individuals (Z= -4.65, p= <0.001). Levels of anti-ABO3 and anti-ABO3b IgG were significantly lower in patients with schizophrenia when compared to control individuals (p= <0.001). Due to multiple testing the Bonferroni correction was applied to set p <0.006 for statistical significance.

Figure 3.2 Comparison of plasma anti-gliadins IgG levels in control subjects and patients with schizophrenia



Levels of IgG against Gliadins in control individuals and patients with schizophrenia. Levels of IgG against AAQ6C were significantly higher in patients with schizophrenia (p= <0.001), while levels of IgG against AL1G1, AL2G1, AL2G2, ABO3a and ABO3b were significantly lower in the schizophrenia group (p= >0.006). Levels of IgG against native gliadins was not significantly different between the two groups (p = >0.05). Case samples are labelled purple.

As shown in *Table 3.5* and *Figure 3.3* circulating levels of IgA against gliadin-derived fragments were all significantly lower in the patient group than the control group (p <0.006), with the exception of anti-AAQ6B IgA (Z= -3.02, p=0.026).

Table 3.5. The levels of plasma IgA against indigestible gliadin derived fragments

Antigen		Control			Case		Z	P
	n	Median	IQR	n	Median	IQR		
AL1G1	222	0.830	0.070	166	0.804	0.080	-4.17	**<0.001**
AL2G1	222	0.965	0.090	166	0.913	0.090	-7.09	**<0.001**
AL2G2	222	1.005	0.060	166	0.973	0.060	-7.2	**<0.001**
AAQ6A	224	1.015	0.150	167	0.928	0.110	-7.28	**<0.001**
AAQ6B	224	0.906	0.100	167	0.885	0.080	-3.02	0.026
AAQ6C	222	1.016	0.100	166	0.965	0.100	-6.82	**<0.001**
ABO3a	221	1.029	0.070	166	0.935	0.060	-12.51	**<0.001**
ABO3b	221	0.919	0.040	166	0.871	0.060	-10.29	**<0.001**

Mann-Whitney U test was applied in order to examine statistical differences in the levels of IgA against indigestible gliadin fragments between schizophrenia cases and control individuals. Levels of IgG against AL1G1, AL2G1, AL2G2 and AAQ6A were significantly lower in patients with schizophrenia when compared to controls (p= <0.001). Levels of IgG against AAQ6B were not significantly different between the groups (p= 0.026), while levels of IgG against AAQ6C, ABO3 and ABO3b were significantly lower in patients with schizophrenia when compared to control individuals (p= <0.001). Due to multiple testing the Bonferroni correction was applied to set p <0.006 for statistical significance.

3.3.2 Levels of circulating Total IgA

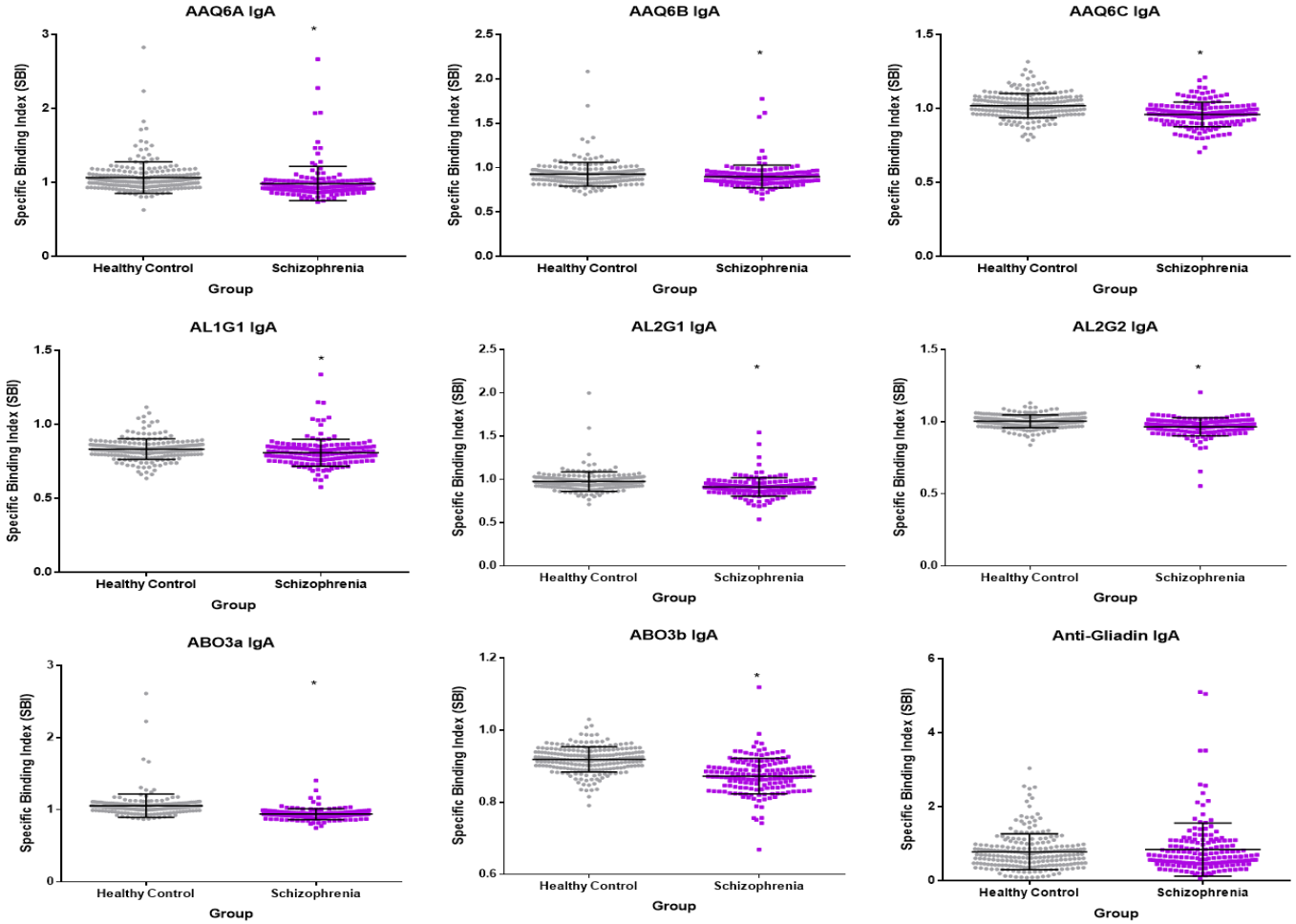
Table 3.6. Total IgA levels are not altered in patients with schizophrenia

	Control			Case			Z	P
	n	Median ng/mL	IQR	n	Median ng/mL	IQR		
Total IgA	225	29.925	31.620	167	30.830	29.410	-0.595	0.552

Mann-Whitney U test was applied in order to examine statistical differences in the levels of total IgA between patients with schizophrenia and control individuals. No significant difference in the level of total IgA was observed between healthy controls and patients with schizophrenia (p >0.05).

In order to test if reduced levels of IgA against gliadin peptides was related to decreases in total IgA, total IgA was measured using ELISA. As shown in *Table 3.6*, the total IgA levels in plasma showed no significant difference between healthy controls and patients with schizophrenia (Z= -0.595, p= 0.552).

Figure 3.3 Graphs of the levels of IgA against Gliadins in controls individuals and patients with schizophrenia



Levels of IgA against Gliadins in control individuals and patients with schizophrenia. Levels of IgA against all linear peptides were significantly lower in patients with schizophrenia (p= <0.006). Levels of IgA against native gliadins was not significantly different between the two groups (p = >0.05). Case samples are labelled purple.

3.3.3 Levels of circulating AGAs

As shown in *Table 3.7*, *Figure 3.2* and *Figure 3.3* there was no significant difference in the levels of plasma AGAs, either IgG (Z= -0.31, p= 0.757) or IgA (Z= -0.22, p= 0.825) against native gliadins, between the patient group and the control group.

Table 3.7. The levels of plasma antibodies against native gliadin.

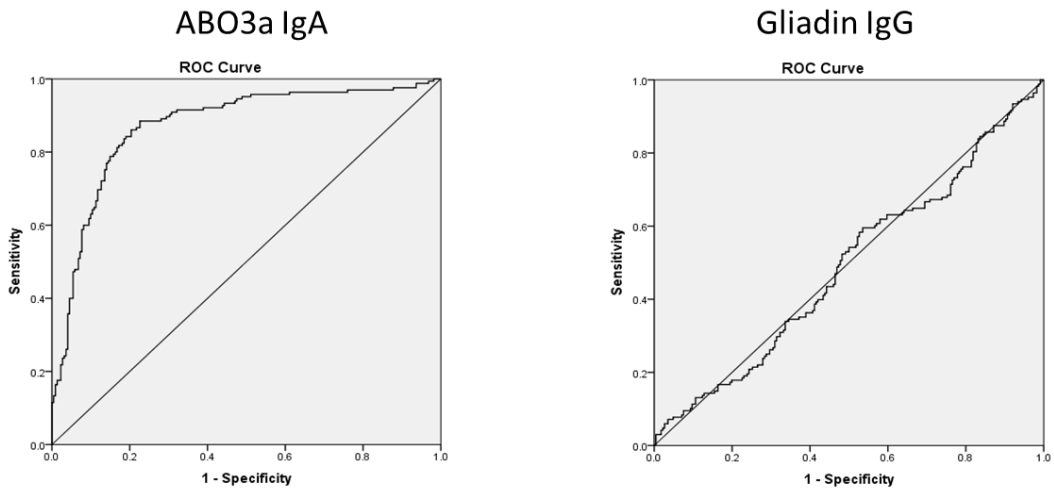
Antibody Class	Control			Case			Z	P
	n	Median	IQR	n	Median	IQR		
AGA IgG	226	0.511	0.470	168	0.477	0.500	-0.31	0.757
AGA IgA	223	0.675	0.510	167	0.647	0.600	-0.22	0.825

Mann-Whitney U test was used in order to examine statistical differences in the levels of AGA-IgG and AGA-IgA in schizophrenia cases and control individuals. There was no significant difference in the levels of *plasma* AGA, IgG or IgA, between healthy controls and patients with schizophrenia (p >0.05).

3.3.4 ROC curve analysis

ROC curve analysis revealed that at a specificity of ≥95% (*Table 3.8*), five assays had a sensitivity of >20%, including anti-AAQ6C IgG assay (20.4%, AUC=0.647), anti-AL2G1 IgG assay (30.7%, AUC=0.758), AL2G2 IgA assay (20.2%, AUC=0.712), anti-ABO3a IgA assay (40.0%, AUC=0.871) and anti-ABOb IgA assay (35.8%, AUC=0.805).

Figure 3.4 ROC Curve Examples



Examples of ROC curves. Based upon Area under the Curve (AUC), the ABO3a IgA (AUC 0.871) test is able to distinguish patients with schizophrenia from control subjects, while the Gliadin IgG test (AUC 0.510) is not. An AUC of 1 is the hypothetical ideal, and would represent a test that is perfectly accurate, while an AUC of 0.5 is statistically and practically useless.

Table 3.8. ROC curve analysis of plasma anti-gliadin antibody levels

Antibody Test	Specificity (%)	Sensitivity (%)	AUC	SE	p	95% CI
IgG						
AAQ6A	95.1	6.0	0.54	0.03	0.243	0.48-0.53
AAQ6B	95.0	8.4	0.58	0.03	0.008	0.52-0.64
AAQ6C	95.1	20.4	0.65	0.03	<0.001	0.59-0.70
AL1G1	95.0	4.2	0.64	0.03	<0.001	0.58-0.70
AL2G1	95.1	30.7	0.76	0.03	<0.001	0.71-0.81
AL2G2	95.1	15.0	0.68	0.03	<0.001	0.63-0.73
ABO3a	95.3	13.8	0.69	0.03	<0.001	0.64-0.75
ABO3b	95.3	7.5	0.66	0.03	<0.001	0.61-0.72
Gliadin	95.1	5.4	0.51	0.03	0.757	0.43-0.55
IgA						
AAQ6A	95.1	18.0	0.72	0.03	<0.001	0.67-0.77
AAQ6B	95.1	6.6	0.59	0.03	0.002	0.53-0.65
AAQ6C	95.0	16.4	0.70	0.03	<0.001	0.65-0.75
AL1G1	95.0	10.9	0.62	0.03	<0.001	0.57-0.68
AL2G1	95.0	14.5	0.71	0.03	<0.001	0.66-0.76
AL2G2	95.0	20.2	0.71	0.03	<0.001	0.66-0.76
ABO3a	95.0	40.0	0.87	0.02	<0.001	0.83-0.91
ABO3b	95.0	35.8	0.81	0.02	<0.001	0.76-0.85
Gliadin	95.1	6.6	0.49	0.03	0.825	0.44-0.55

ROC analysis showed that antibody levels for most of the gliadin derived fragments might be predictive of schizophrenia ($p<0.006$). AUC= Area under the Curve, SE= Standard Error, CI= Confidence Interval

3.3.5 Effects of antipsychotic medication on antibody secretion

Linear regression analysis demonstrated that quetiapine (n=9) was the only antipsychotic drug significantly associated with elevated levels of plasma anti-AAQ6B IgG (adjusted r^2 =0.065, t=3.13, p=0.002), while all eight other antipsychotic drugs did not show a significant association with anti-gliadin IgG levels. Fisher's combining probability suggested that no antipsychotic medication had an overall effect on the secretion of IgG against gliadin-derived fragments.

Table 3.9 *Linear regression analysis of the relationship between antipsychotic drugs and the secretion of plasma IgG against gliadin-derived fragments*

Drug	**Regression**	**AAQ6A** (n=167)	**AAQ6B** (n=167)	**AAQ6C** (n=167)	**AL1G1** (n=169)	**AL2G1** (n=167)	**AL2G2** (n=167)	**ABO3a** (n=161)	**ABO3b** (n=161)	**Combined p-value**
Amisulpride	Adj r^2	0.002	0.006	0.016	-0.007	0.032	-0.003	0.006	0.019	0.460
	t	1.166	-0.394	-0.424	-0.868	2.159	0.055	-1.546	-1.072	
	p	0.246	0.694	0.672	0.387	0.032	0.956	0.124	0.286	
Clozapine	Adj r^2	0.001	0.014	0.019	-0.012	0.003	-0.003	-0.010	0.013	0.961
	t	1.119	-1.167	-0.833	-0.221	0.029	0.088	0.123	-0.509	
	p	0.265	0.245	0.406	0.825	0.977	0.930	0.903	0.612	
Flupenthixol	Adj r^2	-0.002	0.008	0.02	-0.012	0.003	0.007	-0.008	0.015	0.997
	t	0.914	-0.594	-0.884	0.186	0.205	-1.252	0.534	0.713	
	p	0.362	0.553	0.378	0.852	0.838	0.213	0.594	0.477	
Haloperidol	Adj r^2	-0.005	0.005	0.016	-0.012	0.003	0.001	-0.010	0.016	0.999
	t	-0.597	-0.092	0.441	-0.051	0.26	-0.826	-0.121	-0.830	
	p	0.551	0.927	0.660	0.960	0.795	0.410	0.904	0.408	
Olanzapine	Adj r^2	0.006	0.006	0.021	-0.006	0.003	0.006	-0.006	0.011	0.988
	t	-1.424	0.345	1.010	1.008	-0.084	1.207	-0.764	-0.101	
	p	0.157	0.730	0.314	0.315	0.933	0.229	0.446	0.919	
Phenothiazine	Adj r^2	-0.007	0.005	0.017	-0.012	0.006	-0.003	-0.007	0.024	0.998
	t	-0.033	-0.090	-0.568	0.246	-0.681	0.026	0.689	1.411	
	p	0.974	0.928	0.571	0.806	0.497	0.979	0.492	0.16	
Quetiapine	Adj r^2	0.001	0.065	0.023	-0.012	0.011	-0.001	-0.01	0.012	0.250
	t	-1.096	3.132	1.160	0.199	-1.162	0.596	-0.069	-0.239	
	p	0.275	**0.002**	0.248	0.842	0.247	0.552	0.945	0.812	
Risperidone	Adj r^2	-0.007	0.018	0.019	0.006	0.012	0.017	-0.005	0.012	0.399
	t	0.167	1.408	-0.876	-1.694	-1.196	-1.803	0.867	0.276	
	p	0.868	0.161	0.382	0.092	0.234	0.073	0.387	0.783	
Sulpiride	Adj r^2	-0.002	0.019	0.043	-0.011	0.012	0.035	-0.008	0.016	0.218
	t	-0.901	-1.467	-2.135	0.367	-1.206	-2.498	-0.591	-0.827	
	p	0.369	0.144	0.034	0.714	0.230	**0.014**	0.556	0.41	

Regression analysis between levels of IgG against gliadins (n) and medication status in patients with schizophrenia. Fisher's combining probability test revealed that the secretion of plasma IgG against gliadin-derived fragments was not associated with any of the nine antipsychotic medications listed in *Table 3.1* (combined $p<0.05$). Regression analysis revealed that may be predictive of increased levels of anti-AAQ6B IgG ($p= 0.002$). For the combined probability $p<0.05$ was used as the statistical cut-off, while for the individual regression analyses $p<0.006$ was used as the cut-off for statistical significance.

Table 3.10. Linear regression analysis of the relationship between antipsychotic drugs and the secretion of plasma IgA against gliadin-derived fragments

Drug	**Regression**	**AAQ6A** (167)	**AAQ6B** (167)	**AAQ6C** (166)	**AL1G1** (166)	**AL2G1** (166)	**AL2G2** (166)	**ABO3a** (166)	**ABO3b** (166)	**Combined p-value**
Amisulpride	Adj r^2	-0.015	-0.015	-0.009	-0.013	-0.006	-0.003	-0.002	-0.011	0.746
	t	0.182	0.771	-0.592	-0.774	0.441	-0.586	-1.298	-0.884	
	p	0.856	0.442	0.555	0.440	0.660	0.558	0.196	0.378	
Clozapine	Adj r^2	-0.015	-0.005	-0.011	-0.017	-0.007	-0.005	-0.012	-0.015	0.966
	t	-0.376	1.487	0.217	0.155	0.213	-0.102	0.428	-0.392	
	p	0.707	0.139	0.828	0.877	0.832	0.919	0.669	0.695	
Flupenthixol	Adj r^2	0.001	-0.004	-0.009	-0.009	-0.002	-0.002	0.001	-0.015	1.000
	t	-1.610	-1.523	-0.519	-1.052	-0.908	-0.647	-1.485	-0.391	
	p	0.109	0.130	0.604	0.294	0.365	0.518	0.140	0.696	
Haloperidol	Adj r^2	-0.016	-0.012	0.023	-0.017	-0.003	0.007	-0.013	-0.003	0.247
	t	-0.079	-1.071	2.321	0.119	0.824	1.372	0.042	1.391	
	p	0.937	0.286	0.022	0.905	0.411	0.172	0.967	0.166	
Olanzapine	Adj r^2	-0.016	-0.019	-0.010	-0.011	0.002	0.000	-0.009	-0.012	1.000
	t	-0.009	-0.034	-0.414	0.935	1.219	0.870	0.768	0.744	
	p	0.993	0.973	0.680	0.351	0.225	0.385	0.443	0.458	
Phenothiazine	Adj r^2	-0.006	-0.014	-0.011	-0.014	-0.003	0.000	0.001	-0.015	1.000
	t	1.229	0.890	0.156	-0.676	0.806	0.824	1.485	0.253	
	p	0.221	0.375	0.876	0.500	0.422	0.411	0.14	0.800	
Quetiapine	Adj r^2	0.006	-0.016	-0.011	-0.011	-0.007	-0.005	-0.011	-0.016	1.000
	t	1.853	-0.663	0.209	0.911	0.097	-0.093	0.633	0.064	
	p	0.066	0.508	0.835	0.363	0.923	0.926	0.528	0.949	
Risperidone	Adj r^2	-0.014	-0.014	-0.010	-0.016	0.008	-0.001	-0.013	-0.014	1.000
	t	0.478	0.888	0.362	-0.244	-1.540	-0.800	-0.046	0.432	
	p	0.634	0.376	0.718	0.807	0.126	0.425	0.964	0.666	
Sulpiride	Adj r^2	-0.014	-0.018	-0.007	0.007	-0.006	0.000	-0.011	-0.016	1.000
	t	-0.529	-0.401	-0.818	-1.914	-0.430	-0.862	0.624	-0.015	
	p	0.598	0.689	0.415	0.057	0.668	0.390	0.534	0.988	

Regression analysis between levels of IgA against gliadins (n) and medication status in patients with schizophrenia. Fisher's combining probability suggested that there was no association between medication and the levels of plasma IgA against gliadin-derived fragments (combined $p>0.05$). For the combined probability $p<0.05$ was used as the statistical cut-off, while for the individual regression analyses $p<0.006$ was used as the cut-off for statistical significance.

Table 3.11. Linear regression analysis of the relationship between antipsychotic drugs and plasma AGA levels

Drug	**Regression**	**AGA IgG** (168)	**AGA IgA** (167)
Amisulpride	Adj r^2	0.014	0.013
	t	0.201	2.171
	p	0.841	**0.031**
Clozapine	Adj r^2	0.017	0.001
	t	-0.809	-1.679
	p	0.420	0.095
Flupenthixol	Adj r^2	0.018	-0.014
	t	-0.832	-0.647
	p	0.407	0.519
Haloperidol	Adj r^2	0.017	-0.011
	t	-0.723	0.991
	p	0.471	0.323
Olanzapine	Adj r^2	0.014	-0.017
	t	0.215	-0.143
	p	0.830	0.886
Phenothiazine	Adj r^2	0.017	-0.015
	t	-0.755	0.517
	p	0.452	0.606
Quetiapine	Adj r^2	0.018	-0.016
	t	-0.866	0.357
	p	0.388	0.721
Risperidone	Adj r^2	0.019	-0.017
	t	0.867	0.001
	p	0.387	0.999
Sulpiride	Adj r^2	0.014	-0.009
	t	-0.323	-1.087
	p	0.747	0.279

Regression analysis of the relationship between antipsychotic medication and AGA. Only Amisulpride was predictive of increased levels of plasma levels of AGAs in patients with schizophrenia (n= 9, t= 0.201, p= 0.031).

Linear regression analysis showed that antipsychotic medication was not associated levels of plasma AGAs, either IgA or IgG against native gliadins, with the exception of Amisulpride, which was associated with increased levels of AGA IgA (*Table 3.11*).

3.3.6 Relationship between antibodies against gliadin-derived fragments and AGAs

Multivariate linear regression analysis revealed a significant correlation between plasma levels of AGA IgG levels and the IgG against gliadin-derived fragments (*Table 3.12*).

Table 3.12 Regression analysis of the correlation between the AGA IgG levels and the IgG levels for gliadin-derived fragments

Group	r	adj. r2	df	F	p	Antibody	Coefficient β	Standard Error	Standardised Coefficient β	p
Control	0.311	0.059	8	2.540	**0.012**	AL1G1 IgG	0.040	0.210	0.020	0.832
						AL2G1 IgG	0.370	0.130	0.200	**0.004**
						AL2G2 IgG	-0.070	0.150	-0.030	0.633
						AAQ6A IgG	0.060	0.030	0.140	0.051
						AAQ6B IgG	-0.170	0.150	-0.090	0.257
						AAQ6C IgG	0.370	0.240	0.130	0.129
						ABO3a IgG	0.180	0.120	0.110	0.135
						ABO3b IgG	-0.310	0.340	-0.080	0.363
Case	0.230	0.002	8	1.038	0.411	AL1G1 IgG	-0.230	0.240	-0.080	0.333
						AL2G1 IgG	0.140	0.220	0.060	0.524
						AL2G2 IgG	0.120	0.230	0.050	0.609
						AAQ6A IgG	-0.020	0.030	-0.060	0.474
						AAQ6B IgG	0.000	0.050	0.000	0.963
						AAQ6C IgG	0.340	0.160	0.170	**0.037**
						ABO3a IgG	0.390	0.300	0.140	0.202
						ABO3b IgG	-0.800	0.490	-0.180	0.103

Regression analysis was used in order to examine the associations between levels of IgG against gliadin-derived fragments and levels of AGA-IgG. Anti-AL2G1 IgG was the most predictive of AGA IgG in control subjects (p= 0.004), while anti-AAQ6C IgG was the most predictive in patients with schizophrenia (p= 0.037). Levels of IgG against gliadin-derived fragments were predictive of levels of AGA-IgG in the control group (p= 0.012) but not in cases (p= 0.411). ($p < 0.05$).

In the control group, plasma levels of antibodies against gliadin-derived fragments were predictive of AGA levels (F= 2.540, df= 8, p=0.012), although overall IgG levels for gliadin-derived fragments accounted for only 5.9% of AGA variation. Anti-AL1G1 IgG level was the best predictor of AGA IgG level out of all IgG against gliadin-derived fragments tested in the control group (Standardised β= 0.20, p=0.004).

In the case group, plasma levels of AGA IgG were not dependent on the IgG levels for gliadin-derived fragments (df= 8, F= 1.038, p= 0.411), although anti-AAQ6C IgG levels made a significant contribution to AGA IgG levels in the patient group (Standardised β= 0.17, p=0.037).

Table 3.13. Regression analysis between the AGA IgA levels and the IgA levels for gliadin-derived fragments

Group	r	adj. r2	df	F	p	Antibody	Coefficient β	Standard Error	Standardised Coefficient β	p
Control	0.297	0.053	8	2.545	**0.011**	AL1G1 IgA	1.690	0.490	0.250	**0.001**
						AL2G1 IgA	0.060	0.320	0.010	0.851
						AL2G2 IgA	-0.080	0.870	-0.010	0.925
						AAQ6A IgA	0.140	0.160	0.060	0.394
						AAQ6B IgA	-0.280	0.330	-0.060	0.385
						AAQ6C IgA	-0.460	0.430	-0.080	0.287
						ABO3a IgA	0.390	0.210	0.130	0.060
						ABO3b IgA	-0.430	1.090	-0.030	0.693
Case	0.231	0.005	8	1.101	0.365	AL1G1 IgA	0.700	0.710	0.090	0.328
						AL2G1 IgA	0.360	0.810	0.050	0.661
						AL2G2 IgA	-1.290	1.120	-0.110	0.249
						AAQ6A IgA	-0.450	0.370	-0.140	0.221
						AAQ6B IgA	0.220	0.650	0.030	0.742
						AAQ6C IgA	0.030	0.810	0.010	0.973
						ABO3a IgA	2.200	0.930	0.230	**0.019**
						ABO3b IgA	-1.490	1.470	-0.100	0.313

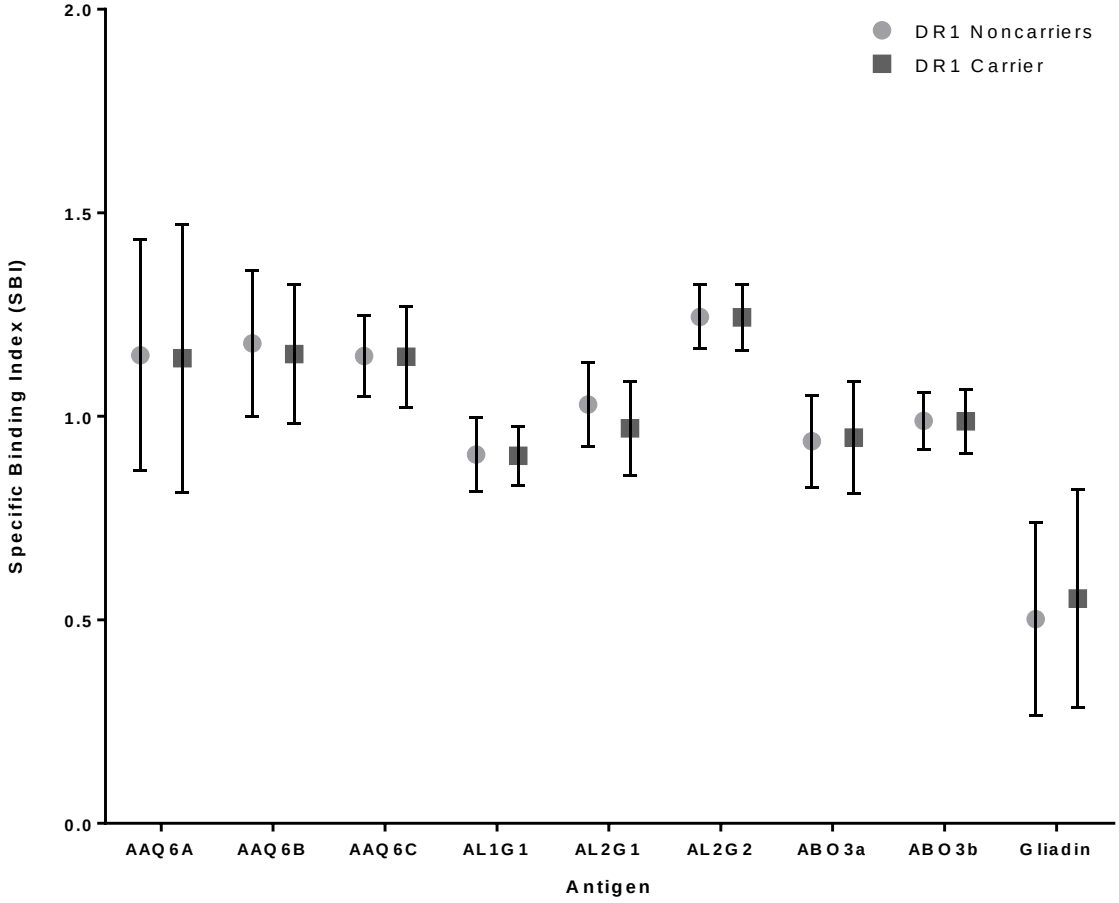
Regression analysis was used in order to examine the associations between levels of IgG against gliadin-derived fragments and levels of AGA-IgG.Anti-AL1G1 IgA level was the most predictive of AGA IgA level in control subjects (p= <0.001), while anti-ABO3a IgA level was most predictive of AGA IgA level in schizophrenia cases (p=0.019). Levels of IgA against gliadin derived fragments were predictive of levels of AGA-IgA in control individuals only (p= 0.011). The cut-off for statistical significance was p <0.05.

Plasma levels of AGA IgA were significantly dependent upon the levels of IgA against gliadin-derived fragments in healthy controls (df= 8, F= 2.545, p= 0.011), although the contribution of IgA against gliadin-derived fragments to AGA IgA levels was small. Plasma anti-AL1G1 IgA levels were the significant contributor to AGA IgA levels in healthy controls (Standardised β= 0.250, p= 0.001). In patients with schizophrenia, plasma AGA IgA levels were not dependent on the IgA levels for gliadin-derived fragments (df= 8, F= 1.101, p= 0.365), although plasma anti-ABOa IgA levels were found to be a significant contributor to the AGA IgA levels in patients with schizophrenia (Standardised β= 0.230, p= 0.019) (*Table 3.13*).

3.3.7 Association between HLA variants and plasma anti-gliadin antibody levels

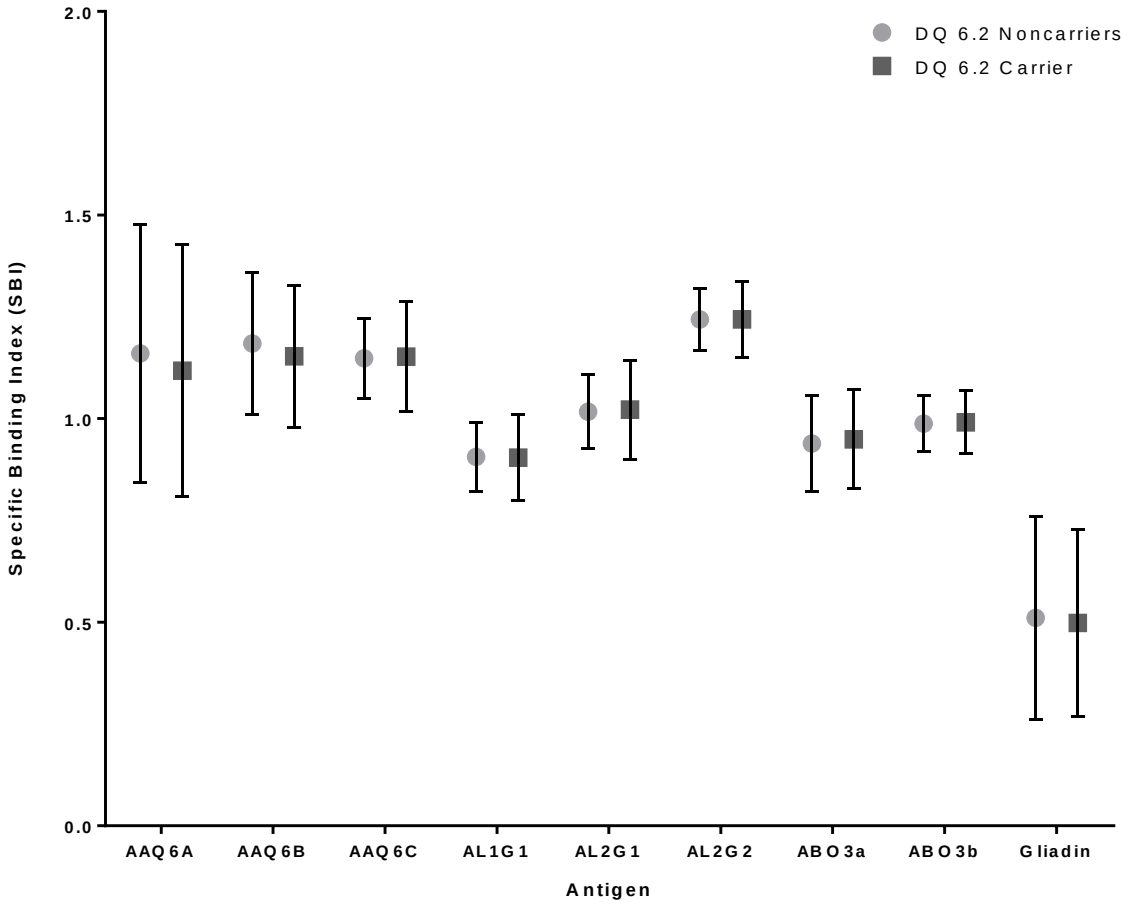
The association between plasma anti-gliadin antibodies and HLA-polymorphisms was examined. In this analysis, cases and controls were grouped together and the genotyping data were split into the presence or absence of a variant of interest. As shown in *Figure 3.5 - Figure 3.9*, there was no significant difference in plasma anti-gliadin IgG levels between the carriers and none carriers of the variants of interest as all statistical tests failed to survive the Bonferroni correction for multiple tests (p>0.006).

Figure 3.5 Comparison of plasma anti-gliadin IgG levels in the DR1 carriers and non-DR1 carriers



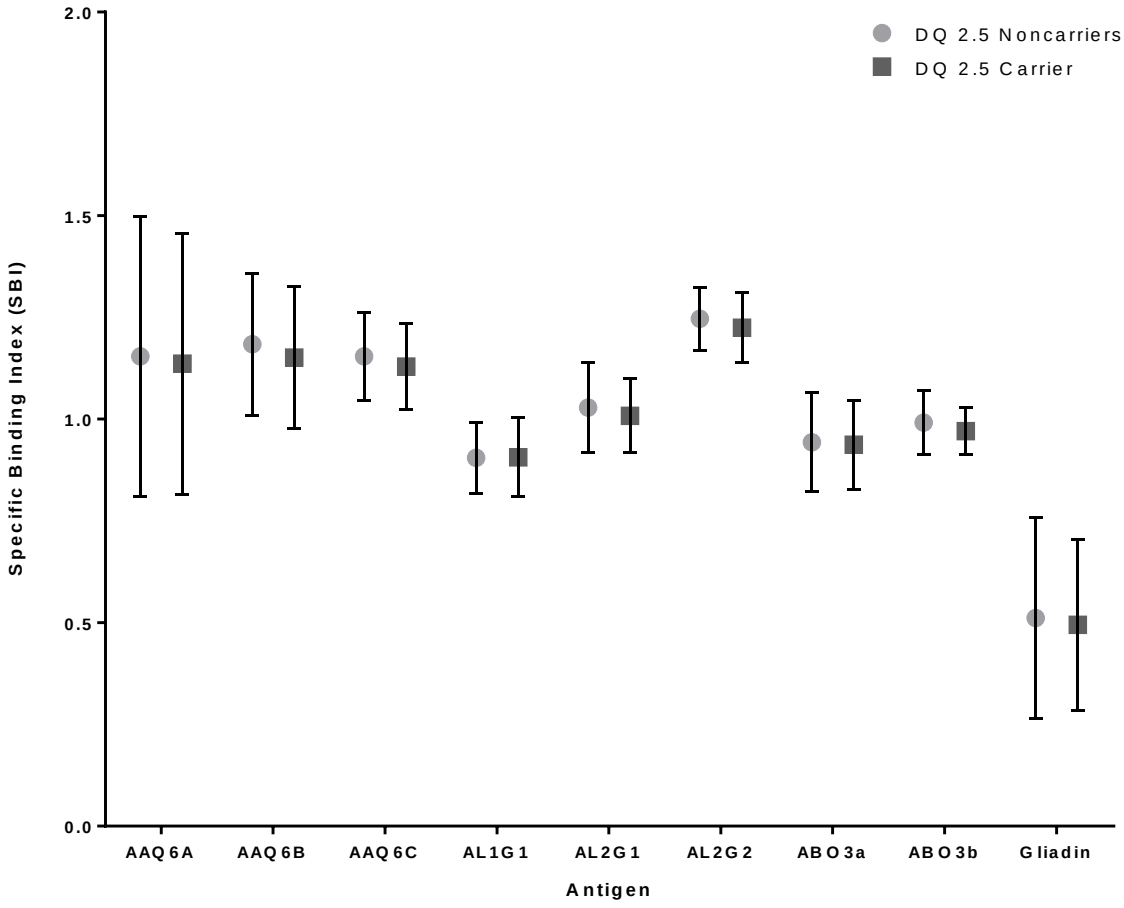
Median and IQR of levels of IgG against gliadins in carriers and non-carriers of DR1. No significant differences were observed in plasma anti-AAQ6A IgG levels between the DR1 carriers (n=77) and non-DR1 carriers (n= 293), anti-AAQ6B IgG levels between carriers (n= 76) and non-carriers (n= 291), anti-AAQ6C IgG levels between carriers (n= 76) and non-carriers (n= 292), anti-AL1G1 IgG levels between carriers (n= 74) and non-carriers (n=291), anti-AL2G1 IgG levels between carriers (n= 75) and non-carriers (n= 294), anti-AL2G2 IgG levels between carriers (n= 75) and non-carriers (n= 294), anti-ABO3a IgG levels between carriers (n= 67) and non-carriers (n= 285), anti-ABO3b IgG levels between carriers (n= 67) and non-carriers (n=285) and anti-gliadin IgG levels between carriers (n=77) and non-carriers (n=296). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014). (p >0.006)

Figure 3.6 Comparison of plasma anti-gliadin IgG levels in the DQ6.2 carriers and non-DQ6.2 carriers



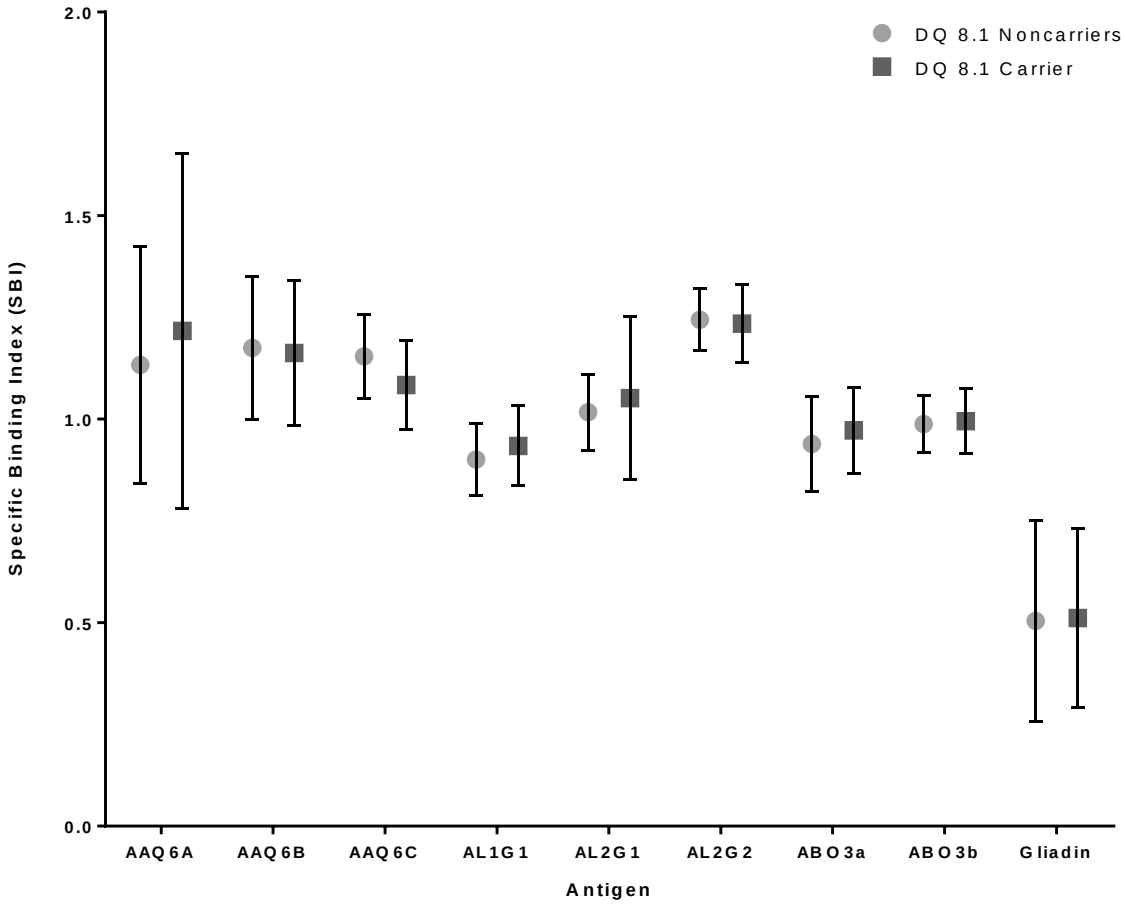
Median and IQR of levels of IgG against gliadins in carriers and non-carriers of DQ6.2. No significant differences were observed in plasma anti-AAQ6A IgG levels between the DQ6.2 carriers (n=109) and non-DR6.2 carriers (n= 260), anti-AAQ6B IgG levels between carriers (n= 109) and non-carriers (n= 257), anti-AAQ6C IgG levels between carriers (n= 109) and non-carriers (n= 257), anti-AL1G1 IgG levels between carriers (n= 108) and non-carriers (n= 256), anti-AL2G1 IgG levels between carriers (n= 111) and non-carriers (n= 257), anti-AL2G2 IgG levels between carriers (n= 111) and non-carriers (n= 257), anti-ABO3a IgG levels between carriers (n= 108) and non-carriers (n= 243), anti-ABO3b IgG levels between carriers (n= 108) and non-carriers (n= 285) and anti-gliadin IgG levels between carriers (n= 109) and non-carriers (n= 263). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014). ($p > 0.006$)

Figure 3.7 Comparison of plasma anti-gliadin IgG levels in the DQ2.5 carriers and non-DQ2.5 carriers



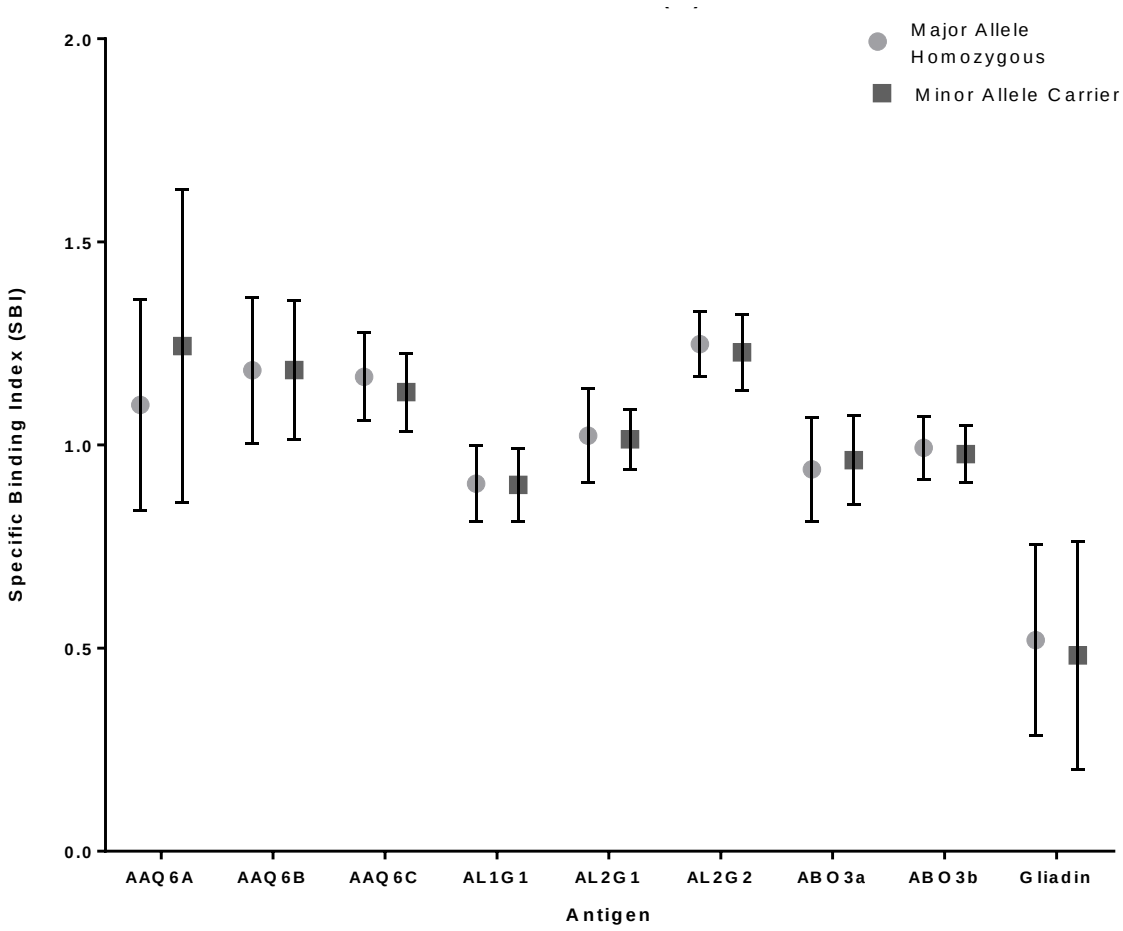
Median and IQR of levels of IgG against gliadins in carriers and non-carriers of DQ2.5. No significant differences were observed in plasma anti-AAQ6A IgG levels between the DQ2.5 carriers (n= 100) and non-DR2.5 carriers (n= 267), anti-AAQ6B IgG levels between carriers (n= 98) and non-carriers (n= 266), anti-AAQ6C IgG levels between carriers (n= 98) and non-carriers (n= 266), anti-AL1G1 IgG levels between carriers (n= 96) and non-carriers (n= 266), anti-AL2G1 IgG levels between carriers (n= 95) and non-carriers (n= 271), anti-AL2G2 IgG levels between carriers (n= 95) and non-carriers (n= 271), anti-ABO3a IgG levels between carriers (n= 90) and non-carriers (n= 259), anti-ABO3b IgG levels between carriers (n= 90) and non-carriers (n= 259) and anti-gliadin IgG levels between carriers (n= 100) and non-carriers (n= 270). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014). ($p > 0.006$)

Figure 3.8 Comparison of plasma anti-gliadin IgG levels in the DQ8.1 carriers and non-DQ8.1 carriers



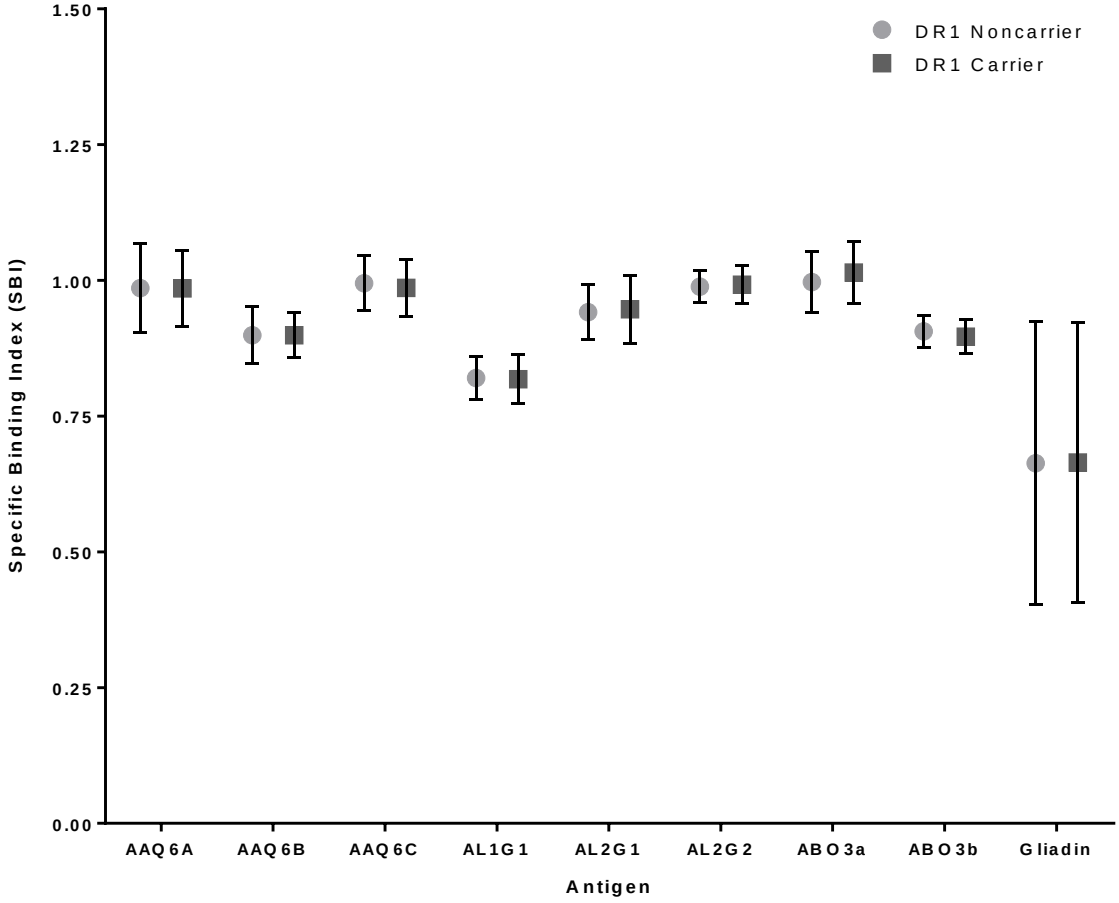
Median and IQR of levels of IgG against gliadins in carriers and non-carriers of DQ8.1. No significant differences were observed in plasma anti-AAQ6A IgG levels between the DQ8.1 carriers (n= 52) and non-DQ8.1 carriers (n= 317), anti-AAQ6B IgG levels between carriers (n= 52) and non-carriers (n= 315), anti-AAQ6C IgG levels between carriers (n= 52) and non-carriers (n= 315), anti-AL1G1 IgG levels between carriers (n= 52) and non-carriers (n= 312), anti-AL2G1 IgG levels between carriers (n= 52) and non-carriers (n= 316), anti-AL2G2 IgG levels between carriers (n= 52) and non-carriers (n= 316), anti-ABO3a IgG levels between carriers (n= 46) and non-carriers (n= 305), anti-ABO3b IgG levels between carriers (n= 46) and non-carriers (n= 305) and anti-gliadin IgG levels between carriers (n= 52) and non-carriers (n= 165). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014). ($p > 0.006$)

Figure 3.9 Genetic association of rs1233578 with plasma anti-gliadin IgG levels



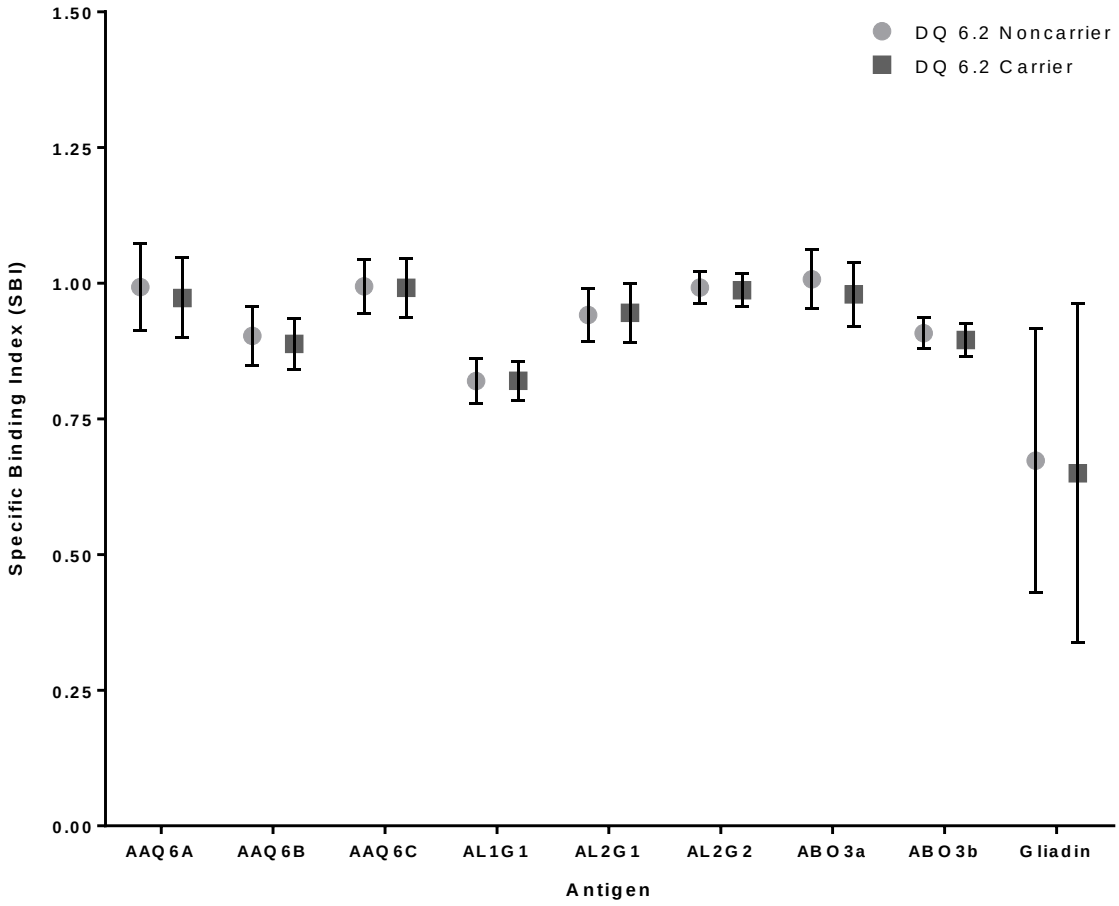
Median and IQR of levels of IgG against gliadins in major allele homozygotes and minor allele carriers of rs1233578. No significant differences were observed in plasma anti-AAQ6A IgG levels between the major allele homozygotes (n= 110) and minor allele (rs1233578-G) carriers (n= 229), anti-AAQ6B IgG levels between minor allele (rs1233578-G) carriers (n= 109) and major allele homozygotes (n= 234), anti-AAQ6C IgG levels between carriers (n= 110) and major allele homozygotes (n= 232), anti-AL1G1 IgG levels between minor allele (rs1233578-G) carriers (n= 110) and major allele homozygotes (n= 229), anti-AL2G1 IgG levels between minor allele (rs1233578-G) carriers (n= 110) and major allele homozygotes (n= 271), anti-AL2G2 IgG levels between minor allele (rs1233578-G) carriers (n= 110) and major allele homozygotes (n= 233), anti-ABO3a IgG levels between minor allele (rs1233578-G) carriers (n= 107) and major allele homozygotes (n= 221), anti-ABO3b IgG levels between minor allele (rs1233578-G) carriers (n= 107) and major allele homozygotes (n= 221) and anti-gliadin IgG levels between minor allele (rs1233578-G) carriers (n= 112) and major allele homozygotes (n= 235). ($p > 0.006$)

Figure 3.10 Comparison of plasma anti-gliadin IgA levels in the DR1 carriers and non-DR1 carriers



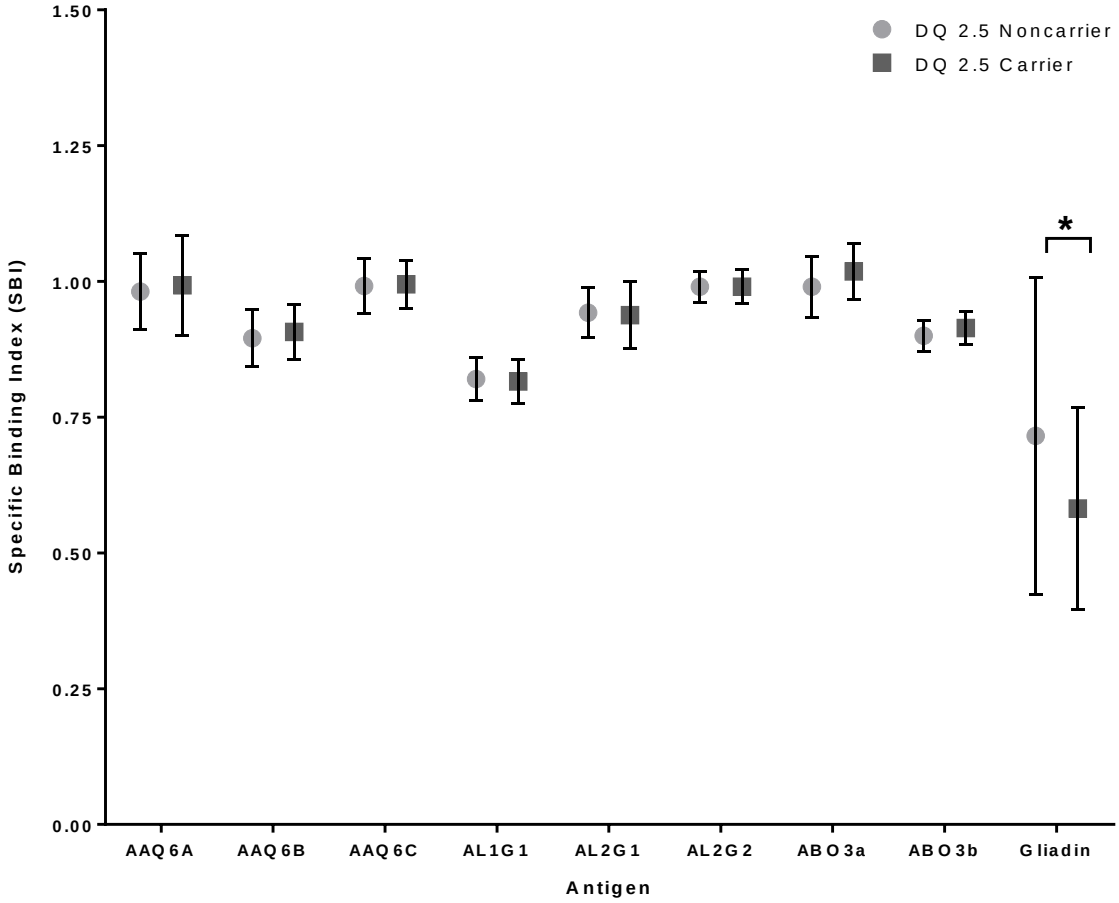
Median and IQR of levels of IgA against gliadins in carriers and non-carriers of DR1. No significant differences were observed in plasma anti-AAQ6A IgA levels between the DR1 carriers (n=77) and non-DR1 carriers (n= 292), anti-AAQ6B IgA levels between carriers (n= 77) and non-carriers (n= 292), anti-AAQ6C IgA levels between carriers (n= 77) and non-carriers (n= 289), anti-AL1G1 IgA levels between carriers (n= 77) and non-carriers (n=289), anti-AL2G1 IgA levels between carriers (n= 77) and non-carriers (n= 289), anti-AL2G2 IgA levels between carriers (n= 77) and non-carriers (n= 289), anti-ABO3a IgA levels between carriers (n= 77) and non-carriers (n= 288), anti-ABO3b IgA levels between carriers (n= 77) and non-carriers (n=288) and anti-gliadin IgA levels between carriers (n=76) and non-carriers (n=293). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014). ($p > 0.006$)

Figure 3.11 Comparison of plasma anti-gliadin IgA levels in the DQ6.2 carriers and non-DQ6.2 carriers.



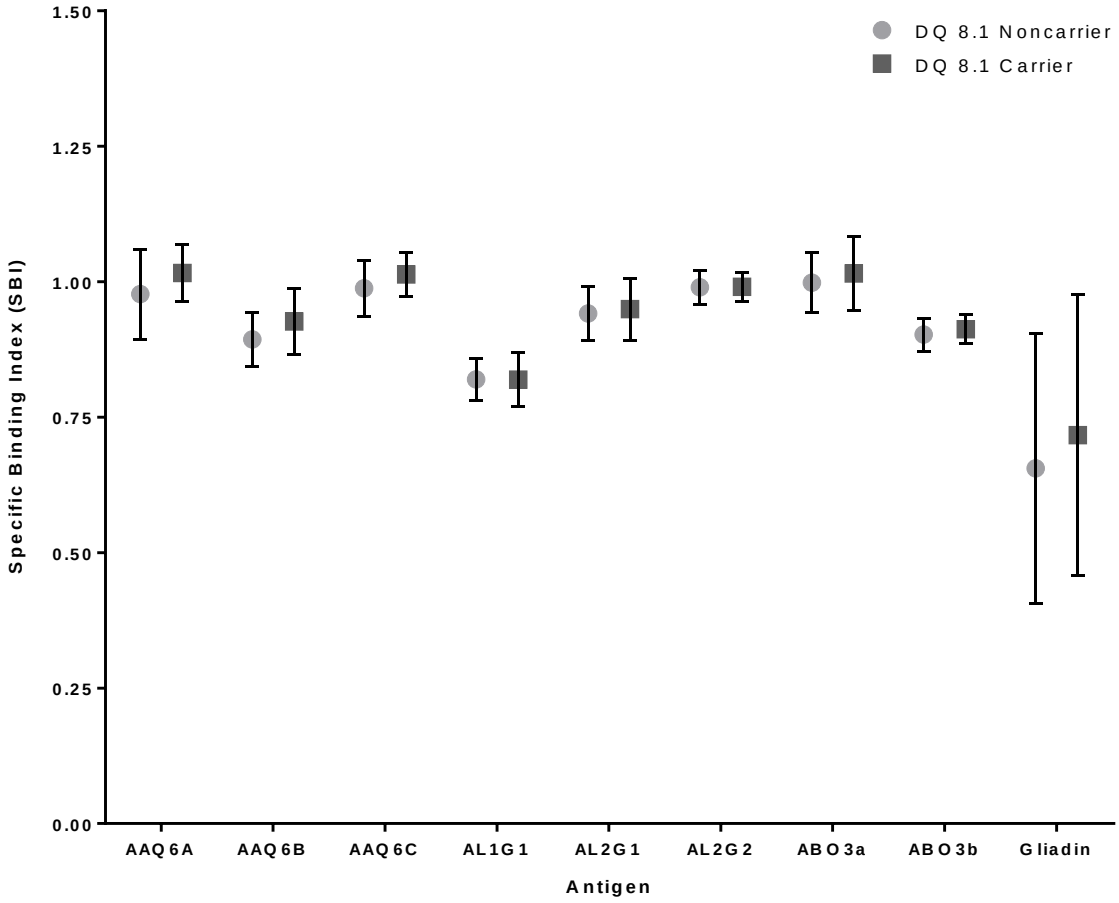
Median and IQR of levels of IgA against gliadins in carriers and non-carriers of DQ6.2. No significant differences were observed in plasma anti-AAQ6A IgA levels between the DQ6.2 carriers (n=109) and non-DQ6.2 carriers (n= 259), anti-AAQ6B IgA levels between carriers (n= 109) and non-carriers (n= 259), anti-AAQ6C IgA levels between carriers (n= 109) and non-carriers (n= 256), anti-AL1G1 IgA levels between carriers (n= 109) and non-carriers (n= 256), anti-AL2G1 IgA levels between carriers (n= 109) and non-carriers (n= 256), anti-AL2G2 IgA levels between carriers (n=109) and non-carriers (n= 256), anti-ABO3a IgA levels between carriers (n= 109) and non-carriers (n= 255), anti-ABO3b IgA levels between carriers (n= 109) and non-carriers (n=255) and anti-gliadin IgA levels between carriers (n= 109) and non-carriers (n=253). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014). ($p > 0.006$)

Figure 3.12 Comparison of plasma anti-gliadin IgA levels in the DQ2.5 carriers and non-DQ2.5 carriers



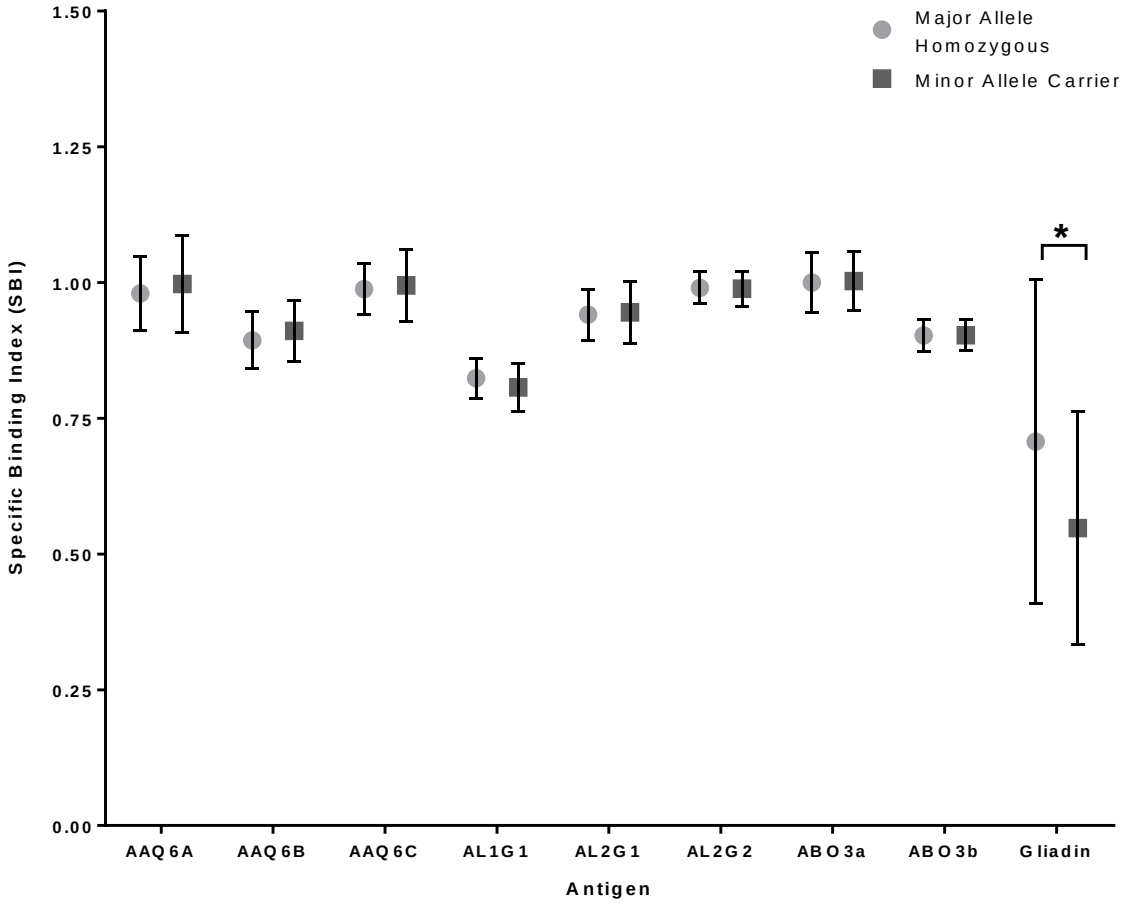
Median and IQR of levels of IgA against gliadins in carriers and non-carriers of DQ2.5. There were no significant differences in the levels of anti-AAQ6A IgA levels between the DQ2.5 carriers (n=99) and non-DQ2.5 carriers (n= 267), anti-AAQ6B IgA levels between carriers (n= 99) and non-carriers (n= 267), anti-AAQ6C IgA levels between carriers (n= 99) and non-carriers (n= 267), anti-AL1G1 IgA levels between carriers (n= 99) and non-carriers (n= 267), anti-AL2G1 IgA levels between carriers (n= 98) and non-carriers (n= 265), anti-AL2G2 IgA levels between carriers (n=98) and non-carriers (n= 265), anti-ABO3a IgA levels between carriers (n= 97) and non-carriers (n= 265) and anti-ABO3b IgA levels between carriers (n= 97) and non-carriers (n=265) ($p > 0.006$). Plasma levels of anti-native gliadin IgA were significantly lower in the DQ2.5 carriers (n= 99) than non-DQ2.5 carriers (n= 267) ($Z = -3.129$, $p = 0.002$). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014)

Figure 3.13 Comparison of plasma anti-gliadin IgA levels in the DQ8.1 carriers and non-DQ8.1 carriers



Median and IQR of levels of IgA against gliadins in carriers and non-carriers of DQ8.1. No significant differences were observed in plasma anti-AAQ6A IgA levels between the DQ8.1 carriers (n=52) and non-DQ8.1 carriers (n= 316), anti-AAQ6B IgA levels between carriers (n= 52) and non-carriers (n= 316), anti-AAQ6C IgA levels between carriers (n= 52) and non-carriers (n= 316), anti-AL1G1 IgA levels between carriers (n= 52) and non-carriers (n= 313), anti-AL2G1 IgA levels between carriers (n= 52) and non-carriers (n= 313), anti-AL2G2 IgA levels between carriers (n=52) and non-carriers (n= 313), anti-ABO3a IgA levels between carriers (n= 52) and non-carriers (n= 312), anti-ABO3b IgA levels between carriers (n= 52) and non-carriers (n= 312) and anti-gliadin IgA levels between carriers (n= 52) and non-carriers (n=164). Genotyping of HLA-tagging SNPs was performed by Dr Lorna Halley (Halley, 2014). ($p > 0.006$)

Figure 3.14 Genetic association of rs1233578 with plasma anti-gliadin IgA levels



Median and IQR of levels of IgA against gliadins in major allele homozygotes and minor allele carriers of rs1233578. No significant differences were observed in plasma anti-AAQ6A IgA levels between the major allele homozygotes (n= 110) and minor allele (rs1233578-G) carriers (n=233), anti-AAQ6B IgG levels between minor allele (rs1233578-G) carriers (n= 110) and major allele homozygotes (n= 233), anti-AAQ6C IgA levels between carriers (n= 109) and major allele homozygotes (n= 231), anti-AL1G1 IgA levels between minor allele (rs1233578-G) carriers (n= 109) and major allele homozygotes (n= 231), anti-AL2G1 IgA levels between minor allele (rs1233578-G) carriers (n= 109) and major allele homozygotes (n= 231), anti-AL2G2 IgA levels between minor allele (rs1233578-G) carriers (n= 109) and major allele homozygotes (n= 231), anti-ABO3a IgA levels between minor allele (rs1233578-G) carriers (n= 108) and major allele homozygotes (n= 231), anti-ABO3b IgA levels between minor allele (rs1233578-G) carriers (n= 108) and major allele homozygotes (n= 231) ($p > 0.006$). Plasma levels of anti-native gliadin IgA were significantly lower in minor allele (rs1233578-G) carriers (110) than major allele homozygotes (233) ($Z = -3.517$, $p < 0.001$).

Of the 5 HLA-variants tested in this study (*Figure 3.5*-*Figure 3.14*), only HLA-DQ2.5 and rs1233578 (G) allele were associated with decreased levels of plasma IgA against native gliadin (*Figure 3.12* and *Figure 3.14*). There was no genetic association between plasma anti-gliadin antibody levels and HLA-DR1 (*Figure 3.5* and *Figure 3.10*), HLA-DQ6.2 (*Figure 3.6* and *Figure 3.11*), HLA-DQ8.1 (*Figure 3.8* and *Figure 3.13*).

In order to examine potential HLA-variant*Schizophrenia interactions on the levels of AGAs, the levels of AGAs in carriers and non-carriers of HLA-alleles were examined in control and schizophrenia subjects (*Table 3.14* - *Table 3.19*)

Table 3.14 Examination of levels of Anti-Gliadin IgGs in carriers and non-carriers of HLA-Variants by schizophrenia status (A)

Genotype	Schizophrenia State	Carrier Status	AL1G1					AL2G1					AL2G2				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
HLA-DR1	Control	Non-carrier	169	0.945	0.16	-0.516	0.606	174	1.056	0.16	-0.839	0.401	174	1.275	0.15	-0.018	0.985
		Carrier	44	0.911	0.14			45	1.032	0.22			45	1.281	0.17		
	Case	Non-carrier	122	0.857	0.20	-0.880	0.379	120	0.941	0.18	-2.128	0.033	120	1.213	0.15	-0.564	0.573
		Carrier	30	0.872	0.19			30	0.891	0.19			30	1.229	0.23		
HLA-DQ 6.2	Control	Non-carrier	149	0.940	0.14	-0.021	0.983	152	1.064	0.16	-0.540	0.589	152	1.266	0.15	-0.813	0.416
		Carrier	63	0.938	0.23			66	1.037	0.20			66	1.295	0.14		
	Case	Non-carrier	107	0.872	0.21	-1.201	0.230	105	0.936	0.18	-0.556	0.578	105	1.221	0.18	-1.109	0.267
		Carrier	45	0.845	0.20			45	0.915	0.29			45	1.197	0.17		
HLA-DQ 2.5	Control	Non-carrier	149	0.933	0.15	-0.065	0.948	155	1.068	0.18	-2.157	0.031	155	1.284	0.14	-1.993	0.046
		Carrier	63	0.945	0.17			63	1.040	0.11			63	1.261	0.16		
	Case	Non-carrier	117	0.857	0.20	-0.719	0.472	116	0.943	0.18	-1.807	0.071	116	1.229	0.15	-0.862	0.389
		Carrier	33	0.872	0.16			32	0.871	0.17			32	1.185	0.21		
HLA-DQ 8.1	Control	Non-carrier	180	0.939	0.16	-0.394	0.694	186	1.044	0.17	-1.098	0.272	186	1.270	0.15	-0.938	0.348
		Carrier	32	0.941	0.17			32	1.088	0.29			32	1.300	0.17		
	Case	Non-carrier	132	0.853	0.19	-1.515	0.130	130	0.925	0.18	-0.050	0.960	130	1.229	0.18	-1.929	0.054
		Carrier	20	0.961	0.23			20	0.925	0.41			20	1.141	0.17		
HLA-ML	Control	Non-carrier	128	0.940	0.16	-0.354	0.724	133	1.078	0.18	-2.395	0.017	133	1.288	0.13	-1.820	0.069
		Carrier	69	0.933	0.17			70	1.030	0.13			70	1.261	0.17		
	Case	Non-carrier	101	0.857	0.20	-0.218	0.827	100	0.927	0.17	-0.521	0.602	100	1.230	0.17	-1.075	0.282
		Carrier	41	0.872	0.22			40	0.922	0.22			40	1.189	0.18		

Table 3.15 Examination of levels of Anti-Gliadin IgGs in carriers and non-carriers of HLA-Variants by schizophrenia status (B)

Genotype	Schizophrenia State	Carrier Status	AAQ6A					AAQ6B					AAQ6C				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
HLA-DR1	Control	Non-carrier	173	1.118	0.74	-0.025	0.980	171	1.148	0.33	-1.352	0.176	172	1.129	0.17	-0.513	0.608
		Carrier	47	1.031	0.54			46	1.086	0.32			46	1.086	0.20		
	Case	Non-carrier	120	1.194	0.49	-0.007	0.994	120	1.203	0.37	-0.601	0.548	120	1.224	0.24	-0.061	0.951
		Carrier	30	1.075	0.75			30	1.220	0.30			30	1.221	0.25		
HLA-DQ 6.2	Control	Non-carrier	155	1.081	0.55	-1.660	0.097	152	1.108	0.33	-1.872	0.061	153	1.114	0.15	-2.373	0.018
		Carrier	64	1.194	0.78			64	1.201	0.31			64	1.131	0.27		
	Case	**Non-carrier**	**105**	**1.200**	**0.56**	**-2.770**	**0.006**	105	1.259	0.32	-2.709	0.007	105	1.233	0.21	-1.651	0.099
		Carrier	**45**	**1.056**	**0.34**			45	1.071	0.48			45	1.170	0.33		
HLA-DQ 2.5	Control	Non-carrier	152	1.125	0.60	-1.910	0.056	151	1.141	0.32	-0.668	0.504	151	1.121	0.19	-0.980	0.327
		Carrier	67	0.997	0.68			65	1.093	0.37			66	1.110	0.18		
	Case	Non-carrier	115	1.109	0.51	-0.445	0.657	115	1.220	0.37	-0.891	0.373	115	1.221	0.24	-1.117	0.264
		Carrier	33	1.300	0.53			33	1.201	0.36			33	1.232	0.27		
HLA-DQ 8.1	Control	Non-carrier	187	1.102	0.61	-0.560	0.575	184	1.137	0.33	-0.426	0.670	185	1.125	0.18	-2.284	0.022
		Carrier	32	1.142	0.76			32	1.094	0.37			32	1.095	0.19		
	Case	**Non-carrier**	**130**	**1.152**	**0.47**	**-2.773**	**0.006**	130	1.211	0.36	-1.592	0.111	130	1.231	0.25	-0.608	0.543
		Carrier	**20**	**1.533**	**0.87**			20	1.130	0.36			20	1.211	0.28		
HLA-ML	Control	Non-carrier	134	1.067	0.55	-1.837	0.066	132	1.136	0.36	-0.483	0.629	132	1.131	0.19	-1.267	0.205
		Carrier	70	1.203	0.76			69	1.099	0.34			70	1.106	0.17		
	Case	Non-carrier	100	1.109	0.50	-1.723	0.085	100	1.214	0.37	-1.665	0.096	100	1.235	0.26	-1.001	0.317
		Carrier	40	1.268	0.52			40	1.201	0.32			40	1.199	0.19		

Table 3.16 *Examination of levels of Anti-Gliadin IgGs in carriers and non-carriers of HLA-Variants by schizophrenia status (C)*

Genotype	Schizophrenia State	Carrier Status	ABO3a					ABO3b					Gliadin				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
HLA-DR1	Control	Non-carrier	167	1.003	0.20	-0.175	0.861	167	1.008	0.15	-0.227	0.820	174	0.511	0.49	-0.021	0.984
		Carrier	41	0.990	0.20			41	1.001	0.16			47	0.621	0.52		
	Case	Non-carrier	118	0.868	0.23	-0.439	0.661	118	0.964	0.16	-0.078	0.938	122	0.510	0.50	-1.000	0.317
		Carrier	26	0.839	0.28			26	0.956	0.12			30	0.553	0.68		
HLA-DQ 6.2	Control	Non-carrier	144	1.004	0.20	-1.236	0.217	144	0.999	0.16	-1.420	0.156	156	0.553	0.49	-0.243	0.808
		Carrier	63	0.995	0.21			63	1.014	0.15			64	0.422	0.42		
	Case	Non-carrier	99	0.869	0.21	-1.276	0.202	99	0.977	0.16	-2.138	0.033	107	0.511	0.57	-0.321	0.748
		Carrier	45	0.830	0.26			45	0.904	0.15			45	0.526	0.48		
HLA-DQ 2.5	Control	Non-carrier	148	1.004	0.20	-1.338	0.181	148	1.016	0.15	-2.167	0.030	153	0.553	0.50	-0.820	0.412
		Carrier	59	0.965	0.21			59	0.969	0.17			67	0.505	0.45		
	Case	Non-carrier	111	0.852	0.23	-1.086	0.277	111	0.956	0.16	-1.072	0.284	117	0.511	0.56	-0.166	0.868
		Carrier	31	0.873	0.29			31	0.991	0.11			33	0.484	0.47		
HLA-DQ 8.1	Control	Non-carrier	180	0.997	0.20	-0.274	0.784	180	1.001	0.15	-0.513	0.608	188	0.541	0.50	-0.586	0.558
		Carrier	27	1.007	0.20			27	1.026	0.22			32	0.460	0.46		
	Case	Non-carrier	125	0.855	0.18	-1.473	0.141	125	0.949	0.14	-0.915	0.360	132	0.491	0.56	-0.970	0.332
		Carrier	19	0.951	0.30			19	0.992	0.22			20	0.712	0.43		
HLA-ML	Control	Non-carrier	125	0.999	0.21	-0.026	0.980	125	1.008	0.15	-0.103	0.918	134	0.520	0.45	-0.322	0.748
		Carrier	68	1.004	0.20			68	0.990	0.17			71	0.510	0.58		
	Case	Non-carrier	96	0.857	0.23	-0.529	0.597	96	0.954	0.14	-0.534	0.593	101	0.511	0.55	-0.178	0.859
		Carrier	39	0.866	0.23			39	0.964	0.16			41	0.511	0.56		

Median and IQR levels of IgG against gliadins in patients with schizophrenia and control individuals by carrier status of HLA-tagging SNPs. Levels of anti-gliadin IgG were, generally, not significantly different between carriers of the HLA-variants of interest and non-carriers when examined by schizophrenia status. However, levels of anti-AAQ6A IgG were significantly lower in patients with schizophrenia who carried HLA-DR6.2 than those who were non-carriers (z= -2.770, p = 0.006), while HLA-DR6.2 carrier status did not significantly alter anti-AAQ6A IgG levels in control individuals. Furthermore, levels of anti-AAQ6C IgG were significantly higher in patients with schizophrenia that carried HLA-DQ 8.1, when compared to non-carriers (z= -2.773, p = 0.006). This effect is not observed in the control group (z=-0.560, p = 0.575). Due to multiple testing the cut-off for statistical significance was p <0.006. Significant associations are highlighted in bold.

Table 3.17 Examination of levels of Anti-Gliadin IgAs in carriers and non-carriers of HLA-Variants by schizophrenia status (A)

Genotype	Schizophrenia State	Carrier Status	AL1G1					AL2G1					AL2G2				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
HLA-DR1	Control	Non-carrier	170	0.8261	0.06	-0.207	0.836	170	0.9521	0.09	-0.906	0.365	170	1.0025	0.06	-0.976	0.329
		Carrier	47	0.8326	0.06			47	0.9772	0.10			47	1.0066	0.07		
	Case	Non-carrier	119	0.8036	0.09	-2.414	0.016	119	0.9155	0.10	-1.103	0.270	119	0.9772	0.06	-1.827	0.068
		Carrier	30	0.7721	0.10			30	0.8754	0.11			30	0.9531	0.07		
HLA-DQ 6.2	Control	Non-carrier	152	0.8362	0.07	-2.024	0.043	152	0.9518	0.09	-0.408	0.683	152	1.0020	0.06	-0.093	0.926
		Carrier	64	0.8190	0.05			64	0.9730	0.09			64	1.0027	0.07		
	Case	Non-carrier	104	0.7855	0.10	-1.972	0.049	104	0.9080	0.10	-0.074	0.941	104	0.9726	0.07	-0.285	0.775
		Carrier	45	0.8206	0.09			45	0.9202	0.11			45	0.9711	0.06		
HLA-DQ 2.5	Control	Non-carrier	150	0.8248	0.06	-0.326	0.744	150	0.9553	0.09	-0.825	0.409	150	1.0027	0.06	-1.399	0.162
		Carrier	66	0.8380	0.07			66	0.9589	0.11			66	0.9995	0.06		
	Case	Non-carrier	115	0.8056	0.09	-1.502	0.133	115	0.9155	0.10	-1.549	0.121	115	0.9736	0.06	-0.967	0.334
		Carrier	32	0.7760	0.07			32	0.8754	0.11			32	0.9619	0.09		
HLA-DQ 8.1	Control	Non-carrier	184	0.8270	0.06	-0.043	0.966	184	0.9561	0.09	-0.880	0.379	184	1.0022	0.06	-1.024	0.306
		Carrier	32	0.8386	0.08			32	0.9681	0.10			32	1.0137	0.06		
	Case	Non-carrier	129	0.7985	0.09	-1.392	0.164	129	0.9092	0.11	-1.314	0.189	129	0.9716	0.07	-0.652	0.515
		Carrier	20	0.7813	0.08			20	0.9200	0.07			20	0.9736	0.03		
HLA-ML	Control	Non-carrier	131	0.8286	0.07	-0.901	0.368	131	0.9573	0.10	-0.031	0.976	131	1.0025	0.06	-0.280	0.779
		Carrier	70	0.8200	0.06			70	0.9561	0.08			70	1.0043	0.06		
	Case	Non-carrier	100	0.8133	0.09	-2.353	0.019	100	0.9187	0.11	-1.711	0.087	100	0.9763	0.06	-1.730	0.084
		Carrier	39	0.7701	0.07			39	0.8781	0.10			39	0.9615	0.07		

Table 3.18 Examination of levels of Anti-Gliadin IgAs in carriers and non-carriers of HLA-Variants by schizophrenia status (B)

Genotype	Schizophrenia State	Carrier Status	AAQ6A					AAQ6B					AAQ6C				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
HLA-DR1	Control	Non-carrier	172	1.0211	0.17	-0.487	0.626	172	0.9107	0.11	-0.544	0.586	170	1.0109	0.10	-0.990	0.322
		Carrier	47	1.0137	0.13			47	0.9029	0.08			47	1.0285	0.10		
	Case	Non-carrier	120	0.9334	0.12	-0.164	0.869	120	0.8862	0.10	-0.146	0.884	119	0.9648	0.10	-1.486	0.137
		Carrier	30	0.9169	0.11			30	0.8838	0.07			30	0.9516	0.11		
HLA-DQ 6.2	Control	Non-carrier	154	1.0176	0.18	-0.810	0.418	154	0.9129	0.10	-0.515	0.606	152	1.0194	0.09	-1.063	0.288
		Carrier	64	1.0136	0.14			64	0.9061	0.11			64	1.0078	0.11		
	Case	Non-carrier	105	0.9273	0.13	-0.482	0.630	105	0.8880	0.10	-0.781	0.435	104	0.9592	0.10	-1.166	0.244
		Carrier	45	0.9361	0.11			45	0.8804	0.07			45	0.9738	0.09		
HLA-DQ 2.5	Control	Non-carrier	151	1.0192	0.15	-0.598	0.550	151	0.9152	0.10	-0.424	0.672	150	1.0148	0.11	-0.118	0.906
		Carrier	67	1.0007	0.19			67	0.9021	0.10			66	1.0225	0.09		
	Case	Non-carrier	116	0.9325	0.11	-0.750	0.453	116	0.8805	0.08	-0.647	0.517	115	0.9650	0.10	-1.718	0.086
		Carrier	32	0.9257	0.13			32	0.9104	0.12			32	0.9598	0.11		
HLA-DQ 8.1	Control	Non-carrier	186	1.0141	0.16	-1.279	0.201	186	0.9020	0.11	-1.400	0.161	184	1.0095	0.10	-1.689	0.091
		Carrier	32	1.0400	0.14			32	0.9464	0.07			32	1.0292	0.10		
	Case	Non-carrier	130	0.9265	0.12	-1.598	0.110	130	0.8841	0.09	-0.995	0.320	129	0.9633	0.10	-0.624	0.533
		Carrier	20	0.9514	0.12			20	0.8896	0.13			20	0.9742	0.06		
HLA-ML	Control	Non-carrier	133	1.0135	0.14	-0.895	0.371	133	0.9055	0.10	-0.669	0.504	131	1.0161	0.09	-0.326	0.745
		Carrier	70	1.0490	0.17			70	0.9203	0.10			70	1.0192	0.11		
	Case	Non-carrier	100	0.9330	0.12	-0.088	0.930	100	0.8812	0.10	-0.770	0.441	100	0.9663	0.08	-2.325	0.020
		Carrier	40	0.9235	0.14			40	0.8909	0.09			39	0.9479	0.10		

Table 3.19 Examination of levels of Anti-Gliadin IgAs in carriers and non-carriers of HLA-Variants by schizophrenia status (C)

Genotype	Schizophrenia State	Carrier Status	**ABO3a**					**ABO3b**					**Gliadin**				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
HLA-DR1	Control	Non-carrier	169	1.0289	0.08	-0.606	0.545	169	0.9170	0.04	-0.497	0.619	172	0.6677	0.49	-1.563	0.118
		Carrier	47	1.0433	0.06			47	0.9167	0.05			46	0.8117	0.58		
	Case	Non-carrier	119	0.9366	0.07	-0.388	0.698	119	0.8703	0.06	-0.265	0.791	121	0.6430	0.61	-0.625	0.532
		Carrier	30	0.9268	0.06			30	0.8671	0.06			30	0.5745	0.48		
HLA-DQ 6.2	Control	Non-carrier	151	1.0316	0.07	-0.952	0.341	151	0.9185	0.04	-1.379	0.168	153	0.6725	0.44	-1.645	0.100
		Carrier	64	1.0291	0.10			64	0.9150	0.05			64	0.7589	0.70		
	Case	Non-carrier	104	0.9341	0.07	-0.447	0.655	104	0.8712	0.05	-0.980	0.327	106	0.6788	0.62	-0.651	0.515
		Carrier	45	0.9354	0.07			45	0.8648	0.06			45	0.6325	0.31		
HLA-DQ 2.5	Control	Non-carrier	150	1.0267	0.08	-1.248	0.212	150	0.9163	0.04	-1.287	0.198	151	0.7505	0.62	-2.254	0.024
		Carrier	65	1.0481	0.07			65	0.9215	0.04			66	0.6061	0.35		
	Case	Non-carrier	115	0.9345	0.06	-0.005	0.996	115	0.8712	0.06	-0.131	0.895	116	0.6496	0.58	-2.642	0.008
		Carrier	32	0.9296	0.08			32	0.8678	0.07			33	0.4783	0.55		
HLA-DQ 8.1	Control	Non-carrier	183	1.0269	0.07	-2.017	0.044	183	0.9162	0.04	-1.645	0.100	185	0.6727	0.52	-0.317	0.751
		Carrier	32	1.0515	0.07			32	0.9231	0.04			32	0.7443	0.46		
	Case	Non-carrier	129	0.9343	0.07	-0.657	0.511	129	0.8705	0.06	-0.707	0.479	131	0.6387	0.58	-0.895	0.371
		Carrier	20	0.9363	0.08			20	0.8702	0.03			20	0.6297	0.59		
HLA-ML	Control	Non-carrier	131	1.0399	0.08	-1.153	0.249	131	0.9185	0.04	-0.150	0.880	**132**	**0.7403**	**0.57**	**-3.033**	**0.002**
		Carrier	69	1.0256	0.06			69	0.9153	0.04			**70**	**0.5732**	**0.47**		
	Case	Non-carrier	100	0.9371	0.07	-1.913	0.056	100	0.8729	0.06	-1.111	0.267	101	0.6586	0.60	-1.921	0.055
		Carrier	39	0.9239	0.06			39	0.8692	0.06			40	0.5208	0.55		

Median and IQR levels of IgA against gliadins in patients with schizophrenia and control individuals by carrier status of HLA-tagging SNPs. As with levels of Anti-Gliadin IgGs, levels of IgAs do not generally vary by carrier status when patients with schizophrenia and healthy controls are analysed separately. However, this analysis reveals that levels of IgA against native gliadin were significantly lower in individuals that carried rs1233578-G; the minor allele at the

HLA-locus and top-hit SNP in the GWA by the Schizophrenia Working Group of the Psychiatric Genomics Consortium, (2014), than those carrying the major allele ($z = -3.033$, $p = 0.002$). There was no significant difference in the levels of IgA against native gliadin by carrier status of HLA-DQ 2.5 in either control individuals ($z = -2.254$, $p = 0.024$) or individuals with schizophrenia ($z = -2.642$, $p = 0.008$). Due to multiple testing the cut-off for statistical significance was $p < 0.006$. Significant associations are highlighted in bold.

3.3.8 Relationship between anti-gliadin antibodies and HLA-Variants

A robust regression analysis was applied to determine if a linear relationship existed between anti-gliadin antibody levels and genetic variants tested, which were designated as false variables: 0, 1 or 2 based on the presence of the number of minor alleles.

Table 3.20. Robust regression analysis of the association between the DR1 variant and plasma anti-gliadin antibody levels

Antigen	Antibody Class	DR1				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	-0.027	0.001	-0.526	0.599	0.602
	IgA	-0.021	0.000	-0.404	0.687	0.690
AAQ6B	IgG	-0.047	0.002	-0.901	0.368	0.361
	IgA	-0.024	0.001	-0.452	0.652	0.651
AAQ6C	IgG	-0.009	<0.001	-0.175	0.861	0.862
	IgA	0.014	<0.001	0.260	0.795	0.799
AL1G1	IgG	-0.020	<0.001	-0.375	0.708	0.706
	IgA	-0.079	0.006	-1.511	0.132	0.128
AL2G1	IgG	-0.064	0.004	-1.221	0.223	0.216
	IgA	0.012	0.000	0.224	0.823	0.821
AL2G2	IgG	-0.032	0.001	-0.616	0.539	0.525
	IgA	0.021	<0.001	0.392	0.696	0.696
ABO3a	IgG	0.014	<0.001	0.262	0.793	0.769
	IgA	0.003	<0.001	0.058	0.954	0.957
ABO3b	IgG	0.018	<0.001	0.331	0.741	0.731
	IgA	0.038	0.001	0.726	0.468	0.466
Gliadin	IgG	-0.018	<0.001	-0.348	0.728	0.726
	IgA	0.077	0.006	1.485	0.138	0.139

Robust regression analysis examining the association between levels of IgG against gliadins and carrier status of HLA-DR1 in combined schizophrenia-control samples. Statistical significance was set at $p<0.05$ and significant associations are highlighted in bold.

Table 3.21. Robust regression analysis of the association between the DQ6.2 variant and plasma anti-gliadin antibody levels

Antigen	Antibody Class	DQ6.2				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	-0.024	0.001	-0.462	0.644	0.649
	IgA	-0.047	0.002	-0.895	0.371	0.376
AAQ6B	IgG	-0.034	0.001	-0.658	0.511	0.532
	IgA	-0.068	0.005	-1.294	0.196	0.190
AAQ6C	IgG	0.057	0.003	1.086	0.278	0.279
	IgA	0.015	<0.001	0.277	0.782	0.776
AL1G1	IgG	-0.062	0.004	-1.174	0.241	0.244
	IgA	0.011	<0.001	0.219	0.827	0.829
AL2G1	IgG	0.000	<0.001	-0.005	0.996	0.996
	IgA	-0.022	<0.001	-0.417	0.677	0.681
AL2G2	IgG	0.000	<0.001	0.008	0.994	0.994
	IgA	0.011	<0.001	0.211	0.833	0.830
ABO3a	IgG	-0.015	<0.001	-0.277	0.782	0.790
	IgA	-0.001	<0.001	-0.026	0.979	0.980
ABO3b	IgG	0.026	0.001	0.493	0.622	0.617
	IgA	-0.100	0.010	-1.905	0.058	0.057
Gliadin	IgG	0.007	<0.001	0.130	0.896	0.898
	IgA	0.046	0.002	0.888	0.375	0.368

Robust regression analysis examining the association between levels of IgG against gliadins and carrier status of HLA-DQ6.2 in combined schizophrenia-control samples. Statistical significance was set at $p<0.05$ and significant associations are highlighted in bold.

As shown in *Table 3.20*and *Table 3.21*, HLA-II variants DR1 and DQ6.2 were not associated with plasma anti-gliadin antibody levels ($p>0.05$).

Table 3.22. Robust regression analysis of the association between the DQ2.5 variant and plasma anti-gliadin antibody levels

Antigen	Antibody Class	DQ2.5				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	-0.036	0.001	-0.685	0.494	0.488
	IgA	0.004	<0.001	0.078	0.938	0.938
AAQ6B	IgG	-0.063	0.004	-1.194	0.233	0.222
	IgA	0.018	<0.001	0.337	0.736	0.741
AAQ6C	IgG	-0.076	0.006	-1.446	0.149	0.150
	IgA	-0.047	0.002	-0.898	0.370	0.380
AL1G1	IgG	0.045	0.002	0.859	0.391	0.398
	IgA	-0.063	0.004	-1.204	0.229	0.230
AL2G1	IgG	-0.057	0.003	-1.088	0.278	0.277
	IgA	-0.017	<0.001	-0.329	0.742	0.740
AL2G2	IgG	-0.082	0.007	-1.570	0.117	0.114
	IgA	-0.043	0.002	-0.814	0.416	0.422
ABO3a	IgG	-0.054	0.003	-1.000	0.318	0.311
	IgA	0.013	<0.001	0.249	0.804	0.811
ABO3b	IgG	-0.086	0.007	-1.599	0.111	0.113
	IgA	0.107	0.011	2.042	0.042	**0.041**
Gliadin	IgG	0.018	<0.001	0.352	0.725	0.719
	IgA	-0.152	0.023	-2.942	0.003	**0.004**

Robust regression analysis examining the association between levels of IgG and IgA against gliadins and carrier status of HLA-DQ2.5 in combined schizophrenia-control samples. Statistical significance was set at $p<0.05$ and significant associations were highlighted in bold. The presence of HLA-DQ2.5 was predictive of increased levels of IgA against ABO3b (t= 2.042, p= 0.041). The presence of HLA-DQ 2.5 was associated with decreased levels of IgA against gliadin (t= -2.942, p= 0.004).

Table 3.22 demonstrates a significant relationship between increased levels of plasma IgA against ABO3b and the presence of HLA-DQ2.5 (r= 0.107, p= 0.041), while HLA-DQ2.5 was found to be associated with decreased levels of plasma anti-gliadin IgA (r= -0.152, p= 0.023).

Table 3.23. Robust regression analysis of the association between the DQ8.1 variant and plasma anti-gliadin antibody levels

Antigen	Antibody Class	DQ8.1				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	0.037	0.001	0.711	0.478	0.487
	IgA	0.020	<0.001	0.391	0.696	0.703
AAQ6B	IgG	-0.058	0.003	-1.107	0.269	0.224
	IgA	0.042	0.002	0.809	0.419	0.417
AAQ6C	IgG	-0.115	0.013	-2.211	0.028	**0.029**
	IgA	0.084	0.007	1.606	0.109	0.111
AL1G1	IgG	-0.002	<0.001	-0.043	0.965	0.966
	IgA	-0.077	0.006	-1.476	0.141	0.140
AL2G1	IgG	0.056	0.003	1.080	0.281	0.264
	IgA	0.031	0.001	0.600	0.549	0.543
AL2G2	IgG	-0.021	<0.001	-0.400	0.689	0.660
	IgA	0.073	0.005	1.394	0.164	0.166
ABO3a	IgG	0.040	0.002	0.751	0.453	0.389
	IgA	0.013	<0.001	0.251	0.802	0.806
ABO3b	IgG	0.072	0.005	1.352	0.177	0.167
	IgA	0.033	0.001	0.637	0.524	0.534
Gliadin	IgG	-0.008	<0.001	-0.155	0.877	0.875
	IgA	-0.025	0.001	-0.473	0.636	0.638

Robust regression analysis examining the association between levels of IgG and IgA against gliadins and carrier status of HLA-DQ8.1 in combined schizophrenia-control samples. The presence of HLA-DQ8.1 was predictive of decreased levels of anti-AAQ6C IgG (t= -2.211, p= 0.029). Statistical significance p<0.05. Significant associations highlighted in bold.

As shown in *Table 3.23*, the presence of HLA-DQ8.1 was associated with decreased levels of anti-AAQ6C plasma IgG (r= -0.115, p = 0.029).

Table 3.24. Robust regression analysis of the association between rs1233578 and plasma anti-gliadin antibody levels

Antigen	Antibody Class	rs1233578 (G)				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	0.087	0.008	1.613	0.108	0.110
	IgA	0.015	<0.001	0.280	0.779	0.774
AAQ6B	IgG	-0.044	0.002	-0.803	0.423	0.437
	IgA	0.094	0.009	1.746	0.082	0.076
AAQ6C	IgG	-0.062	0.004	-1.140	0.255	0.258
	IgA	-0.063	0.004	-1.154	0.249	0.255
AL1G1	IgG	0.054	0.003	0.984	0.326	0.320
	IgA	-0.055	0.003	-1.009	0.314	0.319
AL2G1	IgG	-0.035	0.001	-0.643	0.521	0.517
	IgA	-0.001	<0.001	-0.015	0.988	0.987
AL2G2	IgG	-0.096	0.009	-1.783	0.076	0.074
	IgA	-0.014	<0.001	-0.254	0.800	0.803
ABO3a	IgG	0.019	<0.001	0.349	0.727	0.740
	IgA	-0.075	0.006	-1.389	0.166	0.162
ABO3b	IgG	0.056	0.003	1.014	0.311	0.315
	IgA	0.021	<0.001	0.382	0.703	0.705
Gliadin	IgG	-0.007	<0.001	-0.129	0.897	0.894
	IgA	-0.145	0.021	-2.707	0.007	**0.007**

Robust regression analysis examining the association between levels of IgG and IgA against gliadins and carrier status of rs1233578 in combined schizophrenia-control samples. The presence of the minor allele of rs1233578(G) was predictive of decreased levels of IgA against gliadin (t= -2.707, p=0.007). Statistical significance was set at p<0.05 and significant associations are highlighted in bold.

As shown in *Table 3.24*, minor allele of rs1233578 (G) in the HLA-locus was negatively associated with plasma anti-native gliadin IgA levels (r= -0.145, p= 0.007).

3.4 Discussion

This study was undertaken to investigate circulating levels of antibodies against indigestible gliadin-derived fragments in schizophrenia. The levels of plasma IgG against γ-gliadin derived antigen AAQ6C were elevated in patients with schizophrenia when compared to control individuals (*Table 3.4*). It is possible that an immune response to the AAQ6C antigen is associated with a subgroup of schizophrenia patients, although additional factors, such as their access to the central nervous system, are likely to determine the potential pathological activities of these antibodies in patients with schizophrenia (Hammer et al., 2014). It has been demonstrated that α2-gliadin derived peptides may not be immunogenic in schizophrenia but are likely to be immunogenic in CD patients (Samaroo et al., 2010). A GWA study revealed that the DQA1*0501/DQB1*0201 alleles that encode HLA-DQ2.5 molecules conferring a major risk of CD, were significantly less prevalent in schizophrenia cases than healthy controls; therefore, the decreased levels of circulating antibodies against α-gliadin derived antigens may partially result from the low frequency of the DQA1*0501/DQB1*0201 alleles in the patient group (International Schizophrenia Consortium et al., 2009; Samaroo et al., 2010).

Against all gliadin-derived peptide fragments tested, circulating levels of all IgA antibodies against gliadin-derived fragments were significantly lower in schizophrenia patients than healthy controls (*Table 3.5*). Two studies of IgA in schizophrenia detected an increase and a decrease in global IgA levels in 30 patients with schizophrenia (Delisi et al., 1981; Tiwari et al., 1984). However, global IgA levels in plasma were not found to be significantly different between control individuals and patients with schizophrenia in this cohort, (*Table 3.6*). There is evidence that circulating IgA has an anti-inflammatory role (Russell et al., 1989) and decreased IgA levels are commonly found in patients with autoimmune diseases, possibly related to mechanisms of impaired clearance or impaired antigen-exclusion (Jacob et al., 2008). Accordingly, decreased levels of IgA against gliadin-derived fragments observed in the present

study may reflect dysfunction of immune-regulation and inflammatory processes, in response to these antigens.

Several studies, including a meta-analysis, have suggested an association between increased AGA levels with schizophrenia (Cascella et al., 2011; F. Dickerson et al., 2010; Jackson et al., 2014; Jin et al., 2012; Lachance and McKenzie, 2014; Okusaga et al., 2013). However, the present study failed to show a significant increase in either AGA IgG levels or AGA IgA levels (*Table 3.7*). All native gliadin molecules consist of ~300 amino acid residues and are unlikely to survive degradation in the digestive system. It is possible that multiple AGAs that recognise distinct epitopes exist in the circulation and are different in binding affinities between the case group and the control group. Regression analysis of the correlation between the AGA IgG levels and the IgG levels for gliadin-derived fragments suggests that plasma anti-AAQ6C IgG levels might be the most predictive of AGA IgG levels in patients with schizophrenia and plasma anti-AL2G1 IgG, the most predictive of AGA IgG levels in control subjects, which means that there may be differential epitopes bound to AGA antibodies in schizophrenia patients compared to those in control subjects (*Table 3.12*).

Antipsychotic medication is the first line treatment of schizophrenia but only 50-60% patients show a good clinical response to antipsychotic drugs (Solanki et al., 2009). Consequently, there is an urgent need to identify specific biomarkers for precision treatment of the disease. Of 8 gliadin-derived antigens tested in this study, 4 showed a sensitivity of >20% for the detection of their corresponding IgG in plasma (*Table 3.8*). These 4 tests may have a potential to serve as biomarkers for identification of a gluten-related subgroup of schizophrenia, though further definition of this subgroup is required before the full utility of antibodies against indigestible gliadin-fragments as a test for subgroup identification can be determined. Furthermore, the ability of AGA to distinguish schizophrenia from other closely related disorders must be examined, requiring replication of the work presented in this thesis in patients with other psychiatric illnesses.

Due to the nature of sample collection and the corresponding database information, it was not possible to fully control for potential confounding effects of lifestyle factors - such as alcohol consumption, tobacco use and diets - on the outcomes measured in these case-control samples. Although control subjects were screened for psychiatric illness, there was no additional medical information available; therefore, other confounding factors cannot be excluded. Furthermore, the clinical information for patients did not contain consistent reference to clinical subtypes of schizophrenia and so clinical or symptomatic associations for circulating antibody levels cannot be analysed in this cohort. The lack of antipsychotic-free or drug-naïve patients and incomplete medication histories mean that a potential effect of antipsychotic medication on the secretion of anti-gluten antibodies should be investigated in future study. Fisher's combining probability test failed to detect a significant association between antipsychotic medications and circulating anti-gliadin antibody levels

Previous studies have attempted to examine immunological phenomena observed in schizophrenia in the context of immunogenetics (Avramopoulos et al., 2015; Børglum et al., 2014; Chan et al., 2017). The present results showed that plasma anti-native gliadin IgA levels were significantly lower in the HLA-DQ2.5 carriers and the carriers of minor allele rs1233578 (G) than their non-carriers (Z== -3.129, p= 0.004 and Z= -3.517, p <0.001, respectively) (*Table 3.22* and *Table 3.24*), whereas plasma anti-gliadin IgG levels did not show a significant alteration in the carriers of the target allele of all other genetic variants selected. When examined by schizophrenia state, levels of anti-AAQ6A IgG were significantly lower in schizophrenia carriers of HLA-DQ 6.2 when compared to non-carriers (Z= -2.770, p= 0.006) while levels of anti-AAQ6A IgG were higher in individuals with schizophrenia that carry HLA-DQ 8.1 versus non-carriers (*Table 3.15*). This suggests that HLA-DQ 8.1 may have a role in the production of anti-AAQ6A antibodies in schizophrenia. In light of Alaedini et al., (2007) suggesting that PQ-motif homology may be responsible for AGA cross-reactivity in schizophrenia, this may suggest a subgroup of HLA-DQ 8.1 carrying individuals with schizophrenia may be

predisposed to produce antibodies against AAQ6A, which are more likely to directly cross-react with CNS tissue. However, as neither this HLA-type is more frequent in patients with schizophrenia, nor is there evidence that in CD HLA-DQ 8.1 segregates with extra-intestinal symptoms, and levels of anti-AAQ6A IgG are not elevated in schizophrenia, the clinical relevance of this finding is unclear. It is worth noting that AAQ6A is an amalgamation of two fragments joined by a PQ-motif rich region. However, there is no evidence that in Additionally, it was shown that IgA against native gliadins were significantly lower in control group carriers of rs1233578(G) only and no significant difference in levels of anti-native gliadin IgA was detected between carriers and non-carriers in the control group (Z= -3.033, p= 0.002 and Z= -1.921, p= 0.055, respectively) (*Table 3.19*).

Under robust regression, the HLA-DQ2.5 variant was predictive of decreased levels of anti-ABO3b IgA and anti-native gliadin IgA, although the genotypic contribution to the levels of these antibodies was small, accounting for 1.1% and 2.3% of variation, respectively. The negative association between anti-gliadin IgA and HLA-DQ2.5 is unexpected, since HLA-DQ2.5 is responsible for the presentation of gliadin-derived peptide antigens in coeliac disease and therefore likely responsible for AGA in CD. However, it is likely that no individuals in this study had coeliac disease. Additionally, it has previously been demonstrated that dendritic cells from healthy carriers of HLA-DQ2.5 could have a compensatory, anti-inflammatory response to gliadin incubation, which may result in reduced levels of AGA, although no study appears to have measured circulating anti-gliadin IgA levels in healthy carriers of HLA-DQ 2.5 (Palová-Jelínková et al., 2005). When levels of anti-native gliadin IgA in carriers and non-carriers of HLA-DQ 2.5, levels of anti-native gliadin IgA were not significantly different in either group (control: Z= -2.254, p =0.024; case: Z= -2.642, p= 0.008), however the trend of decreased levels between carriers and non-carriers was observed in both groups (*Table 3.19*). Despite these potential explanations decreased anti-gliadin IgA levels in carriers of HLA-DQ2.5 is nonetheless surprising.

A question to be addressed here is why plasma anti-AAQ6C IgG levels showed a negative association with HLA-DQ8.1 (*Table 3.22*). As reported previously, up to 90% of patients with CD carry HLA-DQ2.5 and the remaining patients carry DQ8.1 (Green and Jabri, 2003; Qiao et al., 2005). Both genetic and serological CD markers have been found to be less prevalent in patients with schizophrenia than in control subjects, including a lower frequency of HLA-DQ 2.5 that has been replicated in this sample biobank (Halley et al., 2013). While there was no association between HLA-DQ 8.1 and schizophrenia in these samples studied, plasma anti-AAQ6C IgG levels were significantly higher in patients with schizophrenia than in controls *Table 3.4*. This alteration raises the possibility that a distinct anti-gliadin immune response may be involved in schizophrenia that is independent from that observed in CD (Halley et al., 2013; Samaroo et al., 2010). The negative relationship between HLA-DQ8.1 and anti-AAQ6C IgG levels is weak. HLA-DQ8.1 accounts for only 1.3% of the anti-AAQ6C IgG variation (*Table 3.22*), suggesting that the production of anti-AAQ6C IgG antibody is not directly regulated by the HLA-DQ 8.1 molecule and that HLA-DQ8.1 is less likely to recognise this γ-gliadin derived fragment. When examined by schizophrenia status neither group showed significantly decreased levels of anti-AAQ6C IgG between carriers and non-carriers of HLA-DQ 8.1, although this effect is likely driven decreased levels of anti-AAQ6C IgG in HLA-DQ 8.1 carriers in the control group, rather than the case group (control: $Z= -2.284$, $p = 0.022$; case: $Z= -0.608$, $p= 0.022$) (*Table 3.15*).

A limitation of the study is the statistical approach to confounding effects; neither the Mann-Whitney U test nor the robust regression analysis was able to account for confounding factors such as age, gender, schizophrenia state. Owing to the non-normal distribution of plasma antibody levels in the study population (Chapter 2), Mann-Whitney U test was used to analyse the differences in antibody levels between the carriers and non-carriers of an allele of interest. Stepwise multilinear regression, including age, gender and schizophrenia status would be an ideal test to determine if plasma anti-gliadin antibody secretion is truly dependent on the

presence of a genetic variant of interest. However, due to the non-normal distribution of antibody measurement data, the statistical output of this test would only be an approximation, and therefore, robust linear regression, being the more rigorous test, was employed to examine the relationship between HLA-alleles and plasma anti-gliadin antibody levels.

In summary, this study demonstrates that altered levels of circulating antibodies against gliadin-derived fragments are associated with schizophrenia. Differential antibody levels may suggest that an immune response against a γ-gliadin derived fragment designated AAQ6C is associated with schizophrenia, however no convincing associations between AAQ6C and any HLA-type examined in this study was observed. In order to further test this hypothesis, disease-specific primary cell-culture models of antigen presenting cells could be employed to examine the immunogenic potential of AAQ6C in patients with schizophrenia. Additionally, animal models immunised with AAQ6C, or purified anti-AAQ6C IgG, could be used to examine the potential functions of anti-AAQ6C IgG.

4. Genetic Variation and Anti-Gliadin Antibodies

4.1 Introduction

It has been reported that, in comparison to controls from the general population, first-degree relatives of patients with schizophrenia have a higher risk of developing schizophrenia, have higher PANSS scores, and are also more likely to develop schizophrenia-related psychiatric disorders such as schizoaffective or bipolar disorder (Hembram et al., 2014; Kendler et al., 1985). These observations suggest that a genetic component predisposes individuals to schizophrenia, in addition to potential environmental risk factors and triggers. There is debate over the level of the contribution of an overall genetic burden to the development of schizophrenia, and to what degree the condition is heritable; however, twin studies suggest that the heritability of the disease is up to 80% (Cardno and Gottesman, 2000).

Schizophrenia is highly heterogeneous in both clinical presentation and genetic transmission. A large number of small studies identified up to 800 loci for associations with schizophrenia, although replication of these initial findings was very poor across studies (Gejman et al., 2010). For example, Sanders and co-workers performed an association study with 1870 cases and 2002 controls to examine 14 most replicable genetic findings, including *DISC1* and *DRD2*, but failed to show an association between these candidate genes and schizophrenia at a genome-wide significance level (Sanders et al., 2008).

In the genetic analysis of schizophrenia, a number of GWA studies have demonstrated genome-wide significant associations between schizophrenia and the HLA region present in the short arm of chromosome 6 (International Schizophrenia Consortium et al., 2009; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The main role of polymorphic HLA molecules is to present protein-derived antigens to T lymphocytes and to genetically influence the functions of antigen-presenting cells (APCs), which include the initiation and regulation of immune responses, as well as the induction of immune tolerance. Since the HLA region is one of the most variable chromosomal regions in the human genome, specific risk alleles, such as HLA-DQ2/DQ8 contributing to both coeliac disease and type-1

diabetes, have yet to emerge as risk factors for schizophrenia. Furthermore, as the HLA-region displays a high density of genes, some non-HLA gene loci that are not involved in antigen presentation may be responsible for the disease-association signal at this locus (Sekar et al., 2016). Additionally, GWA studies that stratified individuals with schizophrenia according to exposure to infectious agents strengthened the association between schizophrenia and previously identified risk loci, such as the *CDH3* and *ARNTL* genes. Additionally, this study was able to identify and replicate novel risk loci in the *CTNNA3* gene coding for catenin alpha-3, involved in cell-cell adhesion, and the *ZEB1* promotor that was associated with the regulation of cell-cell adhesion (Børglum et al., 2014).

In addition to the associations of the HLA locus with schizophrenia, GWA studies have highlighted a number of associations between schizophrenia and immune-related loci, or genes highly expressed by immune cells (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). Apart from the HLA association, there is limited gene-enrichment for immunologically related genes in schizophrenia, when compared to typical autoimmune diseases, though this does not exclude the involvement of the immune system because many of the genes interacting with the identified loci have been confirmed to have functional activity within the CNS (Pouget et al., 2016).

This study was undertaken to genotype some SNPs present in or near to genes related to immunological function, to confirm their association with schizophrenia in a case-control sample set and to examine the association between these SNPs and humoral immune responses to native gliadin and gliadin-derived fragments.

4.2 Materials and methods

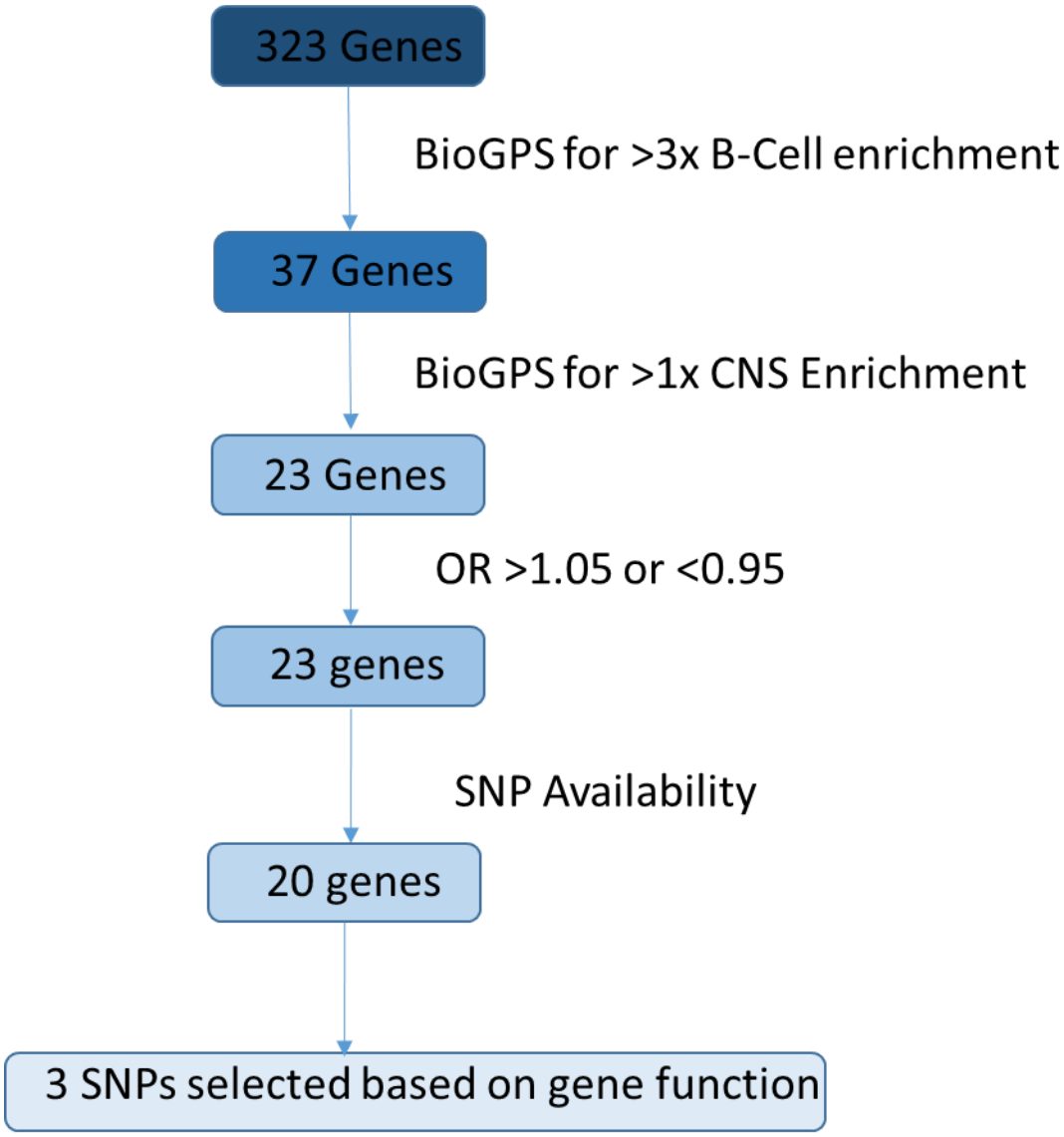
4.2.1 Case and control samples

A total of 957 DNA samples, including 467 schizophrenia cases and 490 healthy controls, were genotyped using the TaqMan protocol as described in Chapter 2. These DNA samples were extracted from white blood cells in whole blood. The antibody levels in plasma are those reported in chapter 3 and therefore were measured using the protocols as described in Chapters 2 and 3.

4.2.2 Selection of SNPs for Genotyping

A GWA-based meta-analysis confirmed 108 genetic loci associated with schizophrenia, in which the index SNPs were identified using a statistical approach with 99% credibility by the Schizophrenia Working Group of the Psychiatric Genomics Consortium (2014). SNPs genotyped in this study were selected based on the following criteria. 1) Genes proximal to SNPs were examined for their enrichment in B-cells. 2) Genes with >3x Enrichment were then examined for their expression levels in the CNS (>1x). 3) From these genes none had an OR >0.95 but <1.05. 4) From the remaining genes three did not have available rs numbers. 5) Three SNPs were then selected based on their gene function and the candidate's opinion of their potential relevance to the pathology of schizophrenia (this process is summarised in *Figure 4.1*).

Figure 4.1 Flow chart summarising the process of SNP selection



The Schizophrenia Working Group of the Psychiatric Genomics Consortium, (2014) identified 108 genetic loci associated with schizophrenia. This included 128 SNPs associated with 323 genes. The genes were examined for their enrichment in B-cells using BioGPS (Wu et al., 2009). Of 323 genes 37 were showed enrichment in B-cells >3x the mean enrichment in other tissues. These genes were also examined for their elevated enrichment in CNS tissues, resulting in 23 genes that had elevated enrichment in B-cells and the CNS. Of these genes none had an OR <1.05 and >0.95. 3 genes were not associated with SNPs, leaving 20 potential genes of interest. The three SNPs used in this study were then selected by the candidate.

Details of the SNPs used in this study are given in *Table 4.1*. HLA-tag SNP rs1233578, which was the most strongly associated SNP identified by the Schizophrenia Working Group of the Psychiatric Genomics Consortium, (2014), was also genotyped for detection of the HLA association with schizophrenia in this study.

Table 4.1. List of SNPs genotyped in this study

SNP ID	Chromosomal location	Base change	Proximal gene	Distance from gene
rs4388249	5:109700365	C/T	*MAN2A1*	Intragenic
rs11139497	9:82125026	A/T	*TLE1*	0.5mb
rs11682175	2:57760458	C/T	*FANCL/VRK2*	0.14mb

Chromosomal location according to UCSC hg19/NCBI Build 37. Mb= megabase pairs

4.2.3 TaqMan® genotyping protocol

The TaqMan® genotyping assay was performed in a 10µl volume, with each reaction well containing 3.25µl of autoclaved water, 0.25µl of Taqman Probe, 5µl of MasterMix and 1.5µl of DNA sample, or 1.5µl of DNA-free water in the case of negative controls. To ensure adequate mixture of samples, the plate was spun for approximately 5-10 seconds. In order to avoid unwanted premature polymerase activity, the reagents were loaded on a frozen block. All probes were supplied by ThermoFisher Scientific (Perth, UK) and the reactions took place in a 96-well MicroAmp Reaction Plate (ThermoFisher Scientific, Perth, UK). The conditions used for the TaqMan® genotyping are given in *Table 4.2*.

Table 4.2. The conditions used for TaqMan genotyping assay

Stage	Temperature	Time (mins)
Pre-PCR Read	60°C	0.5
	95°C	10
Cycling Stage (50 Cycles)	95°C	0.25
	60°C	1
Post-PCR Read	60°C	0.5

Genotype calling was performed automatically based on the allele-specific fluorescent signal, with the calls being made depending on the profile of the fluorescent signal from the two reporter dyes, VIC and FAM, relating to either the two homozygous or the heterozygous groups.

4.2.4 Statistical Analysis

The linkage file format was applied to build a datasheet with the following information, family ID, individual ID, father ID, mother ID, gender, affection status and SNP genotypes. The UNPHASED program (version 3.1.7) was utilised to analyse allelic association between SNPs genotyped and schizophrenia (Dudbridge, 2008), in which a likelihood χ^2 test was performed with 10000 permutations to determine if any allele was associated with risk of schizophrenia.

4.2.5 Sample power test

Based on the results from GWA study, case-control samples always generate a small effect size that is expressed as odds ratio (OR). For this reason, a power test was performed against each SNP genotyped. SPSS SamplePower 2.0 was used to test sample power for detection of OR=1.5 (or 1/1.5=0.67), which is defined as small effect size, based on the Cohen's conventions (Cohen, 1988). The sample power was, therefore, estimated based on a type I error rate of 0.125% (2-tailed) that was corrected for multiple testing due to 4 SNPs genotyped, i.e. the risk of rejecting the null hypothesis when it is true.

4.3 Results

4.3.1 Association of selected SNPs with schizophrenia

Four index SNPs were selected according to a previous GWA study (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014) and genotyped to determine their allelic association with schizophrenia. As shown in *Table 4.3*, none of these 4 SNPs was shown to be associated with the disease.

Table 4.3 Allelic association of 4 index SNPs genotyped with schizophrenia

SNP	Locus	Group	Allele Frequency		X^2	OR	95%CI	p-value
rs4388249	MAN2A1		C	T	0.154	0.952	0.744-1.218	0.695
		Control	848	188				
		Case	616	130				
rs11139497	TLE1		A	T	0.026	0.983	0.803-1.204	0.871
		Control	341	695				
		Case	237	475				
rs11682175	VRK2		C	T	0.629	0.924	0.760-1.123	0.428
		Control	495	523				
		Case	338	330				

The allele frequency of the SNVs of interest was compared between cases and controls from DNA samples in the Aberdeen Schizophrenia Biobank. Allelic frequency was not significantly different between cases and controls for any SNP tested (p= >0.05). The global p-value was 0.894 based on 10,000 permutations performed with a built-in program within UNPHASED.

The power test showed that in detection of OR=1.5 (or 0.67) at the type I error rate of 0.125% (2-tailed), these samples had a power of 83% at rs4388249, 73% at rs1233578, 94% at both rs11139497 94% and rs11682175.

The genotype frequency was also examined in control individuals and patients with schizophrenia. It was found that the distribution of genotype frequency was significantly different in patients with schizophrenia, with individuals homozygous for the major allele being more frequent in patients with schizophrenia than control individuals (X^2 = 11.459, p= 0.003).

Table 4.4 Genotype association of 4 index SNPs genotyped with schizophrenia

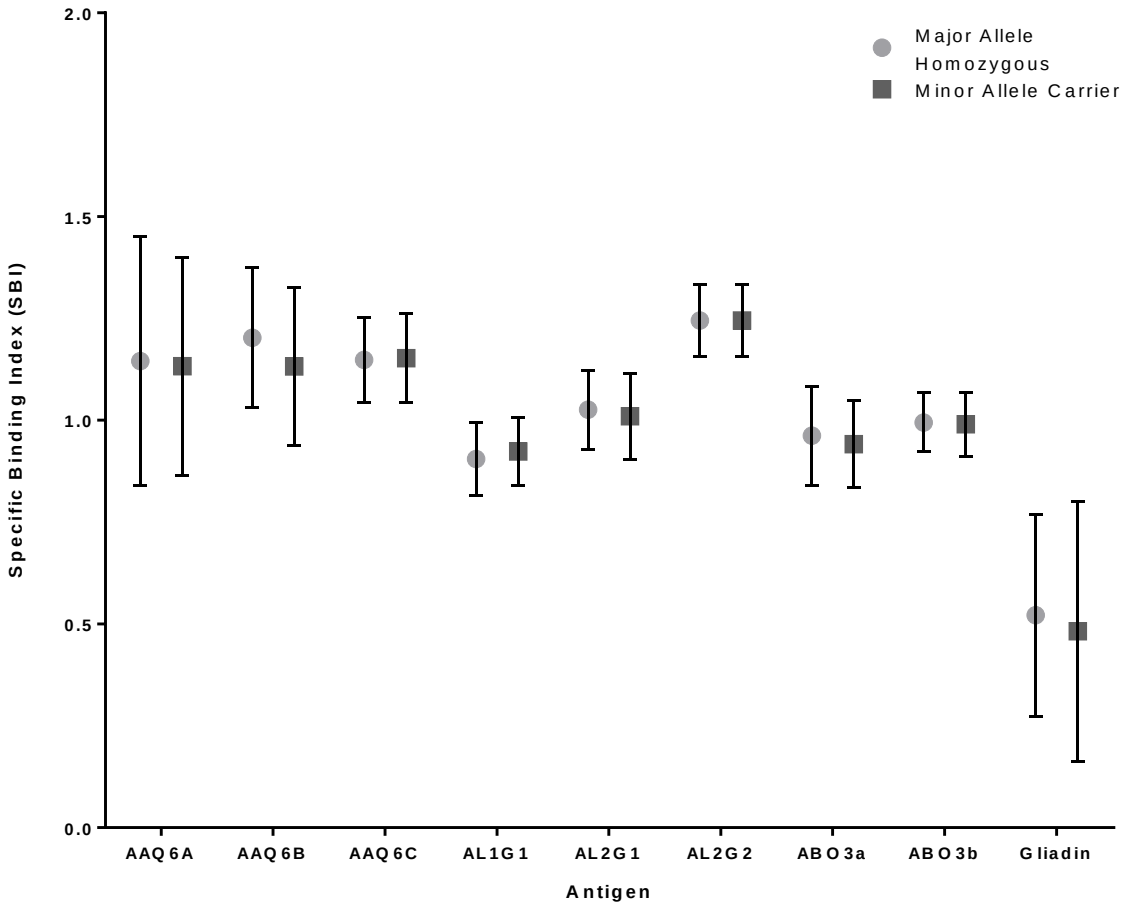
SNP	Locus	Group	Genotype Frequency			X^2	df	p-value
			1/1	1/2	2/2			
rs4388249	MAN2A1	Control	178	76	7	1.404	2	0.495
		Case	129	36	5			
rs11139497	TLE1	Control	55	110	96	2.904	2	0.234
		Case	48	65	57			
rs11682175	VRK2	Control	78	118	65	11.459	2	**0.003**
		Case	78	58	34			

Genotype distribution of selected SNPs in schizophrenia cases and controls was examined using Chi^2 using matched plasma-DNA samples from the Schizophrenia Aberdeen Biobank. The genotype frequency was not significantly different between control individuals and patients with schizophrenia for any SNP with the exception of rs11682175 (X^2 =11.459, p= 0.003). Statistical significance was set at p= <0.005. Significant associations are highlighted in bold.

4.3.2 Association between genetic variants and plasma anti-gliadin antibody levels

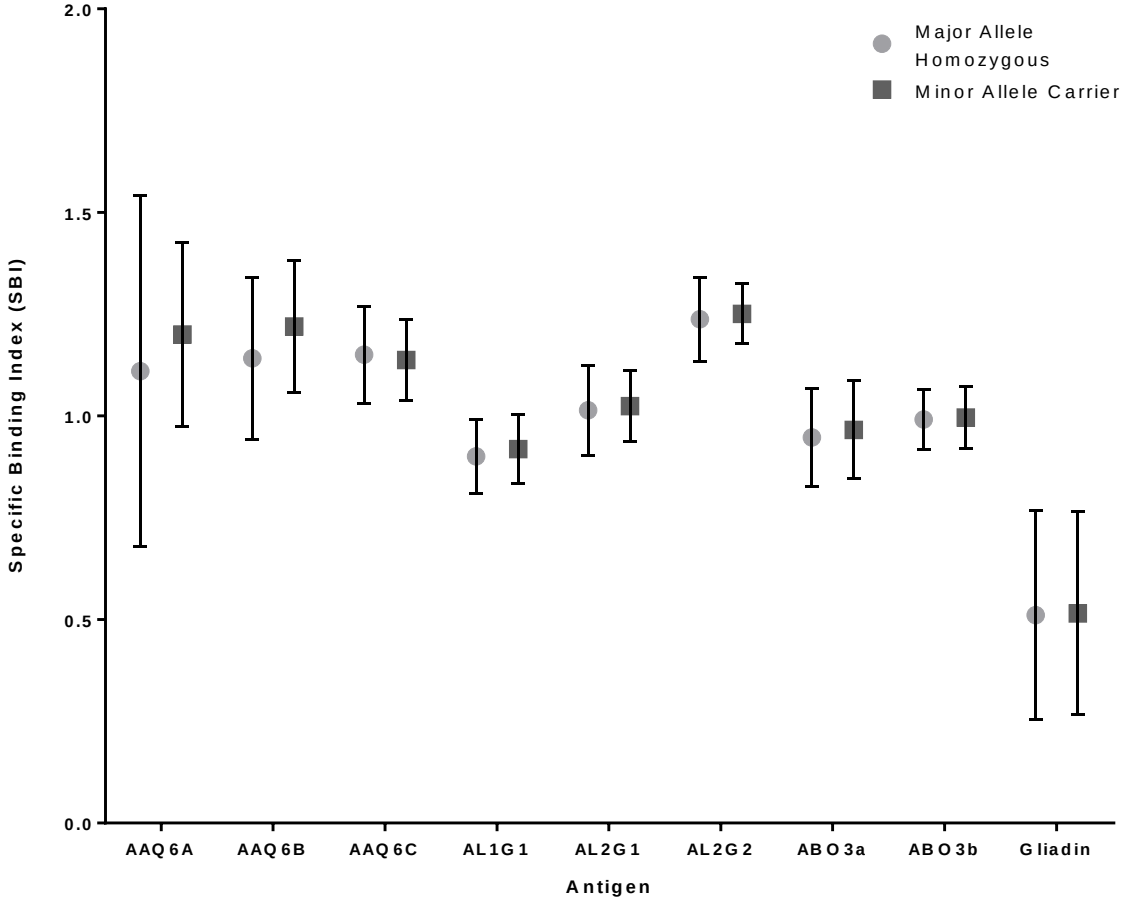
Although no genetic variants were found to be significantly associated with schizophrenia in this study sample, the association between plasma anti-gliadin antibodies and genetic variants was examined. In this analysis, cases and controls were grouped together and the genotyping data were split into the presence or absence of a variant of interest. As shown in *Figure 4.2-Figure 4.4*, there was no significant difference in plasma anti-gliadin IgG levels between the carriers and none carriers of the variants of interest as all statistical tests failed to survive the Bonferroni correction for multiple tests (p>0.006).

Figure 4.2 Genetic association of the MAN2A1 locus with plasma anti-gliadin IgG levels



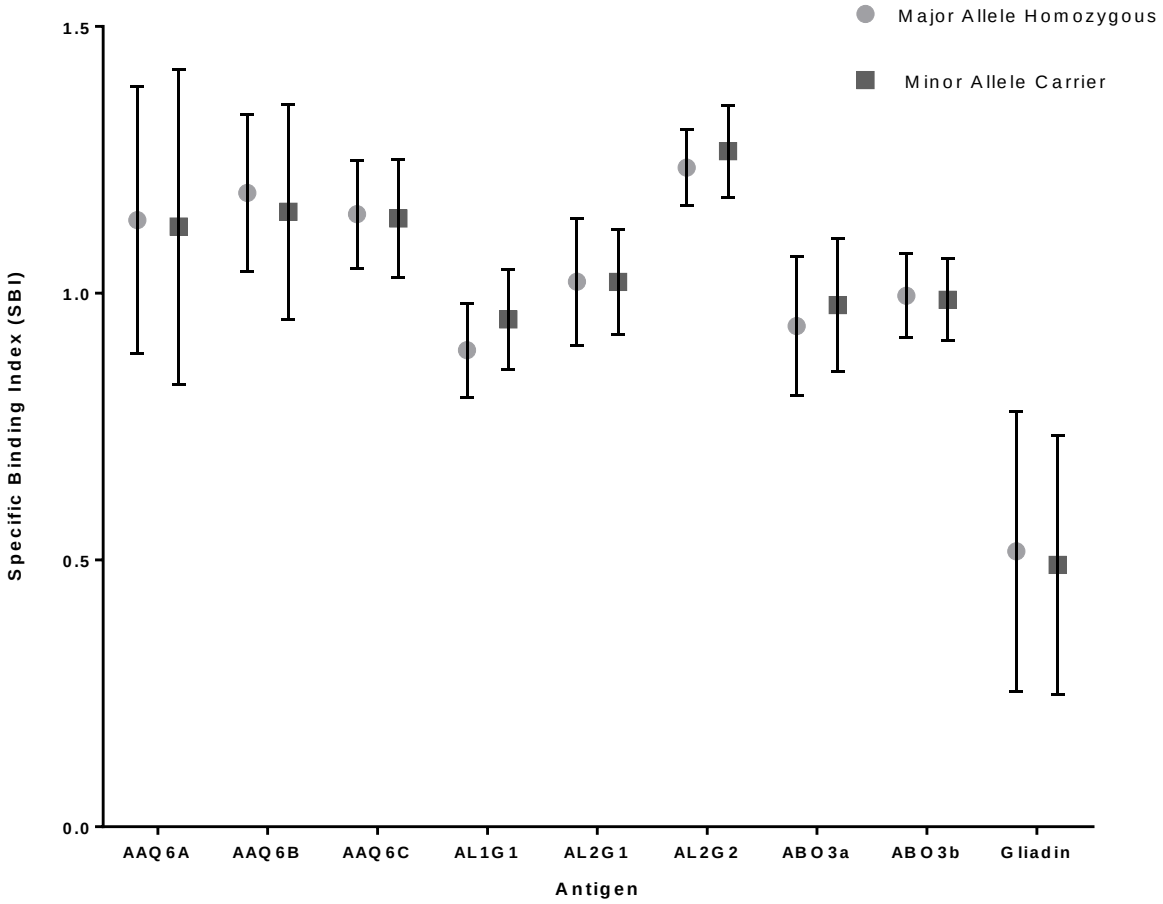
Median and IQR of levels of IgG against gliadins in major allele homozygotes and minor allele carriers of rs4388249(T). No significant differences were observed in plasma anti-AAQ6A IgG levels between major allele homozygotes (n= 209) and minor allele (rs4388249-T) carriers (n= 87), anti-AAQ6B IgG levels between minor allele (rs4388249-T) carriers (n= 87) and major allele homozygotes (n= 209), anti-AAQ6C IgG levels between carriers (n= 87) and major allele homozygotes (n= 209), anti-AL1G1 IgG levels between minor allele (rs4388249-T) carriers (n= 88) and major allele homozygotes (n= 210), anti-AL2G1 IgG levels between minor allele (rs4388249-T) carriers (n= 87) and major allele homozygotes (n= 209), anti-AL2G2 IgG levels between minor allele (rs4388249-T) carriers (n= 87) and major allele homozygotes (n= 209), anti-ABO3a IgG levels between minor allele (rs4388249-T) carriers (n= 87) and major allele homozygotes (n= 204), anti-ABO3b IgG levels between minor allele (rs4388249-T) carriers (n= 87) and major allele homozygotes (n= 204) and anti-gliadin IgG levels between minor allele (rs4388249-T) carriers (n= 88) and major allele homozygotes (n= 210). (p >0.006)

Figure 4.3 Genetic association of the TLE1 locus with plasma anti-gliadin IgG levels



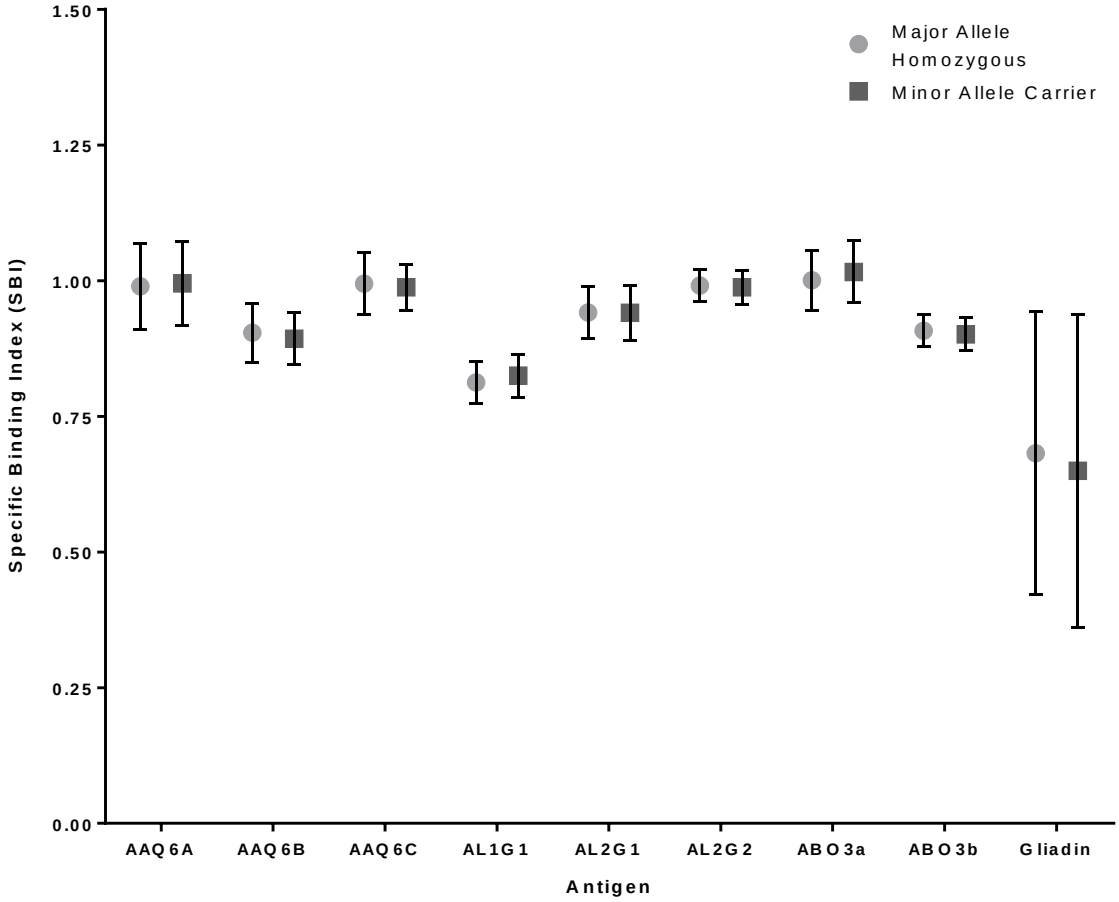
Median and IQR of levels of IgG against gliadins in major allele homozygotes and minor allele carriers of rs11139497(A). No significant differences were observed in plasma anti-AAQ6A IgG levels between major allele homozygotes (n= 124) and minor allele (rs11139497-A) carriers (n= 179), anti-AAQ6B IgG levels between minor allele (rs11139497-A) carriers (n= 179) and major allele homozygotes (n= 124), anti-AAQ6C IgG levels between minor allele (rs11139497-A) carriers (n= 179) and major allele homozygotes (n= 124), anti-AL1G1 IgG levels between minor allele (rs11139497-A) carriers (n= 180) and major allele homozygotes (n= 125), anti-AL2G1 IgG levels between minor allele (rs11139497-A) carriers (n= 179) and major allele homozygotes (n= 124), anti-AL2G2 IgG levels between minor allele (rs11139497-A) carriers (n= 179) and major allele homozygotes (n= 124), anti-ABO3a IgG levels between minor allele (rs11139497-A) carriers (n= 175) and major allele homozygotes (n= 123), anti-ABO3b IgG levels between minor allele (rs11139497-A) carriers (n= 175) and major allele homozygotes (n= 123) and anti-gliadin IgG levels between minor allele (rs11139497-A) carriers (n= 180) and major allele homozygotes (n= 125). ($p > 0.006$)

Figure 4.4 Genetic association of the VRK2/FANCL locus with plasma anti-gliadin IgG levels



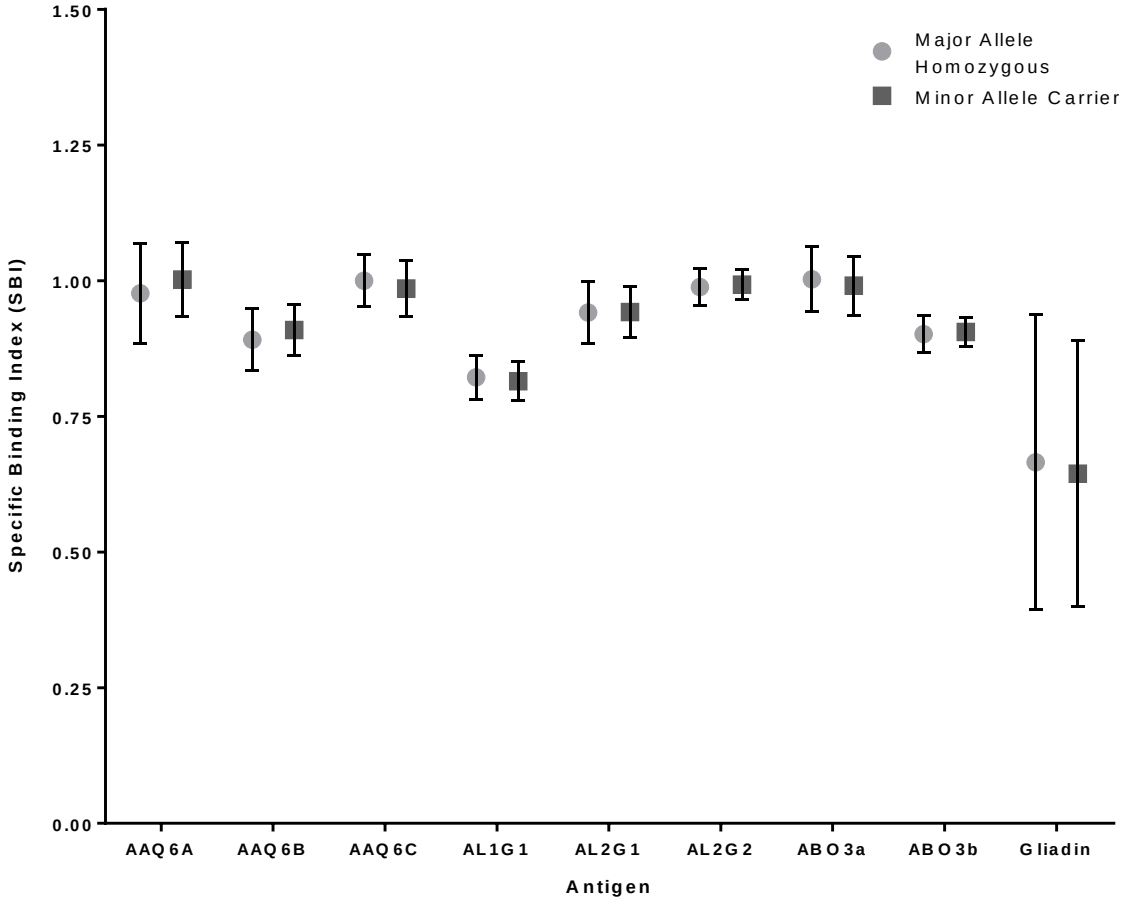
Median and IQR of levels of IgG against gliadins in major allele homozygotes and minor allele carriers of rs11682175(C). No significant differences were observed in plasma anti-AAQ6A IgG levels between major allele homozygotes (n= 80) and minor allele (rs11682175-C) carriers (n= 218), anti-AAQ6B IgG levels between minor allele (rs11682175-C) carriers (n= 216) and major allele homozygotes (n= 80), anti-AAQ6C IgG levels between minor allele (rs11682175-C) carriers (n= 80) and major allele homozygotes (n= 216), anti-AL1G1 IgG levels between minor allele (rs11682175-C) carriers (n= 216) and major allele homozygotes (n= 80), anti-AL2G1 IgG levels between minor allele (rs11682175-C) carriers (n= 216) and major allele homozygotes (n= 80), anti-AL2G2 IgG levels between minor allele (rs11682175-C) carriers (n= 216) and major allele homozygotes (n= 80), anti-ABO3a IgG levels between minor allele (rs11682175-C) carriers (n= 214) and major allele homozygotes (n= 78), anti-ABO3b IgG levels between minor allele (rs11682175-C) carriers (n= 214) and major allele homozygotes (n= 78) and anti-gliadin IgG levels between minor allele (rs11682175-C) carriers (n= 218) and major allele homozygotes (n= 80). ($p > 0.006$)

Figure 4.5 Genetic association of the MAN2A1 locus with plasma anti-gliadin IgA levels



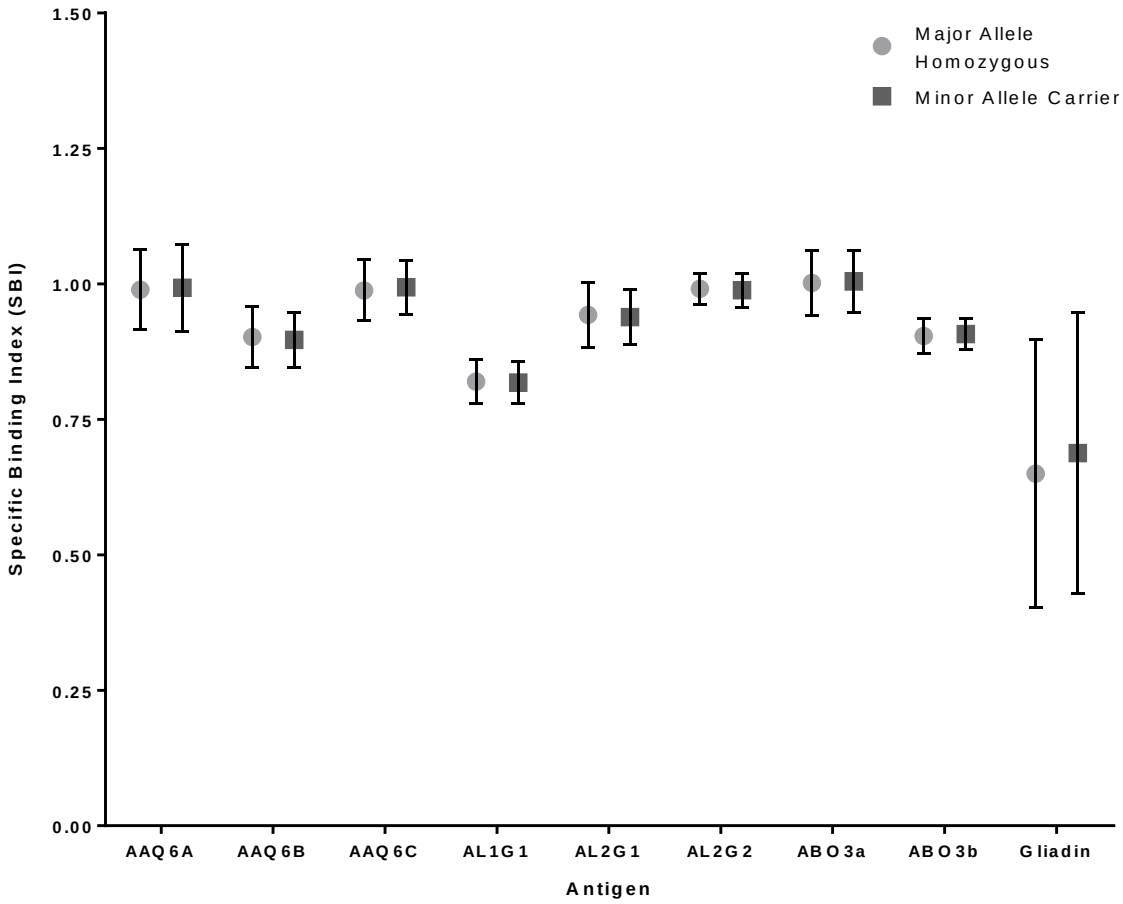
Median and IQR of levels of IgA against gliadins in major allele homozygotes and minor allele carriers of rs4388249. No significant differences were observed in plasma anti-AAQ6A IgA levels between major allele homozygotes (n= 216) and minor allele (rs4388249-T) carriers (n= 92), anti-AAQ6B IgG levels between minor allele (rs4388249-T) carriers (n= 92) and major allele homozygotes (n= 216), anti-AAQ6C IgA levels between carriers (n= 92) and major allele homozygotes (n= 216), anti-AL1G1 IgA levels between minor allele (rs4388249-T) carriers (n= 92) and major allele homozygotes (n= 216), anti-AL2G1 IgA levels between minor allele (rs4388249-T) carriers (n= 92) and major allele homozygotes (n= 216), anti-AL2G2 IgA levels between minor allele (rs4388249-T) carriers (n= 92) and major allele homozygotes (n= 216), anti-ABO3a IgA levels between minor allele (rs4388249-T) carriers (n= 92) and major allele homozygotes (n= 216), anti-ABO3b IgG levels between minor allele (rs4388249-T) carriers (n= 92) and major allele homozygotes (n= 216) and anti-gliadin IgA levels between minor allele (rs4388249-T) carriers (n= 92) and major allele homozygotes (n= 216). ($p > 0.006$)

Figure 4.6 Genetic association of the TLE1 locus with plasma anti-gliadin IgA levels



Median and IQR of levels of IgA against gliadins in major allele homozygotes and minor allele carriers of rs11139497. No significant differences were observed in plasma anti-AAQ6A IgA levels between major allele homozygotes (n= 124) and minor allele (rs11139497-A) carriers (n= 184), anti-AAQ6B IgG levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 124), anti-AAQ6C IgA levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 124), anti-AL1G1 IgA levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 124), anti-AL2G1 IgA levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 124), anti-AL2G2 IgA levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 124), anti-ABO3a IgA levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 124), anti-ABO3b IgA levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 124) and anti-gliadin IgG levels between minor allele (rs11139497-A) carriers (n= 184) and major allele homozygotes (n= 122). ($p > 0.006$)

Figure 4.7 Genetic association of the VRK2/FANCL locus with plasma anti-gliadin IgA levels



Median and IQR of levels of IgA against gliadins in major allele homozygotes and minor allele carriers of rs11682175. No significant differences were observed in plasma anti-AAQ6A IgA levels between major allele homozygotes (n= 84) and minor allele (rs11682175-C) carriers (n= 224), anti-AAQ6B IgA levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84), anti-AAQ6C IgA levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84), anti-AL1G1 IgG levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84), anti-AL2G1 IgA levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84), anti-AL2G2 IgA levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84), anti-ABO3a IgA levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84), anti-ABO3b IgA levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84) and anti-gliadin IgA levels between minor allele (rs11682175-C) carriers (n= 224) and major allele homozygotes (n= 84). ($p > 0.006$)

For all variants tested in this study (*Figure 4.2-Figure 4.7*), there were no significant associations between levels of antibodies against gliadins and the presence of the minor allele for the genotyped SNPs.

In order to detect potential schizophrenia-specific interactions between levels of circulating antibodies against gliadins and selected SNPs, levels of antibodies against gliadins

were examined by carrier status in patients with schizophrenia and control individuals (*Table 4.5- Table 4.7*). As shown in *Table 4.5* levels of anti-AL2G2 IgG were significantly lower in carriers of rs11682175(C), when compared to non-carriers in the control group only (Z= -3.376, p= 0.001).

Table 4.5 Examination of levels of Anti-Gliadin IgGs in carriers and non-carriers of Selected Single Nucleotide Variants by schizophrenia status (A)

Minor allele (gene)	Schizophrenia State	Carrier Status	**AL1G1**					**AL2G1**					**AL2G2**				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
rs4388249-T (MAN2A1)	Control	Non-carrier	123	0.9177	0.17	-2.264	0.024	123	1.0443	0.16	-0.862	0.389	123	1.2716	0.17	-0.886	0.375
		Carrier	47	0.9761	0.15			47	1.0917	0.18			47	1.2827	0.14		
	Case	Non-carrier	87	0.8804	0.20	-0.336	0.737	86	0.9416	0.20	-0.837	0.403	86	1.2293	0.17	-0.192	0.848
		Carrier	41	0.8513	0.17			40	0.9086	0.19			40	1.1870	0.16		
rs11139497-A (TLE1)	Control	Non-carrier	68	0.9423	0.16	-1.011	0.312	68	1.0720	0.21	-0.818	0.413	68	1.3006	0.15	-1.497	0.134
		Carrier	102	0.9329	0.16			102	1.0454	0.14			102	1.2615	0.12		
	Case	Non-carrier	57	0.8831	0.19	-1.123	0.261	56	0.9439	0.22	-0.419	0.675	56	1.2319	0.16	-1.123	0.261
		Carrier	78	0.8544	0.20			77	0.9150	0.18			77	1.2040	0.17		
rs11682175-C (VRK2)	Control	Non-carrier	46	0.9597	0.17	-1.849	0.064	46	1.0980	0.22	-1.559	0.119	**46**	**1.3007**	**0.12**	**-3.376**	**0.001**
		Carrier	124	0.9164	0.16			124	1.0410	0.16			**124**	**1.2452**	**0.17**		
	Case	Non-carrier	34	0.9328	0.17	-1.459	0.145	34	0.9006	0.15	-2.713	0.007	34	1.2269	0.13	-0.161	0.872
		Carrier	94	0.8506	0.19			92	0.9361	0.21			92	1.2108	0.19		

Table 4.6 Examination of levels of Anti-Gliadin IgGs in carriers and non-carriers of Selected Single Nucleotide Variants by schizophrenia status (B)

Minor allele (gene)	Schizophrenia State	Carrier Status	**AAQ6A**					**AAQ6B**					**AAQ6C**				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
rs4388249-T (MAN2A1)	Control	Non-carrier	123	1.1030	0.63	-0.106	0.915	123	1.1281	0.35	-0.031	0.976	123	1.1139	0.19	-0.796	0.426
		Carrier	47	1.1180	0.64			47	1.1804	0.34			47	1.1403	0.17		
	Case	Non-carrier	86	1.1790	0.57	-0.042	0.967	86	1.2477	0.31	-0.409	0.683	86	1.2333	0.22	-1.023	0.306
		Carrier	40	1.1925	0.43			40	1.0895	0.50			40	1.1750	0.30		
rs11139497-A (TLE1)	Control	Non-carrier	68	1.1000	0.77	-0.015	0.988	68	1.2054	0.37	-1.420	0.156	68	1.1191	0.22	-0.183	0.855
		Carrier	102	1.1145	0.56			102	1.1009	0.29			102	1.1233	0.15		
	Case	Non-carrier	56	1.4205	0.91	-0.374	0.709	56	1.2266	0.39	-1.604	0.109	56	1.2197	0.26	-0.124	0.901
		Carrier	77	1.0750	0.26			77	1.1969	0.36			77	1.2361	0.23		
rs11682175-C (VRK2)	Control	Non-carrier	46	1.1055	0.62	-0.047	0.962	46	1.0778	0.27	-0.746	0.456	46	1.1288	0.16	-0.310	0.757
		Carrier	124	1.1070	0.65			124	1.1519	0.37			124	1.1151	0.19		
	Case	Non-carrier	34	1.1430	0.46	-0.390	0.696	34	1.1716	0.30	-0.187	0.852	34	1.2266	0.20	-0.117	0.907
		Carrier	92	1.1865	0.54			92	1.2197	0.40			92	1.2241	0.27		

Table 4.7 Examination of levels of Anti-Gliadin IgGs in carriers and non-carriers of Selected Single Nucleotide Variants by schizophrenia status (C)

Minor allele (gene)	Schizophrenia State	Carrier Status	ABO3a					ABO3b					Gliadin				
			n	Median	IQR	Z	p	N	Median	IQR	Z	p	n	Median	IQR	Z	p
rs4388249-T (MAN2A1)	Control	Non-carrier	123	1.0100	0.20	-0.440	0.660	123	1.0057	0.16	-0.321	0.748	123	0.5532	0.45	-0.479	0.632
		Carrier	47	0.9840	0.20			47	1.0074	0.14			47	0.4442	0.55		
	Case	Non-carrier	81	0.8685	0.29	-0.469	0.639	81	0.9679	0.14	-0.192	0.848	87	0.5107	0.51	-0.742	0.458
		Carrier	40	0.8260	0.17			40	0.9079	0.16			41	0.5320	0.64		
rs11139497-A (TLE1)	Control	Non-carrier	68	1.0265	0.20	-0.189	0.850	68	1.0152	0.17	-0.275	0.784	68	0.5185	0.49	-0.317	0.751
		Carrier	102	0.9945	0.20			102	1.0000	0.15			102	0.5151	0.50		
	Case	Non-carrier	55	0.8785	0.26	-0.391	0.696	55	0.9697	0.13	-0.055	0.956	57	0.5165	0.58	-0.223	0.824
		Carrier	73	0.8385	0.17			73	0.9432	0.15			78	0.5041	0.54		
rs11682175-C (VRK2)	Control	Non-carrier	46	0.9965	0.20	-0.244	0.807	46	1.0217	0.15	-0.396	0.692	46	0.5079	0.53	-0.223	0.823
		Carrier	124	1.0060	0.20			124	1.0038	0.15			124	0.5535	0.46		
	Case	Non-carrier	32	0.8635	0.32	-0.838	0.402	32	0.9656	0.14	-0.311	0.756	34	0.4820	0.63	-0.158	0.875
		Carrier	90	0.8600	0.19			90	0.9432	0.16			94	0.5320	0.52		

Comparison between the median levels of IgG against AL1G1, AL2G1, AL2G2 (A), AAQ6A, AAQ6B, AAQ6C (B), ABO3a, ABO3b and native gliadin (C), in carriers and non-carriers of SNVs of interest in patients with schizophrenia and control individuals. Levels of IgG against gliadins were not significantly different between carriers of SNVs of interest in either cases or controls, with the exception of decreased levels of anti-ABO3a IgG in carriers of rs11682175, proximal to the *VRK2* locus (z= -3.376, p= 0.001). Mann-Whitney U test, p= 0.006 was used as the statistical cut-off for significance.

Generally, decreased levels of plasma IgA against gliadins were not associated with any variant tested in this study in either patients with schizophrenia or control individuals (*Table 4.8* - *Table 4.10*). Levels of IgA against ABO3b were lower in carriers of rs11139497(A), compared to non-carriers, in the control group only (*Table 4.10*).

Table 4.8 Examination of levels of Anti-Gliadin IgAs in carriers and non-carriers of Selected Single Nucleotide Variants by schizophrenia status (A)

Minor allele (gene)	Schizophrenia State	Carrier Status	**AL1G1**					**AL2G1**					**AL2G2**				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
rs4388249-T (MAN2A1)	Control	Non-carrier	134	0.8260	0.07	-0.792	0.428	134	0.9567	0.09	-0.687	0.492	134	1.0022	0.06	-0.335	0.737
		Carrier	56	0.8282	0.06			56	0.9531	0.12			56	1.0034	0.07		
	Case	Non-carrier	82	0.7892	0.08	-0.336	0.737	82	0.9073	0.10	-0.837	0.403	82	0.9695	0.07	-0.192	0.848
		Carrier	36	0.8090	0.09			36	0.9032	0.09			36	0.9695	0.06		
rs11139497-A (TLE1)	Control	Non-carrier	73	0.8262	0.07	-0.322	0.747	73	0.9647	0.08	-1.679	0.093	73	1.0128	0.06	-1.805	0.071
		Carrier	117	0.8279	0.06			117	0.9526	0.10			117	0.9993	0.06		
	Case	Non-carrier	51	0.7805	0.07	-1.123	0.261	51	0.8982	0.08	-0.419	0.675	51	0.9690	0.07	-1.123	0.261
		Carrier	67	0.8056	0.08			67	0.9127	0.10			67	0.9726	0.06		
rs11682175-C (VRK2)	Control	Non-carrier	52	0.8384	0.06	-1.785	0.074	52	0.9581	0.08	-0.902	0.367	52	1.0006	0.05	-0.264	0.792
		Carrier	138	0.8254	0.07			138	0.9520	0.10			138	1.0025	0.06		
	Case	Non-carrier	32	0.7801	0.06	-1.459	0.145	32	0.8712	0.10	-2.713	0.007	32	0.9653	0.06	-0.161	0.872
		Carrier	86	0.8041	0.09			86	0.9126	0.10			86	0.9712	0.07		

Table 4.9 Examination of levels of Anti-Gliadin IgAs in carriers and non-carriers of Selected Single Nucleotide Variants by schizophrenia status (B)

Minor allele (gene)	Schizophrenia State	Carrier Status	**AAQ6A**					**AAQ6B**					**AAQ6C**				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
rs4388249-T (MAN2A1)	Control	Non-carrier	134	1.0214	0.16	-0.315	0.753	134	0.9203	0.12	-0.260	0.795	134	1.0246	0.11	-1.208	0.227
		Carrier	56	1.0170	0.14			56	0.8992	0.09			56	1.0076	0.09		
	Case	Non-carrier	82	0.9361	0.11	-0.042	0.967	82	0.8906	0.09	-0.409	0.683	82	0.9581	0.10	-1.023	0.306
		Carrier	36	0.9183	0.14			36	0.8775	0.09			36	0.9648	0.09		
rs11139497-A (TLE1)	Control	Non-carrier	73	1.0400	0.15	-1.270	0.204	73	0.9217	0.11	-0.542	0.588	73	1.0065	0.10	-1.640	0.101
		Carrier	117	1.0090	0.15			117	0.9085	0.09			117	1.0269	0.10		
	Case	Non-carrier	51	0.9432	0.13	-0.374	0.709	51	0.8993	0.11	-1.604	0.109	51	0.9643	0.10	-0.124	0.901
		Carrier	67	0.9211	0.10			67	0.8767	0.08			67	0.9592	0.09		
rs11682175-C (VRK2)	Control	Non-carrier	52	1.0090	0.14	-0.381	0.703	52	0.8918	0.12	-0.879	0.380	52	1.0089	0.11	-0.119	0.905
		Carrier	138	1.0192	0.15			138	0.9212	0.10			138	1.0185	0.09		
	Case	Non-carrier	32	0.9240	0.18	-0.390	0.696	32	0.8894	0.10	-0.187	0.852	32	0.9617	0.09	-0.117	0.907
		Carrier	86	0.9330	0.10			86	0.8828	0.08			86	0.9602	0.10		

Table 4.10 Examination of levels of Anti-Gliadin IgAs in carriers and non-carriers of Selected Single Nucleotide Variants by schizophrenia status (C)

Minor allele (gene)	Schizophrenia State	Carrier Status	**ABO3a**					**ABO3b**					**Gliadin**				
			n	Median	IQR	Z	p	n	Median	IQR	Z	p	n	Median	IQR	Z	p
rs4388249-T (MAN2A1)	Control	Non-carrier	134	1.0304	0.08	-1.132	0.258	134	0.9171	0.04	-0.443	0.658	134	0.6552	0.51	-1.133	0.257
		Carrier	56	1.0419	0.06			56	0.9136	0.05			56	0.6995	0.57		
	Case	Non-carrier	82	0.9364	0.07	-0.469	0.639	82	0.8705	0.05	-0.192	0.848	82	0.6667	0.57	-0.742	0.458
		Carrier	36	0.9261	0.08			36	0.8690	0.07			36	0.5937	0.54		
rs11139497-A (TLE1)	Control	Non-carrier	73	1.0433	0.08	-0.934	0.350	**73**	**0.9259**	**0.05**	**-2.853**	**0.004**	73	0.6921	0.56	-0.333	0.739
		Carrier	117	1.0291	0.07			**117**	**0.9134**	**0.04**			117	0.6751	0.48		
	Case	Non-carrier	51	0.9253	0.08	-0.391	0.696	51	0.8692	0.06	-0.055	0.956	51	0.6297	0.47	-0.223	0.824
		Carrier	67	0.9363	0.06			67	0.8708	0.06			67	0.6338	0.62		
rs11682175-C (VRK2)	Control	Non-carrier	52	1.0396	0.07	-0.531	0.595	52	0.9141	0.04	-1.071	0.284	52	0.7181	0.49	-1.569	0.117
		Carrier	138	1.0313	0.08			138	0.9171	0.04			138	0.6668	0.53		
	Case	Non-carrier	32	0.9248	0.07	-0.838	0.402	32	0.8618	0.08	-0.311	0.756	32	0.6051	0.55	-0.158	0.875
		Carrier	86	0.9339	0.06			86	0.8729	0.05			86	0.6447	0.58		

Comparison between the median levels of IgA against AL1G1, AL2G1, AL2G2 (A), AAQ6A, AAQ6B, AAQ6C (B), ABO3a, ABO3b and native gliadin (C), in carriers and non-carriers of SNVs of interest in patients with schizophrenia and control individuals. Levels of IgG against gliadins were not significantly different between carriers of SNVs of interest in either cases or controls, with the exception of decreased levels of anti-ABO3b IgA in carriers of rs11139497(A), proximal to the *TLE1* locus (z= -3.376, p= 0.001). Mann-Whitney U test, p= 0.006 was used as the statistical cut-off for significance.

4.3.3 Relationship between anti-gliadin antibodies and genetic variants

A robust regression analysis was applied to determine if a linear relationship existed between anti-gliadin antibody levels and genetic variants tested, which were designated as false variables: 0, 1 or 2 based on the presence of the number of minor alleles.

Table 4.11. Robust regression analysis of the association between the MAN2A1 locus and plasma anti-gliadin antibody levels

Antigen	Ig Class	rs 4388249 (T)				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	0.019	<0.001	0.336	0.737	0.740
	IgA	0.014	<0.001	0.254	0.800	0.799
AAQ6B	IgG	-0.062	0.004	-1.112	0.267	0.269
	IgA	-0.005	<0.001	-0.090	0.928	0.929
AAQ6C	IgG	0.015	<0.001	0.268	0.789	0.784
	IgA	0.000	<0.001	-0.005	0.996	0.996
AL1G1	IgG	0.039	0.001	0.692	0.490	0.485
	IgA	0.006	<0.001	0.112	0.911	0.912
AL2G1	IgG	-0.060	0.004	-1.074	0.284	0.282
	IgA	0.050	0.003	0.896	0.371	0.369
AL2G2	IgG	0.008	<0.001	0.150	0.881	0.886
	IgA	-0.002	<0.001	-0.039	0.969	0.970
ABO3a	IgG	-0.068	0.005	-1.189	0.235	0.230
	IgA	0.016	<0.001	0.281	0.779	0.786
ABO3b	IgG	-0.093	0.009	-1.633	0.104	0.109
	IgA	-0.044	0.002	-0.789	0.431	0.428
Gliadin	IgG	0.015	<0.001	0.269	0.788	0.794
	IgA	0.064	0.004	1.153	0.250	0.250

Robust regression analysis was employed in order to examine the relationship between rs4388249(T) and levels of IgG against gliadins in a grouped schizophrenia-control cohort. Statistical significance was set at $p<0.05$ and significant associations are highlighted in bold. Levels of IgG against gliadins were not significantly associated with rs4388249 (T).

As shown in *Table 4.11*, there was no association found between rs4388249 (T) at the MAN2A1 locus and anti-gliadin antibody levels (*Table 4.11*).

Table 4.12 Robust regression analysis of the association between the TLE1 locus and

Antigen	Ig Class	rs11139497 (A)				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	-0.080	0.006	-1.468	0.143	0.145
	IgA	0.108	0.012	1.993	0.047	**0.044**
AAQ6B	IgG	0.034	0.001	0.615	0.539	0.555
	IgA	0.060	0.004	1.099	0.273	0.274
AAQ6C	IgG	0.009	<0.001	0.157	0.875	0.873
	IgA	0.009	<0.001	0.160	0.873	0.872
AL1G1	IgG	0.009	<0.001	0.159	0.874	0.874
	IgA	0.076	0.006	1.388	0.166	0.168
AL2G1	IgG	-0.069	0.005	-1.275	0.203	0.201
	IgA	0.053	0.003	0.971	0.332	0.335
AL2G2	IgG	-0.026	0.001	-0.477	0.634	0.643
	IgA	-0.039	0.001	-0.705	0.482	0.487
ABO3a	IgG	-0.050	0.003	-0.897	0.370	0.379
	IgA	0.003	<0.001	0.049	0.961	0.959
ABO3b	IgG	-0.032	0.001	-0.570	0.569	0.561
	IgA	-0.093	0.009	-1.711	0.088	0.090
Gliadin	IgG	-0.046	0.002	-0.855	0.393	0.391
	IgA	-0.013	<0.001	-0.240	0.810	0.814

Robust regression analysis was employed in order to examine the relationship between rs11139497(A) and levels of antibodies against gliadins in a grouped schizophrenia-control cohort. Statistical significance was set at $p<0.05$ and significant associations are highlighted in bold. The presence of rs11139497(A) was a significant predictor of levels of anti-AAQ6A IgA.

Table 4.13 Robust regression analysis of the association between the VRK2/FANCL locus

Antigen	Ig Class	rs11682175 (C)				
		r	r2	t	p-value	adjusted p-value
AAQ6A	IgG	0.024	0.001	0.443	0.658	0.668
	IgA	0.018	0.007	0.326	0.745	0.741
AAQ6B	IgG	0.082	<0.001	1.481	0.140	0.146
	IgA	-0.014	0.009	-0.259	0.796	0.801
AAQ6C	IgG	0.004	0.001	0.064	0.949	0.944
	IgA	0.001	0.006	0.014	0.989	0.988
AL1G1	IgG	-0.097	0.002	-1.746	0.082	0.075
	IgA	-0.007	0.005	-0.120	0.904	0.904
AL2G1	IgG	0.035	0.007	0.637	0.525	0.526
	IgA	-0.021	<0.001	-0.378	0.705	0.704
AL2G2	IgG	-0.075	0.006	-1.364	0.174	0.172
	IgA	0.004	<0.001	0.068	0.946	0.948
ABO3a	IgG	-0.041	0.002	-0.725	0.469	0.487
	IgA	0.017	<0.001	0.313	0.754	0.757
ABO3b	IgG	-0.070	0.219	-1.231	0.219	0.215
	IgA	-0.039	0.002	-0.701	0.484	0.480
Gliadin	IgG	0.083	0.130	1.519	0.130	0.129
	IgA	0.032	0.001	0.585	0.559	0.566

Robust regression analysis was employed in order to examine the relationship between rs11682175(C) and levels of antibodies against gliadins in a grouped schizophrenia-control cohort. Statistical significance was set at $p<0.05$ and significant associations are highlighted in bold. Levels of IgG against gliadins were not significantly associated with rs11682175(C).

As shown in *Table 4.12*, robust regression analysis showed that minor allele of rs11139497 (A) in the TLE1 locus was associated with increased anti-AAQ6A IgA levels (r= 0.108, p= 0.044), whereas there was no association between the VRK2/FANCL locus and anti-gliadin antibody levels (*Table 4.13*).

4.4 Discussion

Of the 3 SNPs genotyped in this study, none was shown to be associated with schizophrenia, although the study has enough power to detect a modest OR of 1.5 (or 0.67). These 4 SNPs were selected mainly based on a GWA study that confirmed 108 genetic loci associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014); it is possible that these SNPs could serve as DNA markers without a functional role, which could detect association signals for their linkage disequilibrium with a schizophrenia-underlying variant within a chromosomal region or nearby.

The process of SNP selection (see 4.2.2) led to the selection of rs4388249, rs11139497 and rs11682175 while were intragenic to or proximal to *MAN2A1*, *TLE1* and FANCL/VRK2, respectively (*Table 4.1*). *MAN2A1* encodes alpha-manosidase 2, an enzyme involved in the glycosylation of cell surface proteins. Glycosylation is an important component of self-tissue recognition by the immune system and animal models show that *MAN2A1* knock-out models result in a SLE-like phenotype (Chui et al., 2001). The FANCL/VRK2 locus was of interest due to the role of FANCL as a master regulator of many cellular processes, with variants of this gene associated with developmental problems and cytopenia, while VRK2 may play a role in the cytokine stress response, which has been suggested to be over-active in developmental disorders (Blanco et al., 2008; Esposito et al., 2002; Walden and Deans, 2014). *TLE1* was primarily of interest as it can modulate the activity of NF-κB (Ramasamy et al., 2012, p. 1).

Previous studies have attempted to examine immunological phenomena observed in schizophrenia in the context of immunogenetics (Avramopoulos et al., 2015; Børglum et al., 2014; Chan et al., 2017). In order to examine the impact of genetic variants on anti-gliadin antibodies in this study, cases and controls were grouped together and a difference in plasma antibody levels was then examined between the carriers and non-carriers of an allele of interest.

However, no significant associations were observed between SNVs of interest of antibodies against gliadins, with the exception of rs11139497 (A) being predictive of increased levels of anti-AAQ6C IgA *Table 4.12*.

In conclusion, the study described in this chapter aimed to examine the associations between schizophrenia and genetic variants tested, between plasma levels of the anti-native gliadin antibodies and the antibodies against gliadin-derived fragments as well as between plasma anti-gliadin antibody levels and genetic variants of interest. While no association between schizophrenia and the 4 SNPs selected was found in this study, a genetic component is likely to be involved in the regulation of plasma anti-gliadin antibody levels. Differences in levels of most antibodies in plasma failed to survive the Bonferroni correction, the subsequent association under robust regression suggested that the SNV proximal to TLE1 may be associated with levels of anti-AAQ6A IgA. With hindsight, it is apparent that the selection of SNPs to genotype in this study was flawed. As detailed in section 2.4, this process was biased, particularly in the final stages, based on the candidate's opinion of SNPs that were of interest. In particular, one alteration to the selection criteria that should be adopted in future is that odds ratios should have been the final criterion, with SNPs selected on their OR. This approach would have yielded SNPs proximal to *MEF2C*, *PSMA4* and *TRANK1*. In addition to their elevated ORs (1.085 – 1.101), IgG against TRANK1 protein is elevated in patients in schizophrenia and variants in *PSMA4* result in altered behavioural phenotypes. Another reason for the lack of replication in a smaller cohort may be due to the fact that the odds ratios found by GWAS tend to be modest. The OR for the highest SNP found by the GWAS performed by the Schizophrenia Working Group of the Psychiatric Genomics Consortium, (2014) was 1.23, which individually is unlikely to contribute much to the pathology of schizophrenia. If schizophrenia is multifactorial with multiple aetiologies, it is possible that this will not be

replicated in smaller cohorts. Essentially, this cohort does not include enough patients whose presentation of schizophrenia symptoms is influenced by the SNPs genotyped for this study.

Although no convincing positive associations were found in this current study, the identification of genetic markers that are associated with a gliadin-specific immune response in schizophrenia could lead to the development of genetic tests in order to identify patients that might benefit from a gluten-free diet prior to the onset of symptoms, or even reveal other genetic interactions that might explain increased levels of AGA in patients with schizophrenia.

5. Immune Cell Responses to Gliadin-Derived Peptides

5.1 Introduction

It has long been noted that gluten intake is associated with schizophrenia (Dohan, 1966) but studies of the cellular mechanisms relating to the immune response to wheat gluten have been lacking in the context of schizophrenia research. Attempts to elucidate the possible mechanisms of anti-native gliadin antibodies (AGA) have mainly focused on colocation with complement proteins, associations with other potential schizophrenia-related phenomena or, to a lesser extent, potential AGA cross-reactivity (Alaedini et al., 2007; Okusaga et al., 2016; Rowland et al., 2017; Severance et al., 2012b).

Less often explored is the immunological potential of the gliadin peptides themselves. Although such experiments are scarce in the schizophrenia literature, the immunogenic role of gliadin proteins has been investigated in the context of coeliac disease (CD). An *in vitro* experiment examining antigen loading by dendritic cells (DCs), the archetypical antigen presenting cell, revealed that a mixture of gliadin peptides were able to activate DCs from both healthy controls and patients with schizophrenia, although only DCs from patients with CD were able to subsequently activate T-cells in co-culture (Rakhimova et al., 2009). The stimulatory effect of gliadin was also observed to be independent of genotype, with the demonstration of increased IL-10 production in healthy DQ2 carriers, suggesting the development of a tolerogenic DC phenotype as a compensatory mechanism to alleviate the pathogenicity of gliadin-derived antigens (Palová-Jelínková et al., 2005; Rakhimova et al., 2009). These studies utilised cell models exposed to gliadin peptides that had undergone pepsin-trypsin digestion, resulting in a mixture of indigestible fragments. As a consequence of this experimental design, a differential response to specific gliadin peptides could not be explored.

B-lymphocytes produce antibodies after activation by T-cells that have received antigens presented by DCs. Additionally, B-cells can also be activated through a T-cell

independent pathway either by stimulation of toll-like receptors (TLRs) or via antigen binding to its complementary B-cell receptor (Lydyard et al., 2011b). Most antibodies produced via the T-independent processes are primarily of the IgM class, which is less specific to a target antigen than those produced via the T-dependent responses. HLA class II (HLA-II) molecules are highly expressed in B cells, and studies have suggested that antigen-presentation by B-cells may have important immunological functions such as the upregulation of inflammatory processes, or under steady-state antigen presentation to T-cells, the induction of immune tolerance (Mutnal et al., 2014; Rodríguez-Pinto, 2005). It has also been demonstrated that B-cells can play a role in the regulation of immune responses within the central nervous system (CNS) (Mutnal et al., 2014; Parker Harp et al., 2015). Furthermore, a study of immune cell infiltration into the CNS demonstrated significantly increased numbers of B-cells in the hippocampus of patients with schizophrenia when compared to healthy controls (Busse et al., 2012b). Finally, clinical trials are currently underway to test if monoclonal antibody treatments targeting lymphocytes, particularly B-cells, can effectively treat schizophrenia (Miller, 2017). It is possible that aberrant B-cell activity might play a pathological role in the development of schizophrenia and that targeting B cells could be a promising therapeutic approach in schizophrenia.

Antigen presentation also plays a key role in the induction of immune tolerance. A major contribution to immune tolerance is the wider cellular environment, where anti- or pro-inflammatory processes play an important role in defining the cellular response. Immature DCs (iDCs) are able to present antigen and generally produce anti-inflammatory cytokines such as IL-10. Presentation of antigens to T-cells by iDCs is able to induce differentiation to regulatory T (Treg) cells, and therefore, iDCs are likely to contribute to the development of peripheral immune tolerance (Mahnke et al., 2002). In addition, a subset of B cells known as B-1 cells, some of which have the characteristics of regulatory B (Breg) cells, are important for the

induction of oral tolerance. Studies in mice demonstrated the ability of Breg cells to induce tolerance when they were adoptively transferred into previously responsive mice (De-Gennaro et al., 2009; Margry et al., 2014).

The aim of this study was to test the hypothesis that incubation of antigen presenting cell models, namely B-cells and immature dendritic-like cells, with gliadin-derived peptides would induce the maturation of these cells. Although a T-dependent B-cell response is much more likely to result in plasmablast differentiation in the case of gliadin, a T-independent response cannot be ruled out as it has been theorised that gliadin may act as a toll-like receptor agonist. As a T-independent response is easier to examine in a monoculture, this is the mechanism that was focussed on. Furthermore the study aimed to test the hypothesis that incubation of the cell models with gliadin-derived peptides would result in measurable differential effects on cell apoptosis and the production of the pro-inflammatory cytokine Il-6 in the B-Cell model and the DC model, respectively.

5.2 Materials and methods

Based on the finding outlined in Chapter 3 (i.e. increased levels of plasma IgG against the γ-gliadin fragment AAQ6C and decreased levels of plasma IgG against the α-gliadin fragment AL2G1 in schizophrenia) two cell models were developed. The first, through the incubation of WIL.2.NS B lymphoblast cells, and the second derived from the THP-1 cell line. The effects of incubation with gliadin-derived antigens on cell maturation were examined. To investigate the interaction between gliadin-derived antigens and cultured cells, the synthetic peptides were dissolved in dimethyl sulfoxide (DMSO) (Thermo-Fisher Scientific, USA) to obtain a 20mg/ml stock solution, which was stored at 4°C for use in cell culture. Equivalent concentrations of DMSO were used as a vehicle control (VC) for treatments of cultured cells.

5.2.1 WIL.2.NS B-cell line

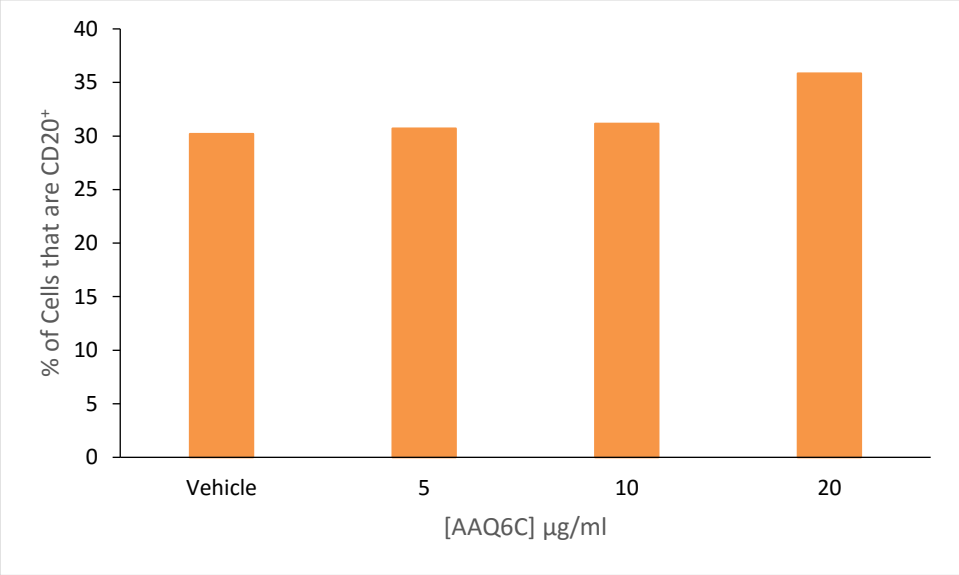
WIL.2.NS B lymphoblast cell line was derived from the spleen of a Caucasian male with spherocytic anaemia (Levy et al., 1968). The WIL.2.NS cells were cultured at 37°C, 5% CO_2 in RPMI1640 (10% FBS, 2mM L-GLUT) and maintained at a density of 0.2-2x10^6 cells/ml, with medium change taking place every other day.

Prior to gliadin-derived peptide challenge, the cell line was characterised by flow cytometry (BD Bioscience, USA) with fluorescence-conjugated monoclonal antibodies (mAbs) against CD5, CD19, CD20, CD27, CD86, HLA-DR and HLA-DQ. Following characterisation of CD20, a pan B-cell marker, expressed widely throughout the lineage, was used as a marker for the gliadin challenge pilots. CD20, CD86 and CD27 were also detected as they are markers for B-cell activation, maturity and differentiation.

5.2.2 B-cell line challenge with gliadin-derived peptides

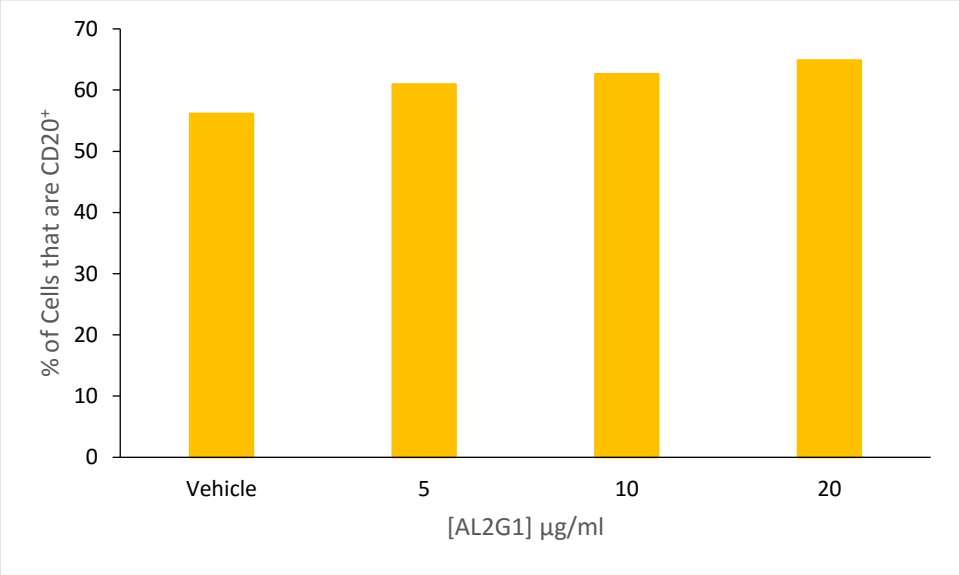
In order to determine the optimal gliadin-treatment dose, $2x10^5$ cells/ml were incubated for 48 hours in complete medium containing 5µg/ml, 10µg/ml or 20µg/ml of the gliadin-derived peptides, respectively (*Figure 5.1*and *Figure 5.2*). The antigen stock solution of 20mg/ml was diluted 1:20 in medium to give an initial antigen dilution of 1mg/ml in 5% DMSO. For a final concentration of 5µg/ml, the initial antigen dilution was further diluted 1:20 in medium containing 0.5% DMSO to give a concentration of 50µg/ml in 1% DMSO, which was then added to the cells in a 1:10 dilution. For a final concentration of 10µg/ml, the initial antigen dilution was further diluted 1:10 in medium containing 0.5% DMSO to give a concentration of 100µg/ml in 1% DMSO, which was then added to the cells in a 1:10 dilution. For a final concentration of 20µg/ml, the initial antigen dilution was diluted 1:5 in medium to produce 200µg/ml in 1% DMSO, which was then added to the cells in a 1:10 dilution. Following the optimisation of antigen concentrations for stimulating B cells, a time-course pilot study was performed with incubation of $2x10^5$ cells/ml with 20µg/ml of antigen for 24, 72 or 120 hours. The antigen-containing medium was changed every 48 hours and the cells were analysed with a flow cytometer at each time point.

Figure 5.1 Optimisation for concentrations of AAQ6C for WIL.2.NS Challenge



Percentage of cells that were $CD20^+$ following AAQ6C challenge at a range of concentrations; 0µg/ml (0.1% DMSO), 5µg/ml, 10µg/ml and 20µg/ml. $CD20^+$% was determined by the percentage of events that registered fluorescence above class-control levels.

Figure 5.2 Optimisation for concentrations of AL2G1 for WIL.2.NS Challenge



Percentage of cells that were $CD20^+$ following AL2G1 challenge at a range of concentrations; 0µg/ml (0.1% DMSO), 5µg/ml, 10µg/ml and 20µg/ml. $CD20^+$% was determined by the percentage of events that registered fluorescence above class-control levels.

For the experiments, $2x10^5$ cells/mL were seeded and incubated with 20µg/mL (0.1% DMSO) of either AL2G1 or AAQ6C over 120 hours under the standard conditions of 37°C and

5% CO_2. Medium containing 0.1% DMSO was used as a vehicle control. Medium containing either the peptides or vehicle control was exchanged approximately every 48 hours.

5.2.3 Fluorescence-activated cell sorting

Prior to analysis with flow cytometry, fluorescence-conjugated monoclonal antibodies (mAbs) were optimised for use on $1x10^6$ cells/ml at a range of antibody volumes. As shown in *Table 5.1*, mAbs against CD20, CD27, CD83 and CD86 were all optimised with 5µl, 10µl or 20µl in order to determine the minimum volume of reagent required.

Table 5.1 Markers for B-cell maturation and plasmablast differentiation

Antibody target and flurophore	Volume (µl)	Dilution	Catalogue Number
CD20 – PE	5	1:100	555623
CD27 – FITC	10	1:50	ab106071
CD83 – APC	10	1:50	551073
CD86 – PE	10	1:50	560957
CD5 - PE	10	1:50	ab106071

Volumes, dilutions and catalogue numbers for antibodies against CD markers used to characterise WIL.2.NS B cells. Volume optimisation was performed using WIL.2.NS cells with 20µl, 10µl and 5µl per test.

Cultured cells were harvested, double washed in PBS and centrifuged at 500*g* for 5 minutes before being re-suspended at a density of $1x10^6$ cells/ml in PBS/azide (100ml PBS containing 1g bovine serum albumin (BSA) and 100µg sodium azide). $1x10^5$ cells in 100µl were added to Eppendorf tubes and then incubated with the optimised volume of mAbs against

CD20, CD27, CD83 or CD86 (*Table 5.1*). Isotype-specific class mAbs conjugated with the same fluorophores were used as control antibodies to assess specific binding. Stained cells were centrifuged at 500*g* for 30 seconds, washed with PBS/Azide, centrifuged again at 500*g* for 30 seconds and resuspended in 500µl for flow cytometry analysis with 10,000 events.

5.2.4 Analysis of cell apoptosis

A commercially available kit for analysis of cell apoptosis was purchased from BD Bioscience (USA). Cultured cells were stained with FITC-conjugated Annexin V and propridium iodide (PI) to measure the percentages of apoptotic cells in response to the treatments with AL2G1 and AAQ6C peptides of 20µg/mL, respectively, compared to 0.1% DMSO vehicle control. The analysis of cell apoptosis was performed with a flow cytometer according to the manufacturer's instructions. In brief, cultured cells were harvested, washed twice in PBS and counted using a haemocytometer. $1x10^6$ cells were pelleted and resuspended in 1mL of Binding Buffer (10x 0.1M Hepes-NaOH, 1.0M NaCl, 25mM CaCl2), diluted 1:10 in deionised water. $1x10^5$ cells in 100µl were transferred to a 1.5mL Eppendorf® tube and 5µl of FITC-Annexin V and 5µl of PI were added. The cells were incubated for 15 minutes in the dark at room temperature and analysed immediately by flow cytometry after 400µl of 1xBinding Buffer was added. The settings for flow cytometry were previously determined for this cell type, as per manufacturer's recommendation described in Chapter 2. Gliadin-antigen treatment experiments were repeated 5 times in sequence for the measurement of apoptotic and dead cells. For example, the first plate was incubated with peptides for five days and the proportion of apoptotic cells was measured. Following this, a new aliquot of cells was thawed, incubated with peptides for five days and the proportion of apoptotic cells were measured. This was repeated to produce five repeated experiments that were as independent from each other as possible.

5.2.5 Differentiation of THP1 cells via gliadin challenge

THP-1 cell line was derived from a 1 year old male with acute monocytic leukaemia. For each new experiment, a fresh aliquot of THP-1 cells was rapidly thawed at 36°C and resuspended at a density of $0.5x10^6$ cells/ml in complete RPMI-1640 containing 2M L-glutamine and 10% FBS (Sigma-Aldrich). Before experiments, the cells were cultured into the growth phase and the number of cultured cells was maintained between 0.5 and $1x10^6$ cells/ml, with medium change every other day. THP-1 cells were seeded at $2x10^5$ cells/mL in a flask and differentiated into immature dendritic-like dells (iDCs) via incubation with RPMI-1640 medium containing 100ng/mL IL-4 and 100ng/mL GM-CSF at 37 ºC and 5% CO_2 for 120 hours (Berges et al., 2005). Immature dendritic-like cells were then differentiated into mature dendritic-like cells via incubation with serum-free RPMI-1640 containing containing 200ng/mL IL-4, 100ng/mL GM-CSF, 20ng/mL TNF-α and 200ng/mL Ionomycin. A flask of THP-1 cells was incubated as control cells under their original subculture conditions. The immature dendritic-like cells were then split into the treatment groups and incubated in a 24-well plate in triplicate. All cells were incubated at a density of $4x10^5$ cells/mL in 500µl of their respective media, detailed in *Table 5.2*. The treatment groups continued to be incubated for 48 hours prior to analysis of cell viability and IL-6 concentration in cell culture supernatants.

Figure 5.3 Plate layout for differentiation of Monocyte-derived THP-1 cells

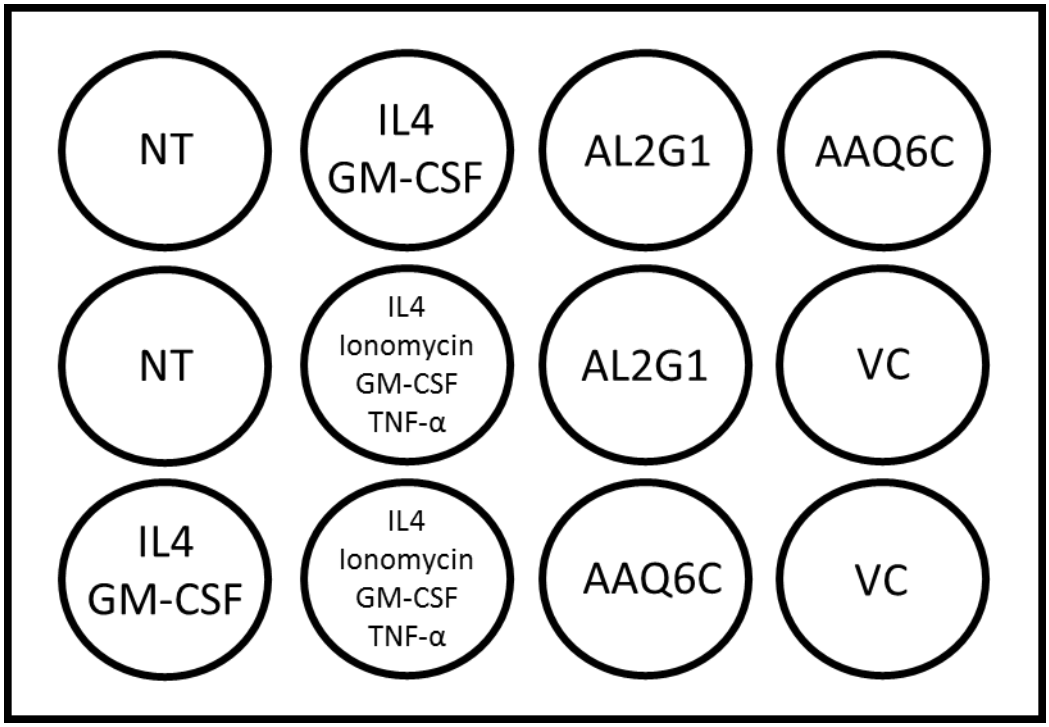


Plate layout showing the treatments for monocyte-derived THP-1 Cells. NT= No treatment – THP1 cells were incubated under standard cell culture conditions. Treatment with IL-4 and GM-CSF is reported to differentiate THP-1 cells into iDC-like cells. Treatment with IL-4, Ionomycin, GM-CSF and TNF-α may differentiate iDC-like cells into MDC-like cells. iDC-like cells were treated with AL2G1 and AAQ6C gliadin peptides. VC= vehicle control.

Table 5.2 Treatment conditions for the THP-1 derived iDCs

Group	Treatment condition
THP-1 cell line	THP-1 cells were incubated with complete RPMI 1640 medium
Immature dendritic-like cells	iDCs were developed with incubation in serum-free RPMI 1640 containing 100ng/mL IL-4 and 100ng/mL GM-CSF
Vehicle control	iDCs were incubated with serum-free RPMI 1640 containing 0.1% DMSO
α-gliadin challenge (AL2G1)	iDCs were incubated with serum-free RPMI 1640 containing the AL2G1 antigen of (20µg/mL)
γ-gliadin challenge (AAQ6C)	iDCs were incubated with serum-free RPMI 1640 containing the AAQ6C antigen (20µg/mL)
Mature dendritic-like cells	iDCs were incubated with serum-free RPMI 1640 containing 200ng/mL IL-4, 100ng/mL GM-CSF, 20ng/mL TNF-α and 200ng/mL Ionomycin

Conditions for immature dendritic-like cell induction and challenge with gliadin. Each condition was treated in duplicate.

5.2.6 Cell viability assay

Since the maturation of immature dendritic-like cells resulted in an adherent phenotype, the ability of cell adherence to the surface of a 24-well plate was used as the primary measurement of mature dendritic-like cell differentiation. This was estimated based on the cell viability detected by a colourimetric CCK-8 assay that relies on the activity of cell dehydrogenases to reduce WST-8, producing an orange formazan product in the presence of viable cells. The assay was performed according to the manufacturer's instructions. Briefly, the medium was removed by pipetting and the plate was washed with 1ml PBS at room temperature, to remove non-adherent cells and producing a quantified measurement of cell adherence in response to each of

the treatment conditions. PBS was then substituted with 450µl complete RPMI 1640. A 50µl volume of the CCK8 solution was then added to each treatment well, and a blank containing medium only. The plate was incubated for 2 hours at 37ºC and the resulting colour change was measured using the Varioskan Lux plate reader at λ=450nm absorbance.

5.2.7 IL-6 assay

In order to measure a pro-inflammatory effect of gliadin-derived peptide stimulation on immature dendritic-like cells, IL-6 concentration in the supernatants was measured using a SimpleStep ELISA kit (Abcam, UK). The assay was performed according to manufacturer's instructions. IL-6 concentration was quantified in the supernatant at 48 hours post-incubation under the conditions given above. The cell culture supernatants were removed from each well, centrifuged at 17,000*g* and frozen at -20ºC prior to the assay. Each treatment was performed in triplicate for three assays for IL-6.

Briefly, the frozen supernatants from all the treatments were thawed on ice; the standard solutions for an ELISA assay were prepared from a 50,000 pg/mL stock solution via a 2-fold serial dilution to generate the following standard curve: 5000 pg/mL, 2500 pg/mL, 1250 pg/mL, 625 pg/mL, 312.5 pg/mL, 156.25 pg/mL, 78.12 pg/mL and a blank sample. 50µl of each sample or standard was loaded onto the plate and tested in duplicate. 50µl of the antibody cocktail, containing both capture and detector antibodies, was added to each well and incubated for 1 hour at room temperature. The plate was washed three times with the wash buffer provided in the ELISA kit and blotted on the final wash step. 100µl of TMB was added to each well and incubated for 10 minutes at room temperature in the dark before the colour development reaction was stopped with 100µl of Stop Solution. The optimal density (OD) for absorbance was read at 450nm for the endpoint reading.

5.2.8 Statistical analysis

Five sequential repeated experiments were used as independent measures for the Annexin V/PI apoptosis detection, in response to gliadin challenge. The experiments were repeated on different plates and days to maximise independence. The percentages of apoptotic cells in the lower right (LR) quadrant and dead cells in the upper right (UR) quadrant, corresponding to Annexin V+ and Annexin V/PI+ cells, respectively, were used to present data, and one-way ANOVA with a Bonferroni *post hoc* test was performed to compare the difference between groups.

The mean±SD in OD was used to present the cell viability data from 3 measurements in response of THP-1 derived iDC to gliadin challenge. The mean±SD in IL-6 concentrations was used to present the IL-6 test data from 3 measurements of each treatment group.

5.3 Results

5.3.1 Characterisation of WIL.2.NS B-cell line

In order to determine the baseline characterisitcs of the WIL.2.NS B-cell line, cells were tested using flow cytometry with mAbs for typical B-cell markers (*Figure 5.4*)

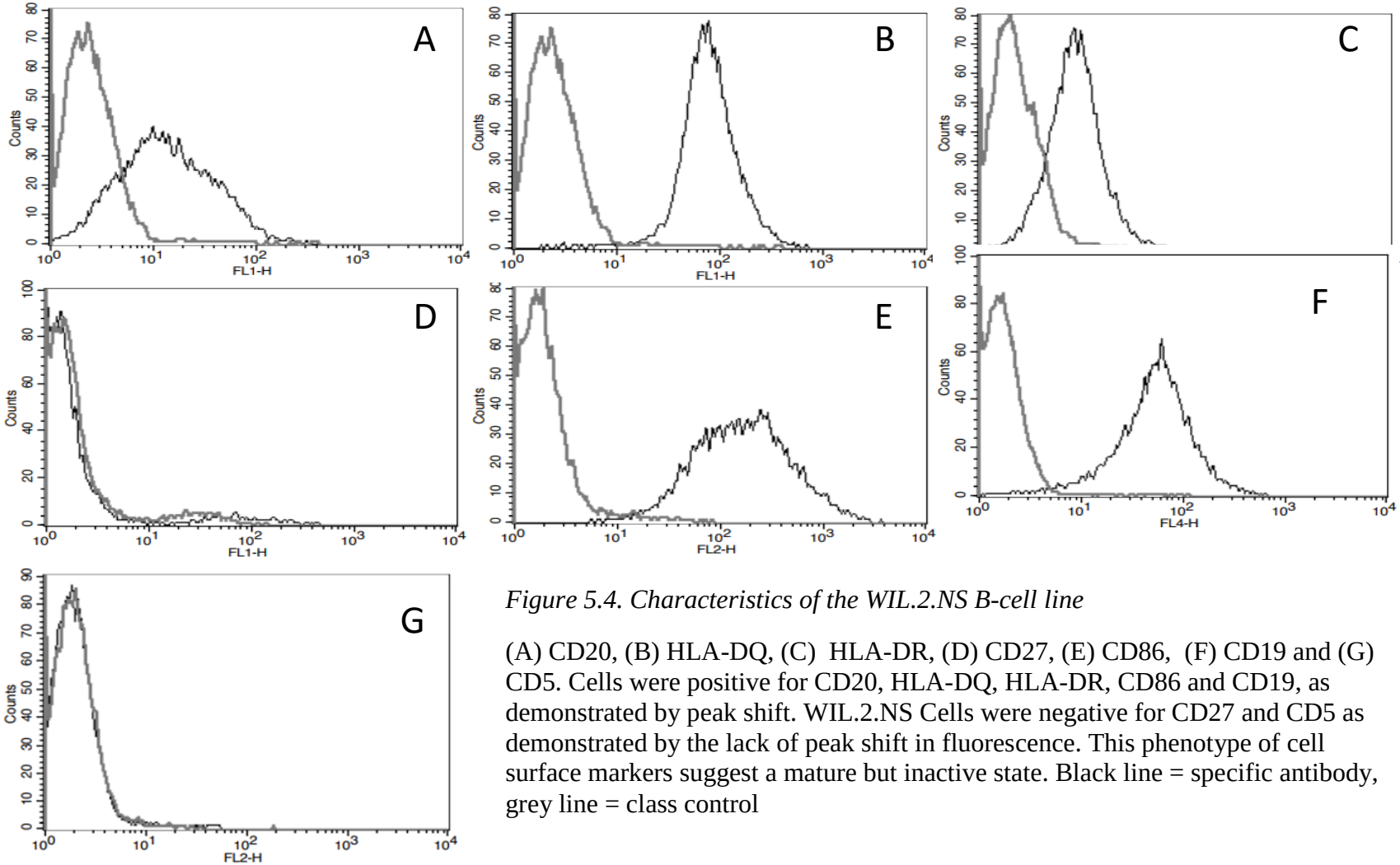


Figure 5.4. Characteristics of the WIL.2.NS B-cell line

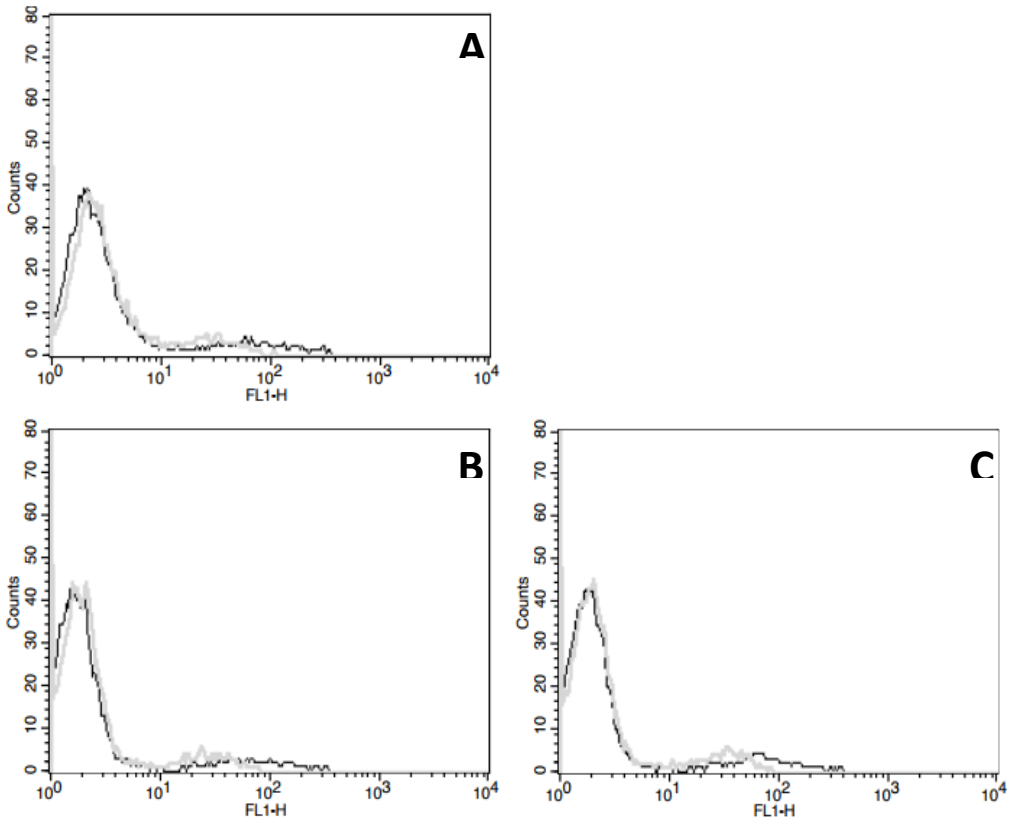
(A) CD20, (B) HLA-DQ, (C) HLA-DR, (D) CD27, (E) CD86, (F) CD19 and (G) CD5. Cells were positive for CD20, HLA-DQ, HLA-DR, CD86 and CD19, as demonstrated by peak shift. WIL.2.NS Cells were negative for CD27 and CD5 as demonstrated by the lack of peak shift in fluorescence. This phenotype of cell surface markers suggest a mature but inactive state. Black line = specific antibody, grey line = class control

As shown in *Figure 5.4*, the WIL.2.NS B-cell line was positive for CD20, HLA-DQ, HLA-DR, CD86 and CD19 but negative for CD27 and CD5.

5.3.2 No differential response of WIL.2.NS cells to α-gliadin or γ-gliadin challenge

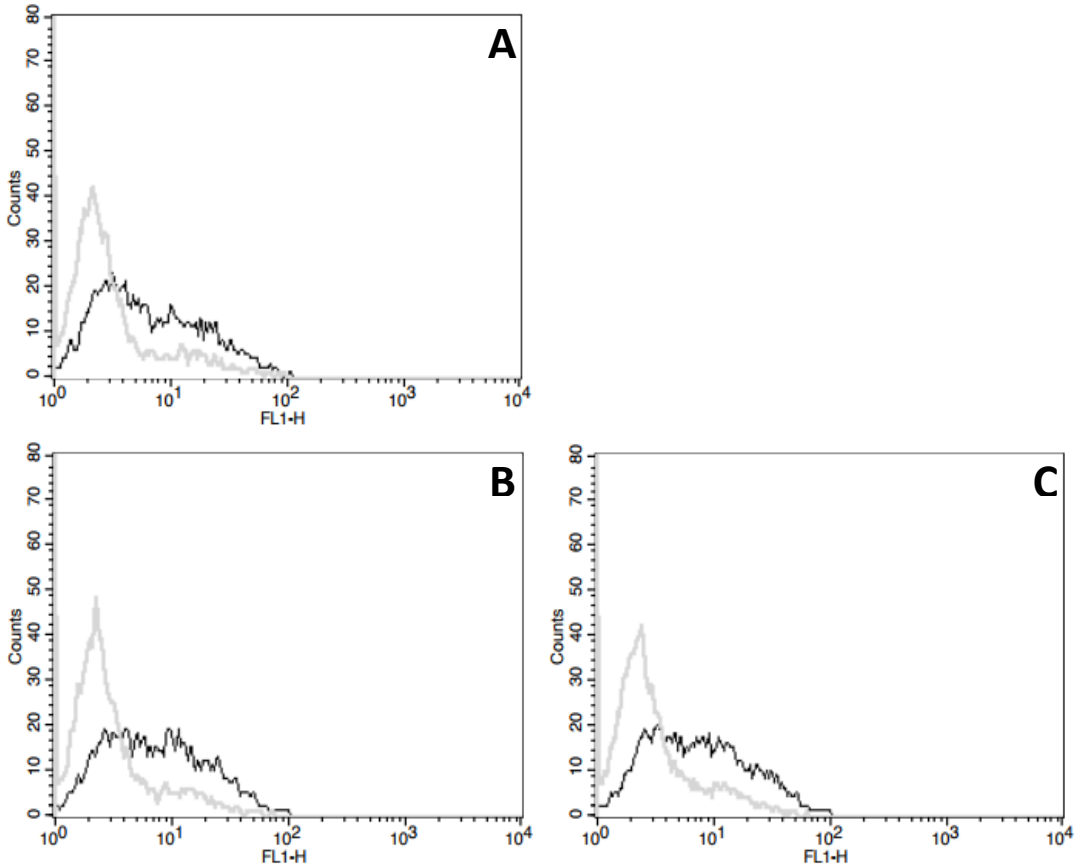
A panel of B-cell markers were examined on WIL.2.NS cells incubated with AAQ6C, AL2G1 or 0.1% DMSO vehicle, respectively. These two gliadin-derived antigens did not appear to alter the expression of CD27 and CD20 (*Figure 5.5-Figure 5.6*).

Figure 5.5 The effects of gliadin peptide challenge on the expression of CD27 in WIL.2.NS cells



This diagrammatic illustration shows the expression of CD27 on WIL.2.NS cells incubated with (A) vehicle control, (B) α-gliadin derived peptide AL2G1 or (C) γ-gliadin derived peptide AAQ6C. Incubation with peptides did not alter the expression of CD27 on WIL.2.NS B cells.

Figure 5.6 The effects of gliadin peptide challenge on the expression of CD20 in WIL.2.NS cells

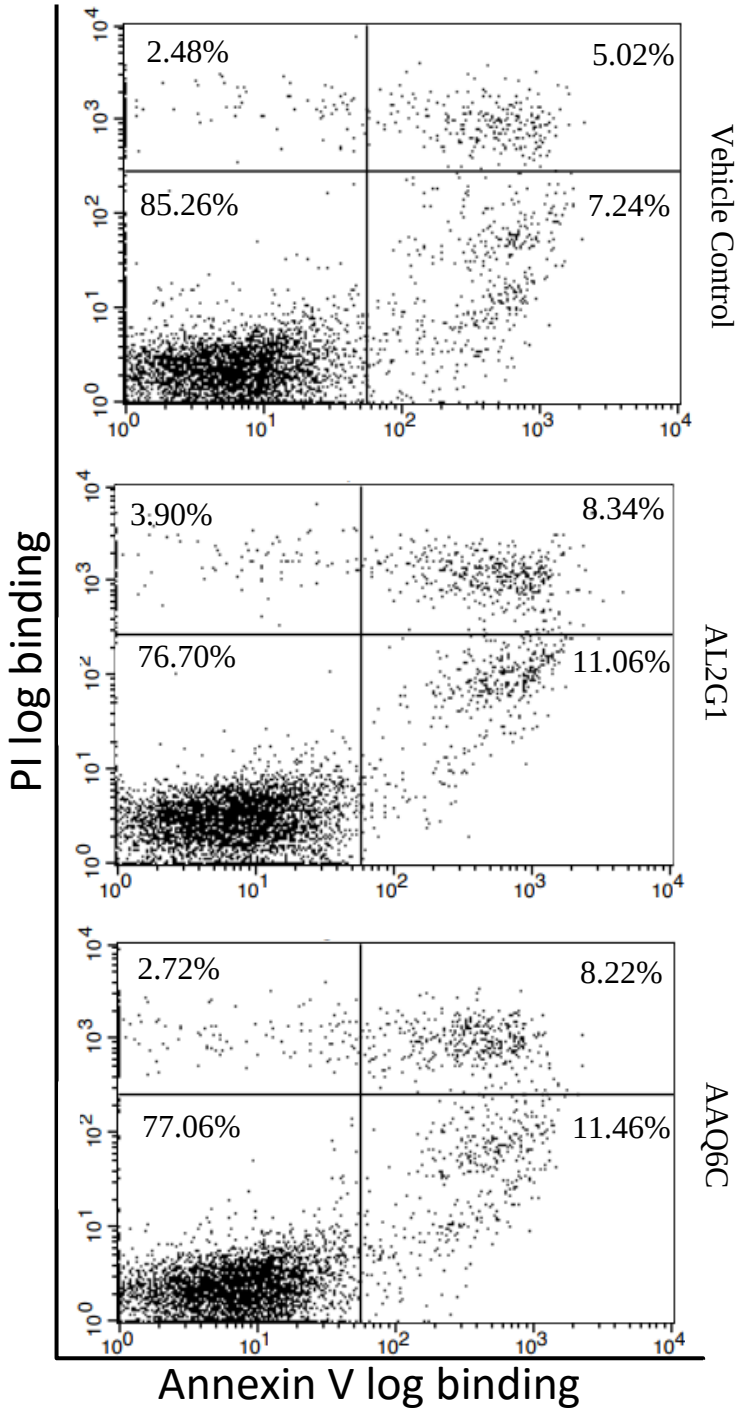


This diagrammatic illustration shows the expression of CD20 on WIL.2.NS cells incubated with (A) vehicle control, (B) α-gliadin derived peptide AL2G1 or (C) γ-gliadin derived peptide AAQ6C. Three repeats were performed independently to confirm the expression of CD20 in WIL.2.NS cells. Peak-shift in fluroescence was not significantly different between peptide and vehicle-treated cells, demonstrating no effect of gliadin-derived peptide incubation on the expression of CD20.

5.3.3 Induction of WIL.2.NS cell apoptosis by gliadin-derived peptides

Incubation with the α- and γ- gliadin derived peptides significantly increased the percentage of Annexin V+ and Annexin V/PI+ cells when compared to the vehicle control (*Figure 5.7*). One-way ANOVA showed a significant difference in percentage of apoptotic and dead cells between 3 treatment groups (F=6.404, df=2,6, p=0.013), in which the Bonferroni post-hot test showed that the percentage of apoptotic and dead cells was significantly higher in both AAQ6C–treated cells (LR+UR=16.17±2.20%, n=5, p= 0.027) and AL2G1-treated cells (LR+UR= 16.14±2.13%, n=5, p= 0.028) than in vehicle-treated cells (LR+UR= 11.83 ±2.30%, n=5), although the percentage of apoptotic and dead cells was not significantly different between AAQ6C-treateed cells and AL2G1-treated cells (p>0.05) (*Figure 5.7*).

Figure 5.7 The effects of gliadin peptide challenge on survival of WIL.2.NS cells



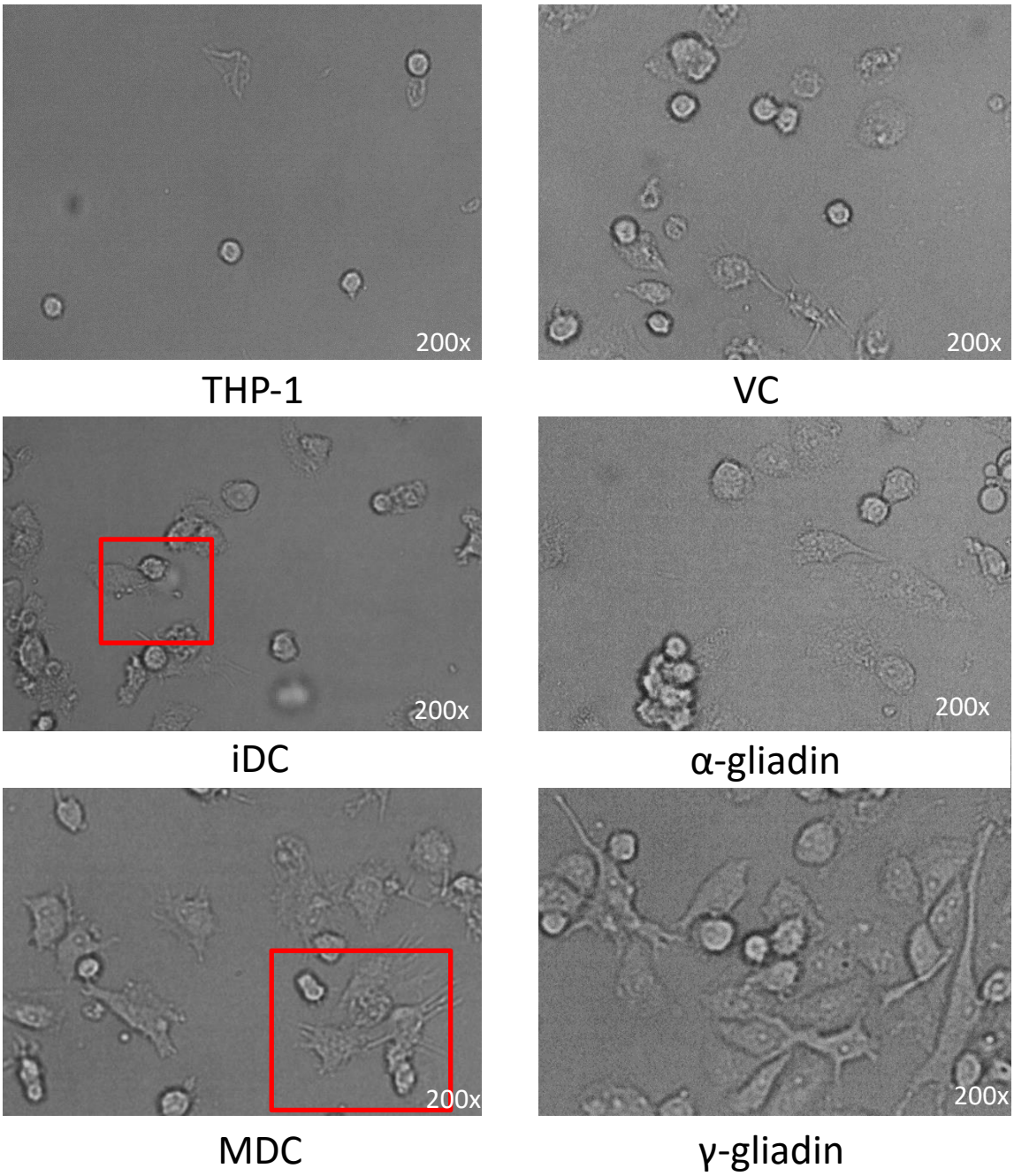
The fate of WIL.2.NS cells incubated with vehicle control was shown in the top, with α-gliadin derived AL2G1 in the middle, and γ-gliadin derived AAQ6C in the bottom. The percentage of Annexin V+ and Annexin V/PI+ cells was significantly higher in both AAQ6C –treated cells (LR+UR= 16.17±%, n=5, p= 0.027) and AL2G1-treated cells (LR+UR= 16.14±%, n=5, p= 0.028) than in vehicle-treated cells (LR+UR= 11.83 ±%, n=5); however, the percentage of Annexin V+ and Annexin V/PI+ cells was not significantly different between AAQ6C-treated cells and AL2G1-treated cells (p>0.05). ANOVA with the Bonferroni *post hoc* test was used to determine significance (p <0.05).

5.3.4 The effects of gliadin peptide challenge on THP-1 derived dendritic-like cells

THP-1 cells underwent challenge with GM-CSF and IL-4 and developed into immature dendritic-like cells as described (Section 5.2.5) according to the method introduced by Berges et al., (2005). The cells were then treated with 20µg/mL AL2G1 peptides, 20µg/mL AAQ6C peptides, 0.1% DMSO as vehicle control or cytokines, respectively, in order to induce the differentiation of immature DCs into mature DCs.

The different treatment groups were examined visually for the typical morphological characteristics of immature and mature dendritic cells. Immature dendritic-like cells displayed a ruffled membrane, and developed into mature dendritic-like cells after treatment with the maturation cocktail (Berges et al., 2005). Morphologically, a visual examination of the three treatment groups revealed that examples of a mature phenotype appeared to be present in all groups (*Figure 5.8*).

Figure 5.8 The effects of gliadin-derived peptide challenge on the development of THP-1 derived DCs

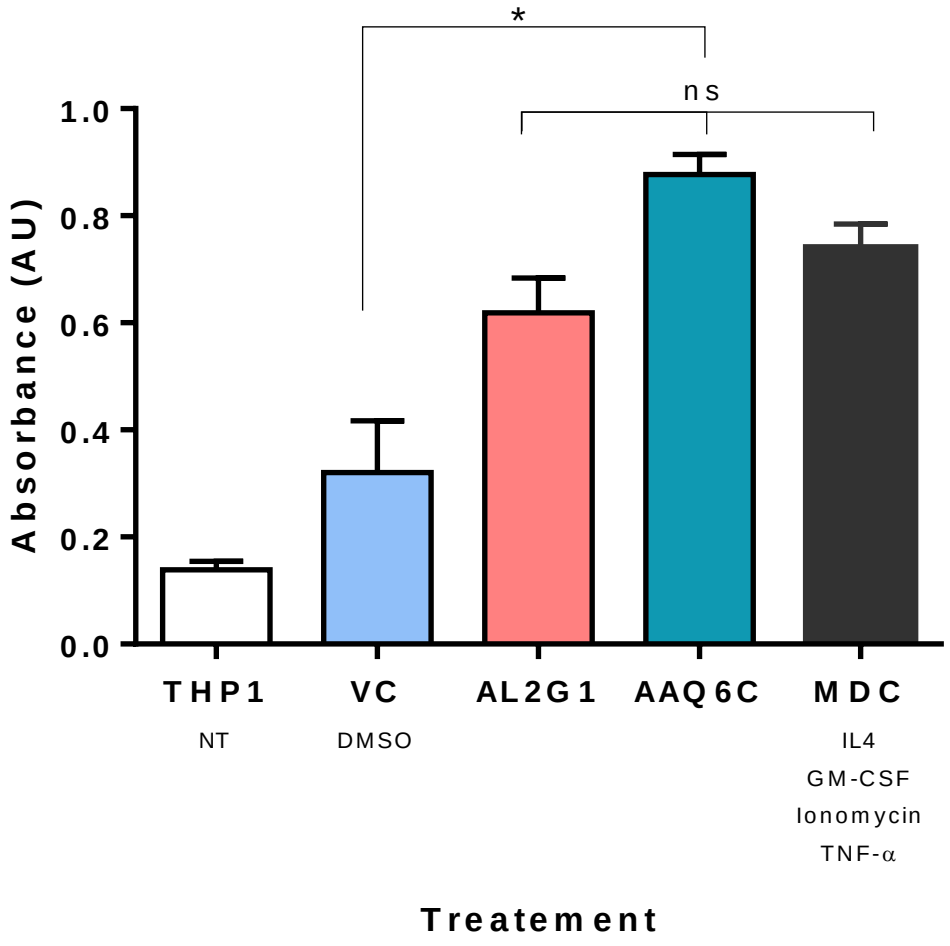


The different cell types derived from THP1-monocytes (left column with examples of an immature dendritic-like cell (iDC) and mature dendritic-like cells (MDC) highlighted with red circles) and examples of cell morphology of iDCs incubated with vehicle control (VC), α-gliadin or γ-gliadin peptides, respectively. Morphologically, each purported cell type appears to be represented to some degree in each of the three treatments (right column).

Given that the mature dendritic-like cells were previously found to be adherent in this model, the ability of cell adherence was quantified using the CCK-8 assay. One-way ANOVA showed a significant difference in cell adherent ability between the 5 treatment groups

following wash with PBS (F= 80.2, df= 4, 10, p<0.001), and the Bonferroni *post hoc* test showed that the untreated THP-1 cells displayed significantly lower adherence than the other 4 groups (p<0.01); when compared to vehicle-treated cells, both AL2G1-treated cells and AAQ6C-treated cells displayed increased adherent ability. As shown in *Figure 5.9*, there was significant difference in adherent ability between AAQ6C-treated and AL2G1-treated cells (p=0.003), but there was no significant difference in adherent ability between mature DCs and gliadin peptide treated cells (p>0.01).

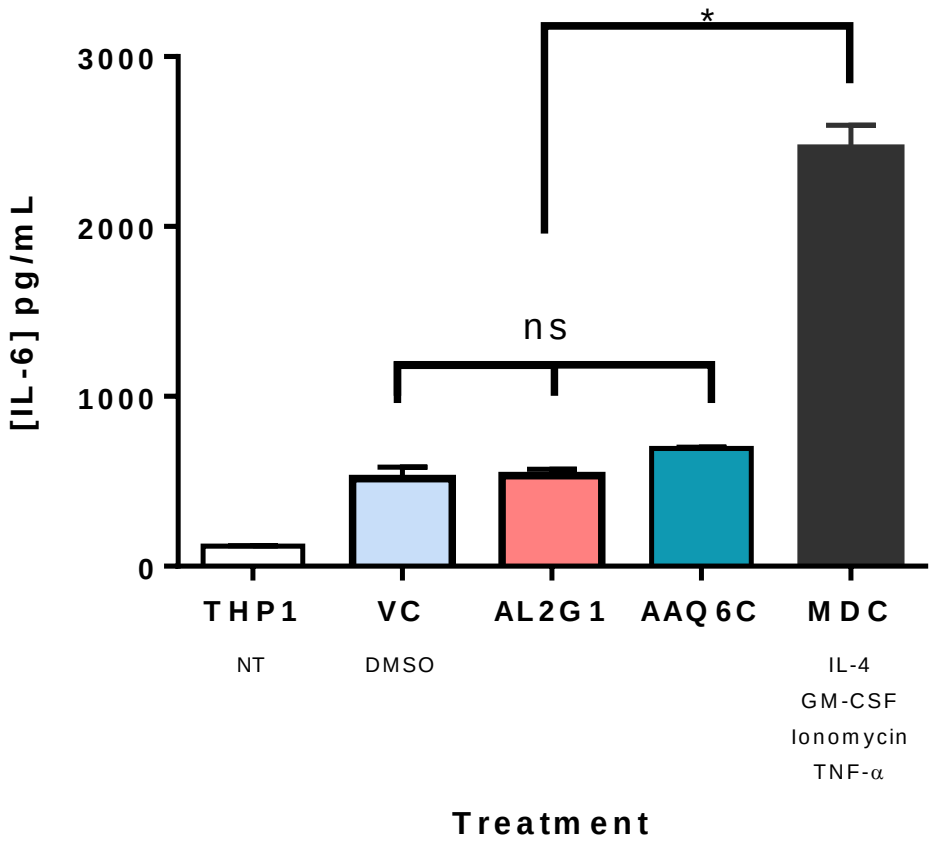
Figure 5.9 The effects of gliadin peptide challenge on adherent ability of immature dendritic-like cells



Mean±SD in OD was taken from three tests with the CCK8 assay. Following wash with PBS, untreated THP-1 cells demonstrated the lowest adherent ability to the 24-well plate (0.139± 0.020). Compared to the vehicle control (VC) (0.320± 0.100), incubation with either AL2G1 peptide (0.618±0.020, p= 0.001) or with AAQ6C peptide (0.877±0.037, p <0.001) increased cell adherent ability; There were no significant difference in cell adherent ability between antigen-treated cells and the cells incubated with the maturation cocktail (MDC) (0.739±0.045, p >0.01). NT= No treatment, VC= Vehicle control. The p-value of <0.01 was set for a statistically significant level as 5 independent tests were performed. * = p<0.01

To investigate the inflammatory response of immature DCs to gliadin-derived peptide incubation, an ELISA was performed to measure IL-6 levels in the cell supernatant at 48 hours post-incubation under the treatment conditions outlined (*Table 5.2*). One-way ANOVA showed a significant difference in supernatant IL-6 levels between five treatment groups ($F= 514.2$, $df= 4,10$, $p <0.001$), and the Bonferroni *post hoc* test showed that THP1-cells had a significant lower IL-6 level than the other 4 groups ($p <0.001$), while the IL-6 levels were significantly higher in mature DCs than in the other 4 groups ($p <0.001$). IL-6 levels did not show a significant change in cells treated with gliadin-derived antigens compared to those treated with vehicle control (*Figure 5.10*).

Figure 5.10 The effect of gliadin peptide challenge on IL-6 production in immature dendritic-like cells



Mean±SD in IL-6 concentrations (pg/mL) was taken from three tests with an ELISA kit. Of all 5 groups, mature DCs showed the highest IL-6 levels in the supernatants (2462±135 pg/mL, $p<0.001$) and untreated THP-1 cells showed the lowest levels (118±3 pg/mL); IL-6 levels in the supernatants were 516±69 pg/mL in vehicle-treated cells, 533±39 pg/mL in AL2G1-treated cells and 694±7pg/mL in AAQ6C-treated cells, with no significant difference observed between AL2G1-treated and AAQ6C-treated cells ($p= 0.185$). MDC= Mature Dendritic Cell, NT= No treatment, VC= Vehicle control. The p-value of <0.01 was set for a statistically significant level as 5 independent tests were performed. *= $p<0.01$.

5.4 Discussion

This study aimed to examine the effects of gliadin-derived peptide incubation on the maturation of professional antigen-presenting cells. Two cell models were employed, THP-1 derived DCs and WIL.2.NS B-cell line.

In order to determine the maturity of WIL.2.NS B-cell line, which was originally derived from the spleen with a mature phenotype (Lydyard et al., 2011b), the cells were characterised using flow cytometry for a number of different B-cell markers (*Figure 5.4*). WIL.2.NS cells did not express CD5, suggesting that they may be derived from bone marrow B2 cells, a subclass of B-cells that are mainly involved in adaptive immune responses to antigenic stimulation. In contrast, B1 cells, which express CD5, are mainly involved in developing immune tolerance and innate immunity such as the production of natural antibodies (Hippen et al., 2000; Lydyard et al., 2011b; Thomas-Vaslin et al., 1992). The expression of CD86 on splenic B-cells has been linked to an increase in IgG production and B-cell activation in response to lipopolysaccharide, suggesting an activated phenotype (Suvas et al., 2002). The expression of CD20 is reduced during plasmablast differentiation; the absence of CD20 from the IgG-secreting plasma cells and consequently variable levels of CD20 expression in WIL.2.NS cells may suggest an activated subpopulation of B-cells. However, WIL.2.NS cells stained negative for CD27, suggesting that untreated cells have not differentiated into short-lived plasmablast cells (Jego et al., 2001; Qian et al., 2010). The pattern of cell surface markers, particularly the absence of CD27 suggests that these cells are derived follicular B-cells, rather than marginal zone B-cells.

WIL.2.NS cells were incubated with either AL2G1 antigen or AAQ6C antigen and vehicle control (0.1% DMSO) to determine if these cells could be differentiated into plasmablast cells in a T-cell independent manner. Based on flow cytometry analysis with mAbs against CD27 and CD20, the expression profiles of CD27 or CD20 showed no difference between AL2G1-treated and AAQ6C-treated cells (*Figure 5.5-Figure 5.6*), demonstrating that

challenge with gliadin peptide did not result in the differentiation of WIL.2.NS cells into plasmablasts. Although anti-CD27 mab nonspecific binding was controlled for, the lack of a positive control stain for CD27 means that a problem with the anti-CD27 mab cannot be excluded as an alternative explanation for these results.

Since the majority of plasmablasts are short-lived and survive only a few days post-differentiation, an increase in the proportion of apoptotic cells would be consistent with differentiation into plasmablasts. This study showed that incubation with either AL2G1 or AAQ6C antigens increased the number of apoptotic WIL.2.NS cells (*Figure 5.7*), but such an effect does not appear to be specific to either gliadin-derived peptide. In the absence of CD27 expression or a decrease in CD20 expression. Therefore, it can be concluded that this effect is not driven by WIL.2.NS cell differentiation into short-lived plasmablasts. Possibly, gliadin peptides are slightly cytotoxic, as previously demonstrated in a colorectal cancer cell line (Giovannini et al., 2000). It has been shown that EBV-transformed B-Cells are resistant to apoptosis and therefore it is possible that the true cytotoxicity of gliadin peptides on these cells has been understated or masked by the use of EBV-transformed cells (Price et al., 2017.). However, a small but significant increase was observed specific to gliadin peptides although the use of an EBV-transformed cell line means that the physiological implications of this are unclear.

It is possible that peripheral blood mononuclear cells (PBMCs) may have been a better model to examine plasmablast differentiation as the cells are more physiological. The availability of schizophrenia patient-derived lymphoblast cell lines would have allowed for an examination of this effect in a disease-relevant model. However, WIL.2.NS cells represented a mature but inactive phenotype (*Figure 5.4*) and so were considered a good candidate cell for plasmablast differentiation. To fully examine the potential for gliadin to induce plasmablast differentiation PBMCs should be collected in order to examine if gliadin can induce plasmablast differentiation and then, if they can, a disease-specific effect can be examined.

THP-1 cells were differentiated into immature dendritic-like cells according to Berges et al., (2005). For this reason, the effect of incubation with gliadin-derived peptides was examined in a THP-1 derived DC model. Mature Dendritic-like Cells have adherent ability in this model and therefore adherence to the cell culture plate was used as the primary measure of iDC maturation. Morphologically, examples of cells displaying mature DC traits could be found in each treatment group. However, the results of the CCK8 assay suggested that iDC-like cells incubated with gliadin-derived peptides display more adherent phenotypes than those incubated with vehicle control (*Figure 5.8* and *Figure 5.9*). It is worth noting that AAQ6C treatment was more likely to induce adherent ability of iDC-like cells than AL2G1 treatment.

The results of testing IL-6 concentrations in the cell supernatants suggest that incubation of iDCs with the two gliadin-derived peptides did not significantly enhance IL-6 levels in the cell supernatants and IL-6 levels in response to peptide incubation were lower than the levels observed when the cells were stimulated with the cytokine cocktail directly (*Figure 5.10*). These two assays therefore suggest that AAQ6C is more likely to be able to induce a MDC-like phenotype than AL2G1, but the phenotype is less pro-inflammatory than fully activated mature DCs in this model (*Figure 5.10*). These results are consistent with studies in coeliac disease that show gliadin-peptides are able to induce maturation of DCs regardless of disease state, though the lack of anti-inflammatory cytokine assays is a limitation in this current study (Rakhimova et al., 2009).

Another point that should be taken into account is the HLA. As demonstrated in Chapter 4, no HLA variants genotyped demonstrated a positive correlation with anti-AAQ6C antibodies. A previous study showed that THP-1 cells were positive for HLA-DR1, HLA-DR15, HLA-DQ6 and HLA-DQ5, though serotyping was not able to fully resolve specific alleles (Battle et al., 2013). The minor allele of rs6457614 (G) is tagged to the HLA-DR1 and HLA-DQ5 haplotypes, in a Caucasian population (de Bakker et al., 2006). However, there was no association observed between this SNP and altered levels of antibodies against peptides used

in this study (see Chapter 2). Furthermore, the minor allele of rs3135388(T) is tagged to the HLA-DR15 and HLA-DQ6 haplotypes (de Bakker et al., 2006), but again the levels of plasma antibodies against both AL2G1 or AAQ6C peptide antigens were not associated with this SNP (see Chapter 2). It is interesting to note, however that AL2G1 and AAQ6C were predicted to bind to HLA-DR1, while AAQ6C was also predicted to bind to HLA-DR15 (*Table 2.1*). It can be speculated that, since AAQ6C may be presented by two HLA molecules on THP-1 cells, this could explain the trend of increased adherence observed in AAQ6C-treated cells (*Figure 5.9*). Assuming the findings in this cell model are replicated in primary cell cultures, with different HLA variants, it is possible that in a subgroup of monocyte-derived DCs with the HLA variants that recognise the AAQ6C peptide, their maturation and production of pro-inflammatory cytokines would be promoted in response to AAQ6C incubation. Furthermore, if this response to AAQ6C peptide was more common in patients with schizophrenia, it would demonstrate a potential immunological link between AAQ6C immunogenicity and schizophrenia. In future studies, the potential of DCs from schizophrenia patients to present gliadin-derived antigen to autologous T-cells should also be examined.

The major limitation of the iDC model study presented here is the lack of data on the cell surface characteristics, which could be measured by flow cytometry. It has been found that the majority of the MDC-like cells in this model, which were incubated with IL-4, GM-CSF, TNF-α and ionomycin, had strongly adherent ability and the yield of cells removed by versene, cell scraping or shear was very low, requiring up to $4x10^6$ cells for a yield of $\sim 1x10^4$ or fewer. This number of cells was able to be used to successfully stain MDC-like cells however an antibody recall meant that these results were invalid and so quantification of adherence of MDC-like cells was focussed on as the primary outcome measure.

In conclusion, the gliadin peptides used in this study were not found to differentiate WIL.2.NS cells into plasmablasts, although they were able to induce an increase in cell apoptosis. As there is no evidence to suggest that gliadin binds to toll-like receptors (Moossavi,

2014) and the expansive repertoire of B-cell receptors suggests that the BCR on WIL.2.NS cells are unlikely to specifically take up either AL2G1 or AAQ6C peptides, in the absence of T-cell co-stimulation, it is likely that this cell model is too simplistic to elicit plasmablast differentiation. In order to fully examine the effects of gliadin incubation on the differentiation of B-cells into plasmablasts, it is likely that co-culture using PBMCs would be required, rather than EBV-transformed B-cell lines. Despite the limitations of the THP-1 derived DC model outlined in the previous paragraph, this study raised the possibility that both peptides could induce the adhesion and possibly maturation of THP-1 derived iDCs. The incubation of iDC-like cells with gliadin peptides was not associated with an increase in pro-inflammatory IL-6, but the secretion of anti-inflammatory cytokines cannot be ruled out as they were not measured in this study.

6. Discussion and Conclusions

The aims of the research presented in this thesis were to further characterise the anti-gliadin immune response that was previously observed in patients with schizophrenia. To this end, the levels of circulating antibodies against indigestible gliadin-derived fragments were measured in plasma samples from patients with schizophrenia and control subjects using an in-house ELISA, with differential levels of IgG and IgA antibodies against gliadin-derived fragments observed. Due to the role of HLA-II molecules in the presentation of protein-derived epitopes, the relationship between plasma antibodies against gliadin-derived antigens and previously genotyped HLA-II variants was examined, in addition to potential associations with schizophrenia-associated SNPs confirmed by a recent GWA-based meta-analysis (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). This was done in order to examine the possible mechanism by which gliadin-derived fragments might trigger abnormal immune responses in schizophrenia. Finally, based on the antigens that were most likely to be involved in alteration of anti-gliadin antibody levels in schizophrenia, both B-cell and DC models were used to test the interactions between the immune cells and gliadin-derived antigens.

In summary, these studies demonstrated that the levels of plasma IgG against a γ-gliadin fragment, designated AAQ6C, were significantly higher in patients with schizophrenia, while the levels of plasma IgG against α-gliadin fragments and IgA against all gliadin-derived fragments tested in this study were significantly lower in patients with schizophrenia than control subjects (Chapter 3). None of the 4 SNPs tested was found to be significantly associated with schizophrenia in this study cohort although a negative correlation was observed between anti-gliadin antibodies and HLA-II variants, with HLA-DQ 8.1 being predictive of lower anti-AAQ6C IgG levels (Chapter 4). Challenge with gliadin-derived peptides could induce a mature phenotype in an immature dendritic cell model with regards to cell adherence, albeit with a much reduced pro-inflammatory profile (Chapter 5).

6.1 Possible roles of plasma antibodies against native gliadin and gliadin-derived antigens

There is a difficulty in performing an intervention study on antibodies directed against gliadin-derived fragments due to the lack of available monoclonal antibodies, and the antibodies specific to gliadin-derived fragments of interest are not commercially available. Potential cross-reactivity between anti-native gliadin antibodies (AGAs) and antigens derived from brain molecules is one obvious mechanism by which AGA may contribute to the pathology of schizophrenia. CD patients with neurological symptoms demonstrated cross-reactivity between AGAs and synapsin I, a synapse-related protein involved in vesicle formation and neurotransmitter release (Alaedini et al., 2007). An immune response against this protein could conceivably disrupt neuronal connectivity. Additionally, the hypothesis that schizophrenia may be related to antibodies against CNS antigens has been explored. Studies demonstrating that plasma from patients with schizophrenia was better able to stain CNS tissue, derived from human and non-human primates, than plasma from healthy controls (Margari et al., 2013, 2015). Other studies identified potential targets, such as dopamine receptor 2 (DRD2) and controversially, the NMDA receptor (Lennox et al., 2017; Masopust et al., 2015; Pathmanandavel et al., 2015; Pollak et al., 2014). One of the main theories regarding AGA cross-reactivity is the high density of PQ motifs present in gliadin, which may promote cross-reactivity to proteins rich in proline and glutamine. Although plasma anti-AAQ6C IgG levels were found to be increased in schizophrenia, this γ-gliadin derived antigen lacks such PQ motifs. Interestingly, α-gliadin derived fragments are comparatively rich in these motifs, but they did not appear to provoke an increase in their respective immunoglobulins in schizophrenia, and indeed levels of these antibodies were the highest in control individuals when compared to patients with schizophrenia. It is possible that in the case of a gliadin-sensitive schizophrenia subgroup, the pathological activity of AGAs may not be related to cross-reactivity, but instead could be related to the activation of the complement system in the circulation as it has been demonstrated that AGAs are co-located with C1q complexes

(Severance et al., 2012b). Moreover, increased maternal activation of C1q could increase the odds of psychosis in offspring, with significant associations observed between AGAs and C1q complexes in cases only (Severance et al., 2014). The levels of plasma AGAs were found to be increased in animal models exposed to *Toxoplasma gondii*, suggesting a relationship between these two immunological findings in schizophrenia (Xiao et al., 2016). In addition, anti-*Toxoplasma gondii* IgG was also associated with increased cerebral C1q (Xiao et al., 2016). Complement proteins in both the CNS and the periphery fulfil an important immunological role, supporting the adaptive immune system, or directly clearing pathogens, while within the CNS itself, complement proteins are expressed by neurons in an activity-dependent manner, tagging synapses for subsequent microglia-mediated elimination (Mastellos, 2014; Perez-Alcazar et al., 2014). A genetic link between complement, schizophrenia and aberrant synapse elimination suggests that increased expression of C4 may lead to an increase in synaptic pruning in patients with schizophrenia (Sekar et al., 2016). Therefore, inappropriate complement activation may contribute to alteration of cortical connectivity, leading to the development of schizophrenia (Fields et al., 2015; Forsyth and Lewis, 2017; Narr and Leaver, 2015). As the assumption that PQ-motif homology is integral to AGA cross-reactivity does not appear to be true for schizophrenia-related AGAs, the complement-mediated pathways may be more relevant to the role of AGA activity in schizophrenia, than cross-reactivity with CNS antigens. Additionally, circulating IgA antibodies are weak activators of the complement pathway and may even inhibit activity of complement proteins. Consequently, decreases in specific IgA against gliadin-derived peptides may be enable the formation of complement complexes in the presence of gliadin-derived fragments (Russell et al., 1989). It is worth noting that the lack of PQ-motifs in AAQ6C is only suggestive of a lack of cross-reactivity with schizophrenia-associated AGAs and a study seeking to nullify this hypothesis would require purified antibodies specifically binding to AAQ6C. Additionally, an appropriate assay performed with antibody-binding studies in CNS-receptor transfected HEK-cells or primary tissue would be the gold standard (Margari et al., 2013; van Mierlo et al., 2015). Further studies are required to

investigate the mechanism behind the pathological role of anti-AAQ6C IgG in animal models with a range of behavioural, electrophysiological and histological measures.

One implication of AGA involvement in schizophrenia is the use of a gluten-free diet (GFD) in the treatment of schizophrenia. It has recently been proposed that targeting B-cells with monoclonal antibodies may ameliorate immunological phenomena in patients with schizophrenia. Should AGAs play a role in a subgroup of schizophrenia, it is possible that treatments with natalizumab and ritulzimab could lead to a reduction in symptoms, although the elimination of gluten from the diets would likely result in fewer side-effects and therefore, if viable, is more likely to be adopted. Previous studies have suggested that the GFD may be beneficial for a small number of patients, a recent case-study with a double-blind gluten challenge demonstrated that a patient displayed psychiatric symptoms in response to gluten and amelioration of symptoms upon gluten-withdrawal with no persistent adverse effects (Lionetti et al., 2015). In a separate case-study, psychiatric symptoms in a patient with schizophrenia were alleviated with the GFD, but this patient subsequently developed autoimmune-related hearing loss (Eaton et al., 2015b). It should be noted, however, that these interventions were given early in adolescent patients; if, as hypothesised based on the above results, AGAs are able to cause immune-mediated changes in neuronal connectivity, then the possibility that these alterations would permanently alter neuronal circuits cannot be excluded. It is therefore imperative that GFD interventions should be given as early as possible in order to circumvent the risk of permanent structural alterations and also that studies of GFD are performed on first-episode or preferably early onset candidates.

In the broad context of the study of AGA in schizophrenia, these results suggest that a compliment-mediated mechanism is more likely than the more simplistic cross-reactivity mechanism, although it is important to acknowledge that the two are not mutually exclusive as IgG-Ag complexes are required for complement activation and therefore a cross-reactive AGA bound to a target CNS protein could also promote complement activation. Putatively, decreased

levels of specific serum IgA against gliadin peptides may also support the complement activation model, which provides an intriguing mechanism by which AGA could contribute to alterations of neuronal connectivity. One problem with this proposed mechanism is that an antibody response against any antigen, in the context of the CNS, could then conceivably result in a schizophrenia-like pathology. Although this may seem unlikely, this thesis details a body of literature that includes a number of antibodies against a broad array of targets derived from various sources associated with increased risk of developing schizophrenia.

6.2 Genetic contributions to the secretion of anti-gliadin antibodies

The study presented in this thesis failed to demonstrate dependence of anti-AAQ6C levels on the presence of any HLA-II variants, instead showing that the presence of the HLA-DQ 8.1 variant was predictive of decreased anti-AAQ6C IgG levels. It is not surprising that none of HLA-II variants was found to be associated with plasma anti-AAQ6C antibody levels, because the HLA-region is one of the most gene-dense in the human genome and contains highly polymorphic loci of the HLA genes, with 4230 named HLA-II alleles in 2013 (Robinson et al., 2013) and 4700 identified to date (https://www.ebi.ac.uk/ipd/imgt/hla/). In the absence of an immune-dominant HLA variant associated with schizophrenia, unlike CD, detection of potential associations between HLA-II variants and anti-AAQ6C IgG a large sample cohort would be required, allowing for the relationship between significant HLA-II associations and schizophrenia to be examined. As such, a study would likely reveal primarily statistical rather than biological associations, and experimental validation in cell culture models would be required. Taken together, the identification of specific HLA-variants for schizophrenia is important, as genotyping individuals could identify potential risk factors for schizophrenia and a gluten-sensitive subgroup.

HLA-allele binding prediction was based upon HLA-DRB alleles, while the minor alleles of genotyped HLA-tagging SNPs were HLA-DQ variants. However, HLA-DR alleles are also tagged to some of these SNPs also tagged to HLA-DRB alleles, forming DQ-DRB haplotypes. The minor allele of rs6457614 (G) is tagged to the HLA-DR1 and HLA-DQ5 haplotypes, in a Caucasian population (de Bakker et al., 2006). However, there was no association observed between this SNP and levels of antibodies against gliadins (see Chapter 2). Furthermore, the minor allele of rs3135388(T) is tagged to the HLA-DR15 and HLA-DQ6 haplotypes (de Bakker et al., 2006) (see Chapter 2). Levels of antibodies against AAQ6A were increased in carriers of this SNV in patients with schizophrenia, however HLA-DR15 was not predicted to bind to this fragment *in silico* (Chapter 2 and Chapter 3).

The detection of genetic association between plasma antibodies against gliadin-derived antigens and schizophrenia could link anti-gliadin antibodies and other schizophrenia-related loci, and potentially other schizophrenia mechanisms. SNP rs4388249, for example, is proximal to the MAN2A1 gene, and knockouts of its homologue in murine models induced a systemic lupus-like pathology; therefore examining the association between the two factors could provide insight into the secretion of antibodies against gliadin-derived antigens. However, none of 4 SNPs detailed in Chapter 4 showed any association with plasma anti-gliadin antibody levels. It is possible that the genes tagged to these 4 SNPs are not involved in alteration of anti-gliadin antibodies. It is possible that alternative selection criteria of SNPs or employing an unbiased approach to SNP selection may have yielded more promising associations.

6.3 The immunogenic potential of AAQ6C and AL2G1 antigens

Two cell-line models were used to examine the immunological effects of AAQ6C and AL2G1 antigens in this study. These antigens were selected based on the levels of IgG against them as levels of IgG showed the greatest differences between case and control samples. The immature dendritic cell model demonstrated that challenge with both AAQ6C and AL2G1 was able to increase the adherence of immature dendritic-like cells, consistent with dendritic cell

maturation and confirming that maturation does not depend upon the specific peptide present (Palová-Jelínková et al., 2005). This study also demonstrated that AAQ6C and AL2G1 had immunological effects, independent of disease state and likely independent of HLA-II genotypes. These observations suggest that there may be no differential effects of gliadin-derived antigens on DC maturation although anti-inflammatory cytokines should be measured in future experiments with this cell model.

There were a number of technical issues in this DC model. The low yield of cells likely due to the strong adherence, meant that cell surface characterisation was a particular problem. Induction of DCs from monocytes isolated from peripheral blood mononuclear cells is a routine technique, compared to the THP-1 cell-line, and the resulting DC-like cells are characterised as 'mildly adherent', making flow cytometry characterisation of the cells much less challenging (Nair et al., 2012). There are a number of other advantages in utilising primary cells, Firstly, primary cells are more likely to reflect the typical physiological function of DCs; secondly, there are more options for outcome measurement in primary cells including the use of flow cytometry; finally, the use of primary cells is suitable for the examination of immune responses to gliadin-derived peptides in schizophrenia populations. Based on the results reported in this thesis, therefore, the use of primary cells appears to be superior in almost all measures than the THP-1 derived DC-like cells. In general, a main advantage of cell-line based models over primary tissues is that they are comparatively easy to handle and grow, which removes tissue availability as the primary limiting step. Despite this, it is recommended that future studies of DCs, in the context of schizophrenia or otherwise, should utilise primary cell cultures rather than this cell model.

Although there were some limitations of the study with the cell models employed here, this chapter presented useful preliminary results demonstrating that gliadin-derived peptides have biological effects on antigen-presenting cells, suggesting that these gliadin-derived peptides are not simply inert molecules, ignored by the immune system. The broader

implications of this point must be assessed with more comprehensive studies of the immunological response to these cells in the context of schizophrenia, which could eventually provide direct evidence to address the interaction between gliadin-derived antigens and immune cells.

6.4 Future work and Outlook

The work presented in this thesis has further characterised the anti-gliadin immune response in schizophrenia, although it was not conclusive in clarifying whether an immune response against wheat gluten contribute to schizophrenia. With case studies demonstrating that gluten could trigger the symptoms of schizophrenia, a gluten-sensitive subgroup is likely to exist among patients with schizophrenia although the proportion of this subgroup is unknown. While this and other subgroups become better defined and quantified in the future, it remains to be seen whether schizophrenia survives as a clinical classification or whether various potential subgroups become clinical classifications within their own right (Lionetti et al., 2015; Os, 2016b).

Studies of circulating antibodies against native gliadins have produced almost consistent outcomes that AGAs were elevated in patients with schizophrenia; recent studies have substantiated this claim although the results presented in this thesis failed to replicate the elevated AGA IgG levels in patients with schizophrenia (Čiháková et al., 2017; Lachance and McKenzie, 2014). Increased levels of IgG antibody against AAQ6C peptide derived from a γ-gliadin are consistent with previous studies showing elevated AGA IgG levels in patients with schizophrenia, but this finding has yet to be validated in the same way as antibodies against native gliadins; therefore, further replication of these initial results are required in independent case-control samples. Additionally, the inclusion of a drug-naïve or unmedicated group would be useful in fully addressing the role of antipsychotic medication on the production of anti-gliadin antibodies.

The cell culture study presented here developed cell models using cell-lines derived from peripheral immune cells; while the peripheral immune system may play a role in both schizophrenia and immune activity in the CNS, cell culture studies in primary and disease relevant tissue are desirable. Under inflammatory conditions, a subset of peripherally derived monocytes migrate into the CNS and undergo phenotypic and functional differentiation, resembling microglia. Although microglia have often been termed CNS-resident macrophages, this is an oversimplification and important differences exist between these two populations of immune cells (London et al., 2013). Ohgidani et al. (2015) developed a protocol to induce peripherally derived monocytes into microglia-like cells using a cytokine cocktail comprising IL-4 and GM-CSF; when applied to the psychiatric disorder Nasu-Hakola disease, a difference in microglia function between cases and controls was observed (Ohgidani et al., 2014).

This microglia model represents an opportunity to study the effects of a gliadin-specific immune response in cells that are pathologically relevant to schizophrenia. This cell model could be investigated using gliadin-peptide challenge in microglia to examine a difference in the immunogenic effects between AAQ6C and AL2G1 with a similar experimental design to the DC model as described in Chapter 5. Alternatively, since antibodies against AAQ6C may be more likely to play a role in susceptibility to schizophrenia, AAQ6C antigen-IgG complexes could be utilised for an experimental design. In the absence of commercially available antibodies, this would be achieved using plasma rich in anti-AAQ6C IgG. Autologous microglia could then be incubated with such plasma after heat-inactivation. The outcome measure of this study needs to be carefully considered and should probably focus on the cellular response rather than products excreted into the supernatant. One recent outcome measured in microglia from an animal model of maternal immune activation showed the upregulation of the C4 receptors, suggesting that C4 may contribute to an increase in synaptic pruning in schizophrenia, although an unbiased exploratory study with the differential gene expression in response to plasma incubation may also be a valid approach (Mattei et al., 2017; Sekar et al.,

2016). One advantage of using short linear peptides to identify antibodies is that antibodies against them should be epitope-specific, and therefore, the effects of anti-AAQ6C IgG incubation on microglia could be achieved using healthy control individuals as an initial experiment, followed by experiments using samples from patients with schizophrenia to examine a differential microglial response between cases and controls.

6.5 Concluding remarks

The results presented in this thesis have further characterised the altered levels of a range of specific anti-gliadin antibodies in patients with schizophrenia, and then attempted to investigate these observations through analysis of genetic associations and cell models. However, there were no association between genetic variants tested and anti-AAQ6C IgG secretion nor was a specific peptide effect convincingly demonstrated in cell models. This thesis fulfilled the aims set out in section 1.7, despite the lack of associations observed between genotype, antibodies and schizophrenia and with no observed difference in cell response to gliadin peptides. The potential reasons for this have been discussed in their relevant sections and the work in this thesis can be built upon to more definitively examine the anti-gliadin immune response in patients with schizophrenia.

The study of the anti-gliadin antibody contribution to schizophrenia has the potential to lead to a relatively economical and benign treatment for a gluten-sensitive subgroup of the disease. Although the link between gluten consumption and schizophrenia was noted several decades ago, the study of the precise mechanism behind this phenomenon is still in its infancy. As clinical trials with a GFD in treatment of schizophrenia are imminent, it is important to categorise which patients would benefit from this treatment and to clarify by which mechanism gliadin may contribute to the disease. The results in this thesis require further replication although they have raised interesting questions regarding the nature of the anti-gliadin immune

response and pointed to new avenues of research in this field. The above studies provided a new understanding of the anti-gliadin immune response in schizophrenia by showing that different gliadin-derived peptides provoke different antibody responses. To the candidate's knowledge, this thesis contains the first studies attempting to address the possible mechanism by which gliadin-derived peptide antigens may trigger a pathological immune response in the context of schizophrenia. Future studies following this thesis should focus on the mechanism, if any, by which these anti-gliadin antibodies may contribute to the pathogenesis of schizophrenia.

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Appendix 1

Output from Peptide Cutter Software

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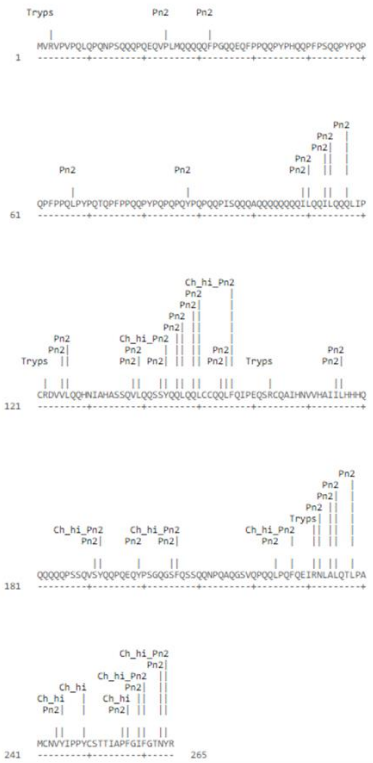
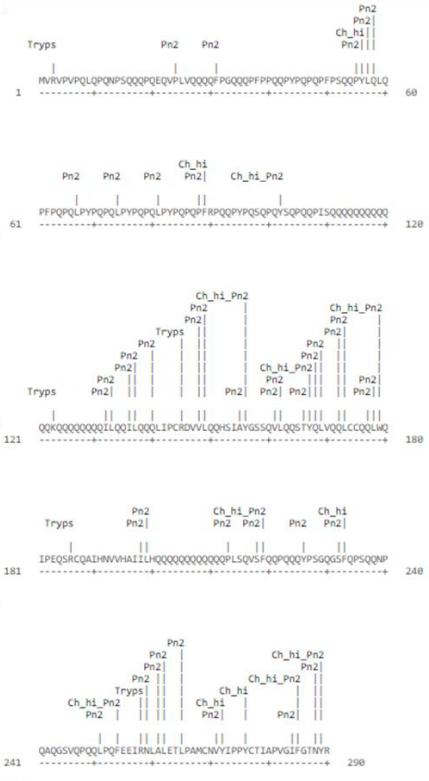


CAB76964



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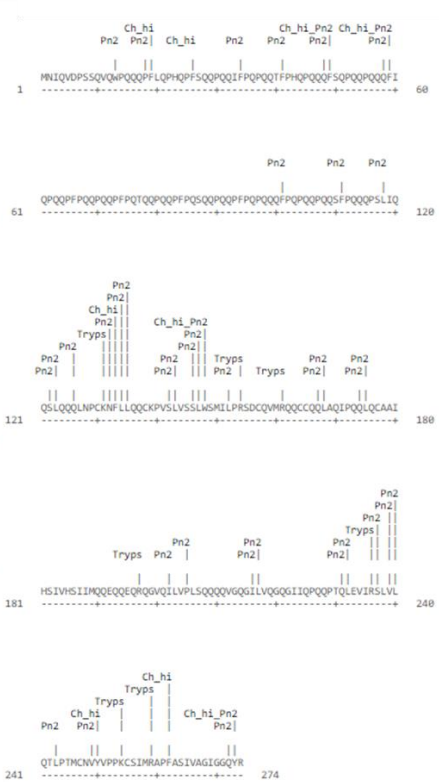
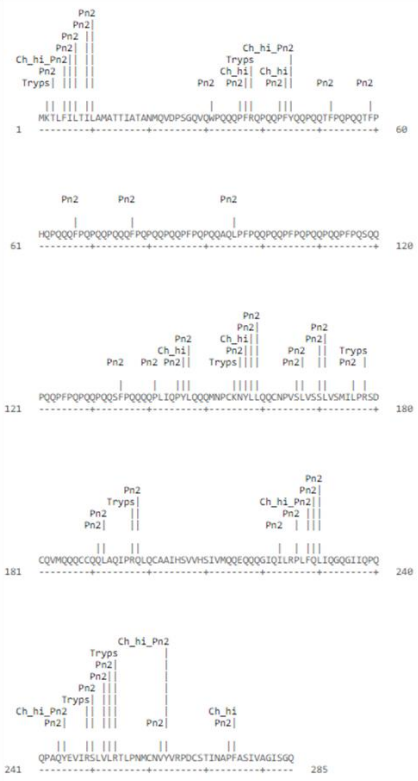


ABO37962



CAB76957

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SRCQAIHNVVHAIIL

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RSDCQVMQQQCCQQL

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LQCAAIHSVVHSIVMQQEQQQGIQI

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LIQGQGIIQPQQPAQ

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Outputs from IEDB HLA-Recognition

CAB76957

allele	seq_num	start	end	peptide	smm_align_core	smm_align_ic50	smm_align_rank
HLA-DRB1*07:01	5	1	15	LQQHNIAHASSQVLQ	IAHASSQVL	49	0.37
HLA-DRB1*01:01	5	1	15	LQQHNIAHASSQVLQ	IAHASSQVL	60	11.59
HLA-DRB1*07:01	6	1	15	SRCQAIHNVVHAIIL	IHNVVHAII	101	0.92
HLA-DRB1*13:02	6	1	15	SRCQAIHNVVHAIIL	AIHNVVHAI	105	2.82
HLA-DRB1*01:01	6	1	15	SRCQAIHNVVHAIIL	IHNVVHAII	212	29.49
HLA-DRB1*13:02	5	1	15	LQQHNIAHASSQVLQ	IAHASSQVL	399	10.7
HLA-DRB1*01:01	4	2	16	QPQQPISQQQAQQQQ	ISQQQAQQQ	464	44.13
HLA-DRB1*01:01	4	1	15	PQPQQPISQQQAQQQ	QQPISQQQA	469	44.34
HLA-DRB1*15:01	5	1	15	LQQHNIAHASSQVLQ	IAHASSQVL	496	9.64

CAB76964

allele	seq_num	start	end	peptide	smm_align_core	smm_align_ic50	smm_align_rank
HLA-DRB1*07:01	5	1	15	RCQAIHNVVHAIILH	HNVVHAIIL	101	0.92
HLA-DRB1*13:02	5	1	15	RCQAIHNVVHAIILH	AIHNVVHAI	104	2.79
HLA-DRB1*01:01	5	1	15	RCQAIHNVVHAIILH	IHNVVHAII	216	29.81

AAQ63857

allele	seq_num	start	end	smm_align_core	smm_align_ic50	smm_align_rank
HLA-DRB1*07:01	3	1	15	IHSIVHSII	84	0.76
HLA-DRB1*07:01	3	2	16	IHSIVHSII	84	0.76
HLA-DRB1*01:01	6	1	15	FASIVAGIG	84	15.38
HLA-DRB1*07:01	3	3	17	IHSIVHSII	87	0.79
HLA-DRB1*01:01	3	3	17	IHSIVHSII	152	24.07
HLA-DRB1*01:01	3	2	16	IHSIVHSII	154	24.27
HLA-DRB1*01:01	3	1	15	IHSIVHSII	156	24.47
HLA-DRB1*07:01	3	4	18	HSIVHSIIM	160	1.62
HLA-DRB1*07:01	1	1	15	IQVDPSSQV	196	2.11
HLA-DRB1*03:01	1	1	15	IQVDPSSQV	206	0.1
HLA-DRB1*01:01	5	1	15	QGIIQPQQP	252	32.48
HLA-DRB1*07:01	3	5	19	IHSIVHSII	256	2.87
HLA-DRB1*13:02	1	1	15	IQVDPSSQV	263	7.44
HLA-DRB1*01:01	3	4	18	IHSIVHSII	310	36.24
HLA-DRB1*04:04	1	1	15	IQVDPSSQV	366	4.11
HLA-DRB1*13:02	3	2	16	AIHSIVHSI	369	10.03
HLA-DRB1*13:02	3	1	15	AIHSIVHSI	391	10.52
HLA-DRB1*01:01	3	5	19	IHSIVHSII	444	43.29
HLA-DRB1*01:01	4	1	15	LSQQQQVGQ	466	44.21
HLA-DRB1*04:01	1	1	15	IQVDPSSQV	481	5.26
HLA-DRB1*15:01	6	1	15	FASIVAGIG	481	9.34

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allele	seq_num	start	end	peptide	smm_align_core	smm_align_ic50	smm_align_rank
HLA-DRB1*01:01	8	8	22	INAPFASIVAGISGQ	FASIVAGIS	32	6.21
HLA-DRB1*01:01	8	6	20	STINAPFASIVAGIS	INAPFASIV	33	6.42
HLA-DRB1*01:01	8	7	21	TINAPFASIVAGISG	FASIVAGIS	33	6.42
HLA-DRB1*07:01	6	1	15	LQCAAIHSVVHSIVM	IHSVVHSIV	103	0.94
HLA-DRB1*07:01	6	2	16	QCAAIHSVVHSIVMQ	IHSVVHSIV	104	0.95
HLA-DRB1*07:01	6	3	17	CAAIHSVVHSIVMQQ	IHSVVHSIV	105	0.96
HLA-DRB1*01:01	8	3	17	PDCSTINAPFASIVA	INAPFASIV	105	18.34
HLA-DRB1*01:01	8	2	16	RPDCSTINAPFASIV	CSTINAPFA	108	18.73
HLA-DRB1*07:01	6	4	18	AAIHSVVHSIVMQQE	IHSVVHSIV	111	1.02
HLA-DRB1*01:01	8	5	19	CSTINAPFASIVAGI	INAPFASIV	120	20.28
HLA-DRB1*01:01	6	4	18	AAIHSVVHSIVMQQE	IHSVVHSIV	126	21.05
HLA-DRB1*01:01	6	1	15	LQCAAIHSVVHSIVM	IHSVVHSIV	127	21.19
HLA-DRB1*01:01	8	4	18	DCSTINAPFASIVAG	INAPFASIV	128	21.32
HLA-DRB1*01:01	6	3	17	CAAIHSVVHSIVMQQ	IHSVVHSIV	128	21.32
HLA-DRB1*01:01	6	2	16	QCAAIHSVVHSIVMQ	IHSVVHSIV	130	21.56
HLA-DRB1*07:01	8	6	20	STINAPFASIVAGIS	INAPFASIV	190	2.03
HLA-DRB1*07:01	1	1	15	AMATTIATANMQVDP	TIATANMQV	212	2.31
HLA-DRB1*07:01	1	2	16	MATTIATANMQVDPS	TIATANMQV	213	2.33
HLA-DRB1*07:01	1	3	17	ATTIATANMQVDPSG	TIATANMQV	221	2.42
HLA-DRB1*07:01	6	5	19	AIHSVVHSIVMQQEQ	IHSVVHSIV	225	2.47
HLA-DRB1*01:01	6	5	19	AIHSVVHSIVMQQEQ	IHSVVHSIV	261	33.11
HLA-DRB1*07:01	8	2	16	RPDCSTINAPFASIV	DCSTINAPF	271	3.07
HLA-DRB1*07:01	8	5	19	CSTINAPFASIVAGI	INAPFASIV	293	3.34
HLA-DRB1*07:01	8	3	17	PDCSTINAPFASIVA	INAPFASIV	295	3.36
HLA-DRB1*07:01	8	7	21	TINAPFASIVAGISG	FASIVAGIS	299	3.41

HLA-DRB1*07:01	8	8	22	INAPFASIVAGISGQ	FASIVAGIS	302	3.45
HLA-DRB1*04:01	8	8	22	INAPFASIVAGISGQ	FASIVAGIS	303	3.19
HLA-DRB1*04:01	8	7	21	TINAPFASIVAGISG	FASIVAGIS	307	3.24
HLA-DRB1*07:01	8	4	18	DCSTINAPFASIVAG	INAPFASIV	310	3.56
HLA-DRB1*04:01	8	6	20	STINAPFASIVAGIS	PFASIVAGI	317	3.34
HLA-DRB1*07:01	6	6	20	IHSVVHSIVMQQEQQ	IHSVVHSIV	325	3.75
HLA-DRB1*13:02	6	3	17	CAAIHSVVHSIVMQQ	AIHSVVHSI	346	9.49
HLA-DRB1*13:02	6	1	15	LQCAAIHSVVHSIVM	AIHSVVHSI	349	9.56
HLA-DRB1*13:02	6	2	16	QCAAIHSVVHSIVMQ	AIHSVVHSI	350	9.59
HLA-DRB1*15:01	8	6	20	STINAPFASIVAGIS	NAPFASIVA	357	6.85
HLA-DRB1*01:01	6	6	20	IHSVVHSIVMQQEQQ	IHSVVHSIV	364	39.37
HLA-DRB1*01:01	1	1	15	AMATTIATANMQVDP	TIATANMQV	370	39.69
HLA-DRB1*11:01	8	8	22	INAPFASIVAGISGQ	FASIVAGIS	373	0.82
HLA-DRB1*11:01	8	7	21	TINAPFASIVAGISG	FASIVAGIS	377	0.84
HLA-DRB1*11:01	8	6	20	STINAPFASIVAGIS	STINAPFAS	394	0.9
HLA-DRB1*01:01	1	2	16	MATTIATANMQVDPS	TIATANMQV	398	41.1
HLA-DRB1*15:01	8	5	19	CSTINAPFASIVAGI	NAPFASIVA	407	7.84
HLA-DRB1*15:01	8	3	17	PDCSTINAPFASIVA	INAPFASIV	408	7.86
HLA-DRB1*15:01	8	7	21	TINAPFASIVAGISG	NAPFASIVA	415	8.01
HLA-DRB1*13:02	1	1	15	AMATTIATANMQVDP	TIATANMQV	424	11.25
HLA-DRB1*13:02	1	2	16	MATTIATANMQVDPS	TIATANMQV	424	11.25
HLA-DRB1*13:02	1	3	17	ATTIATANMQVDPSG	TIATANMQV	426	11.3
HLA-DRB1*15:01	8	4	18	DCSTINAPFASIVAG	NAPFASIVA	433	8.37
HLA-DRB1*04:04	8	6	20	STINAPFASIVAGIS	INAPFASIV	487	5.54
HLA-DRB1*01:01	1	3	17	ATTIATANMQVDPSG	TIATANMQV	496	45.43