A Genetic Analysis of the Relationship between Gluten Intolerance and Schizophrenia

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A Genetic Analysis of the Relationship between Gluten Intolerance and Schizophrenia

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Declaration

This is to certify that this thesis has been composed by the author named, Matilda M.J. Bradford. It has not been accepted in any previous application for a degree, and all work described is my own unless clearly stated otherwise. All quotations have been distinguished by quotation marks and all sources of information specifically acknowledged.

Signed…….

(Matilda M.J. Bradford)

Date……..

Abstract

A relationship between schizophrenia and coeliac disease (CD) has long been postulated. However, despite the substantial genetic contribution defined for both conditions, the possibility of shared genetic risk factors has not previously been addressed. In this study, key CD-associated loci were tested in a schizophrenia cohort. Where association was found, functional studies were performed; including gene expression analysis, screening for functional variants, and correlation of alleles with antibody and cytokine levels.

Two principle findings were drawn from the data produced. Firstly, there was no statistically significant association between schizophrenia, and the CD-associated loci; MYO9B, IL-2/21, or HLA-DQ2. Secondly, a robust association was found for 3 SNPs located in the 5' region of the gene encoding TGM2; the gluten-modifying enzyme targeted by autoantibodies in CD. Functional studies provided evidence of decreased serum IL-2, in correlation with a schizophrenia-associated SNP located in the TGM2 gene. In addition, cell culture work showed altered expression of the TGM2 gene, following treatment with 1 μg/ml of the neuroleptic drug clozapine.

This study concludes that the major genetic factors underlying schizophrenia and CD are distinct, and that the gluten response observed in schizophrenia does not follow the classical CD pathway. Furthermore, this study provides evidence of a novel candidate gene for schizophrenia, TGM2, and explores how it may contribute to neurological aspects of the disease.

Publications

Concepts and data within this thesis have appeared previously in the following publications:

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BRADFORD, M., LAW, M. H., MEGSON, I. L. & WEI, J. 2011. The functional significance of the TGM2 gene in schizophrenia: a correlation of SNPs and circulating IL-2 levels. *J Neuroimmunol,* 232**,** 5-7.

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*These two authors contributed to this work equally.

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Nomenclature

CD = coeliac disease

cDNA = copy deoxyribonucleic acid

CNS = central nervous system

DMSO = dimethyl sulfoxide

DSM-III/DSM-IV = diagnostic and statistical manual of mental disorders

DNA = deoxyribonucleic acid

ELISA = enzyme-linked immunosorbent assay

EMA = endomysial antibody

GWAS = genome wide association study

GWLS = genome wide linkage study

HLA = human leukocyte antigen

HUVEC = human umbilical vein endothelial cells

HWE = Hardy-Weinberg equilibrium

IL-2 = interleukin 2 (gene or protein)

LD = linkage disequilibrium

LPS = lipopolysaccharide

MHC = major histocompatibility complex

mRNA = messenger ribonucleic acid

MYO9B = myosin IXB gene

NMDA = N-methyl-D-aspartate

PCP = phencyclidine (phenylcyclohexylpiperidine)

PCR = polymerase chain reaction

RFLP = restriction fragment length polymorphism

RNA = ribonucleic acid

RT-QPCR = reverse transcription- quantitative polymerase chain reaction

SNP = single nucleotide polymorphism

TDT = transmission disequilibrium test

TF = transcription factor

TFBS = transcription factor binding site

TGM2 = tissue transglutaminase 2 (gene or protein)

THP1 = human acute monocytic leukemia cell line

U937 = human leukemic monocyte lymphoma cell line

Background and Objectives

Schizophrenia is a chronic and debilitating psychiatric illness, with a median incidence of 15.2/100,000 (McGrath, 2006) across populations. The World Health Organisation (WHO) estimates that around 24 million people are affected by schizophrenia worldwide. The condition is usually lifelong, with substantial medical, financial and social impacts for sufferers. In societal terms, the cost is equally high; a study of the data from 2002 estimated the annual cost to the US at $62.7 billion, ranking schizophrenia in the top ten diseases for expense to society (Knapp et al., 2004, Wu et al., 2005).

Although a variety of psychotic disorders are recorded in medical histories, schizophrenia itself was not defined until the 19th century, when Emil Kraepelin described the condition using the term "dementia praecox". This name referred to the incapacitating nature of the condition, and distinguished it from dementia occurring in the elderly. Eugen Bleuler later renamed the disorder with its modern term: schizophrenia, meaning "fragmented mind", referring to the variety of states that a sufferer may experience as a result of the illness.

Schizophrenia is now widely accepted to be a condition determined by both genetic and environmental factors, with genetics constituting the greatest risk; twin studies estimate the condition to be being 80-85% heritable (Cardno et al., 1999, Cardno and Gottesman, 2000). The search for genetic variation contributing to schizophrenia, has encompassed a range of approaches, including genome-wide analyses (GWA) and a host of hypothesis-driven research, such as candidate gene studies. Despite this, very little of the genetic contribution to schizophrenia has been defined (Tiwari et al., 2010). The presentation of schizophrenia suggests that a large number of genes may be involved, acting in combination to produce a predisposition to the illness (Purcell et al., 2009).

Although the figure for heritability is high, it is not thought to reflect a simple causative relationship between genes and disease; rather, the condition is a result of complex gene-gene and gene-environment interactions (Jaffee and Price, 2007, Tsuang et al., 2004). In considering the relationship between genetic and environmental factors, discussion has arisen regarding the role of the immune system in schizophrenia. A number of hypotheses have been put forward regarding schizophrenia and autoimmunity, of which the association between schizophrenia and coeliac disease is one of the best known (Kalaydjian et al., 2006, West et al., 2006). Coeliac disease is perhaps the best-characterized autoimmune condition, with defined genetic variation and dietary risk factors (Mowat, 2003). As such, analysis of the relationship between coeliac disease and schizophrenia could offer novel insights into the pathology of schizophrenia, for which causative factors are currently far less clear. This is not a novel hypothesis - research has been performed for several decades aimed at defining the relationship in terms of shared phenotypes (primarily antibody levels) (GRAFF and HANDFORD, 1961, Reichelt and Landmark, 1995, De Santis et al., 1997, Peleg et al., 2004). However, little has been done to investigate the possibility of shared genetic factors between the conditions. This project aimed to perform such investigations, searching for common factors that might shed light upon the relationship between coeliac disease and schizophrenia, and so the aetiology of schizophrenia itself.

1 Introduction

1.1 Literature review

1.1.1 Schizophrenia: Definitions and incidence

Primarily a psychotic disorder, schizophrenia is characterized not by a single phenotype, but by a number of clinical symptoms appearing in combination (though not necessarily at the same time point). The majority of symptoms are defined as either "positive" or "negative". Positive symptoms are those which present with an increase or distortion of normal experience, such as hallucinations, delusions and thought disorders, while negative symptoms are those involving a loss of function, such as apathy or social withdrawal. Symptoms relating to disorganization are also a feature, with patients often experiencing thought disorder, disorientation or cognitive impairments. Current diagnoses are usually made according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV), the criteria for schizophrenia from which are summarized in Table 1-1. It should be noted that samples collected for this research were from patients diagnosed according to the criteria set out in the earlier edition, DSM-III-R, but these two versions are considered to be relatively consistent in their criteria for diagnosis (Weinberger and Harrison, 2011).

Table 1-1 DSM-IV diagnostic criteria for schizophrenia

Category	Description	Duration
Characteristic symptoms	Two or more of: - Delusions - Hallucinations - Disorganized speech - Grossly disorganized or catatonic behaviour - Negative symptoms	Present for a significant proportion of time during a one month period.
Social or occupational dysfunction	Markedly below the level prior to onset in at least one of: - Work - Interpersonal relations - Self-care	For a significant proportion of the time since symptoms began.
Duration		Continuous signs of disturbance for at least 6 months. Characteristic symptoms may be attenuated or not present at times within this.
Schizoaffective and mood disorder exclusion	These have been ruled out.	
Substance or general medical condition exclusion	The symptoms do not result from the physiological effects of either a substance or other medical condition.	
Relationship to a pervasive developmental disorder.	If there is a history of autism of another pervasive developmental disorder, schizophrenia should only be diagnosed if there are: - Prominent hallucinations or delusions	Symptoms must be present for at least one month.

Although the symptoms described in Table 1-1 must be present for a diagnosis of schizophrenia to be made, the nature and presentation of the symptoms can vary widely between patients. Schizophrenia may share symptoms and biological causes with a number of closely related diseases, such as bipolar disorder and schizoaffective disorders.

Individuals who develop schizophrenia usually do so in early adulthood, although there are exceptions with some adults developing the illness later in life, and a small number of children presenting with a juvenile-onset form (Kydd and Werry, 1982).

There has been a concept of schizophrenia as a disease which is present equally across regions and cultures (Sartorius et al., 1986). Re-examination of the literature in recent years, however, has highlighted the degree to which its incidence varies. A review of 158 studies concerning schizophrenia incidence found that conservative estimates (including the central 80% of medians) produced a range of figures from 7.7 to 43.0 cases per 100,000 (McGrath et al., 2004). Interestingly, another review, which took into account economic status of the countries included, reported no significant link between this factor and the incidence of schizophrenia (Saha et al., 2006b). It is notable though, that the range of data for the least developed countries was from 0.4 to 35.0 per 100,000, despite only three studies being available for this group, compared to 42 studies were available for the developed countries.

Several factors are consistently reported to increase the risk of developing schizophrenia; winter birth/latitude (Davies et al., 2003, Saha et al., 2006a), male gender (Aleman et al., 2003), urban dwelling (Cantor-Graae, 2007), and migrant status (Cantor-Graae and Selten, 2005, McGrath et al., 2004). All contribute small relative risks, but have effects that have been successfully replicated. These factors, alongside the variance in geographical incidence, confirm the relevance of environmental factors for schizophrenia. Whilst consistent, however, these factors all represent broad categories within which a host of mechanisms might operate, and considerable research is still needed to define the specific elements that contribute to schizophrenia. When

taken in combination with the substantial heritability observed for schizophrenia, this evidence of environmental input validates the search for gene-environment interaction in the condition.

1.1.2 Brain-centred models of pathology

1.1.2.1 Neurotransmitters in schizophrenia

While this project is not intended to review or expand specifically the neurobiological understanding of schizophrenia, a brief overview of the hypotheses in this area will be discussed.

For many years, the dopaminergic hypothesis, which emphasizes over-activity of dopaminergic neurotransmission in the brain, has been the most widely accepted theory to explain the aetiology of schizophrenia. Originally derived from observations of drug effects - namely that dopamine agonists induced paranoia, while dopamine antagonists improved the symptoms of psychosis (CARLSSON and LINDQVIST, 1963) - it has since been expanded and refined, and continues to be a central part of schizophrenia theory. The dopamine hypothesis was partly developed through the concept of "motivational salience", which refers to the process of external stimuli being linked to rewarding events, and so translated into something of motivational significance. This concept was combined with the theory of dopamine dysregulation, with dopamine postulated as the facilitator of stimulus translation. It was suggested that the frequent and dysregulated firing of dopamine neurones in schizophrenia might result in aberrant salience, with patients therefore misattributing significance to inappropriate stimuli or internal experiences, and so displaying psychotic symptoms (Berridge and Robinson, 1998, Heinz and Schlagenhauf, 2010, Kapur, 2004). This concept has since been reinforced by biological evidence from neuroimaging studies: patients with schizophrenia show increased dopamine transmission in the ventral striatum, the primary region for dopaminergic reward processing (Abi-Dargham et al., 1998, Howes et al., 2009, Schmitt et al., 2009, Jensen et al., 2008).

Abnormal dopamine activity may also explain other aspects of schizophrenia, such as difficulties relating to cognition and working memory. In this case, it is the balance and interaction between dopamine D_1 and D_2 receptors which is hypothesised to influence symptoms (Durstewitz and Seamans, 2008). Twin studies into schizophrenia appear to confirm the relevance of dopamine, and furthermore to identify it as a genetic risk factor. Hirvonen *et al* found that in monozygotic twins discordant for schizophrenia, the unaffected twin (like their affected sibling) had increased D_2 receptor density in the caudate, a feature not reflected in the unaffected dizygotic twins and sib-pairs (Hirvonen et al., 2006a, Hirvonen et al., 2006b).

While the dopamine hypothesis is well established and convincing in its explanation of positive symptoms, schizophrenia encompasses a much wider range of effects and excess dopamine alone seems unlikely to account for all of these. Utilising a similar approach to that used to derive the dopamine hypothesis, the concept of aberrant neurological signalling has since been extended to the glutamatergic system. Treatment with phencyclidine (phenylcyclohexylpiperidine, PCP) causes not only psychosis but also the negative symptoms and cognitive impairments seen in schizophrenia (Tamminga, 1998). These symptoms are thought to result from impaired glutamatergic transmission, which may be caused by blockage of key ion channels in the N-methyl-D-aspartate (NMDA) receptors (Tamminga et al., 2003). A study by Pilowsky *et al.* (2006) provides *in vivo* evidence for this reduction of glutamatergic transmission, via an imaging study performed in medication-free patients. Perhaps most importantly, the symptoms observed through PCP treatment cover a wider range of those seen in schizophrenia, not focusing primarily on the positive symptoms associated with dopamine overactivity (Javitt and Zukin, 1991). The glutamate theory, however, is not a replacement for the dopamine hypothesis, but more likely a complement to it. The physiological structures of the glutamatergic and dopaminergic systems converge in the cortex, and evidence suggests that they

may interact with one another postsynaptically, and in a site-specific manner (Otani et al., 1999, Stone et al., 2007).

1.1.2.2 Neuro-anatomy and morphological change

While the pattern of morphological change in the brain is far from agreed for schizophrenia, there is a large volume of data showing alterations in morphology of some description. Some studies concern a very basic change in morphology with loss of volume/density, especially decreased grey or white matter in the prefrontal cortex. The results of such studies have yielded heterogeneous results, possibly because this alteration in brain structure is an age-sensitive process (Bose et al., 2009) or similarly, only present in latter stages of the illness (Moriya et al., 2010). Lack of replication is a pattern which has frustrated understanding in this field, likely influenced by the small sample sizes used in many studies. The pattern and timing of morphological change are important in characterising schizophrenia, and especially so in defining the relevance of neurodegenerative or neurodevelopmental processes to the condition. A review by Rund (2009) highlights the fact that while structural changes reminiscent of neurodegeneration, occur following disease onset, patients' cognitive functioning appears stable; the disconnect between these two factors prompts the question of which morphological changes drive the disease, and which result from it.

A recent study (Nenadic et al., 2010) showed impressive results for assigning specific morphological changes to subsyndromes within schizophrenia. With a cohort of 99 patients and 113 controls, the authors achieved >95% accuracy in predicting individuals status and subsyndrome from their scan alone. This more refined approach is likely to be important in generating consistent results for morphological change, and even more so for understanding the basis of symptoms from that morphology.

As imaging techniques have improved, research into brain structure has also focused on neuronal connectivity, with suggestions that decreased connectivity and synaptic reorganization

are features of schizophrenia. Evidence of altered connectivity is also important in the theory that plasticity (the brain's ability to adapt) is altered in schizophrenia. The nervous system appears to be capable of adapting to and, in some cases, compensating for, a great deal of change and disturbance, an ability often referred to as plasticity. Some researchers have postulated that impaired synaptic plasticity may be a major factor in the development of schizophrenia, and may explain the accompanying changes observed in brain structure (Stephan et al., 2006).

1.1.3 Genes and schizophrenia

Of the pathological factors contributing to schizophrenia, the bulk are thought to be genetic, with twin studies giving an estimated heritability of up to 85% (Cardno et al., 1999). Schizophrenia is a broad condition comprising many different phenotypes, with symptoms present to different degrees in individual patients. Such phenotypic heterogeneity can arise solely due to moderating environmental factors, and in some conditions a single gene mutation can exert a variety of effects - a process known as pleiotropy. In the case of schizophrenia, however, a single gene model would fail to explain the patterns of disease transmission, for which the best models implicate not only multiple genes, but a variation in which genes are affected in any given case, (ie – schizophrenia involves substantial genetic heterogeneity). While a number of genes have been associated with schizophrenia, their individual impact on susceptibility is uniformly small. As such, it is believed that the condition is influenced by multiple variants, each contributing a relatively small effect (Freedman et al., 2001). In addition to the cumulative effect of many small-impact gene mutations, there may also be epistatic effects, epigenetic effects, rare but high impact variants and a range of environmental factors, all acting in combination.

1.1.3.1 Linkage and HapMap

When searching for new genetic associations, the principle of linkage disequilibrium (LD) can be exploited. LD describes the situation whereby alleles at two or more loci associate with each other, due to less than average recombination having occurred between them historically. Regions of loci which segregate together in this way are referred to as LD blocks. Knowledge of LD blocks can be utilised to minimise the genotyping needed in association analyses; common DNA polymorphisms, such as single nucleotide polymorphisms (SNPs), can be used to "tag" the block they are located in, with the genotype determined for that SNP then being used to infer the variants present at other polymorphic loci within that block (Neale, 2010).

The HapMap project (Consortium, 2003, Tanaka, 2005, Thorisson et al., 2005) is an international collaboration, designed to investigate genetic variation across populations. All data from the HapMap project are available in the public domain, and the database can be used to aid researchers in selecting tag SNPs, identifying them based on location and heterozygosity (specific to ethnicity), to ensure they are sufficiently informative in a given sample. Both case-control and familial studies are suitable for this approach; family studies, have the benefit of being free from the confounding effects of stratification, but are associated with recruitment difficulties.

Sequences of alleles which are inherited together on the same chromosome are referred to as haplotypes. Haplotype analysis can be used in much the same was as allelic analysis when testing for association, with the added advantage that this approach can increase statistical power (Liu et al., 2008). Without sequencing, however, the phasing (which alleles belong on the same chromosome, for bi-allelic loci) of haplotypes is uncertain. The exception to this is when both parents and offspring are genotyped, with heterozygosity in the offspring allowing determination of which allele came from which parent. Where samples are unrelated or phase cannot be

determined by the alleles inherited, haplotypes can be mathematically inferred, by methods such as the maximum-likelihood algorithm used in the UNPHASED program (Dudbridge, 2008).

1.1.3.2 Whole Genome Studies

Since the advent of the human genome project, it has been possible to perform wide-scale genetic analyses such as genome-wide association (GWA) studies and genome-wide linkage scans (GWLS). GWA studies use DNA-chip technology to survey markers across the genome, comparing the frequency of distributions between groups of cases and controls. GWLS use pedigree samples to test spaced, informative DNA markers that can track a disease-causing mutation as it segregates within a family. For common diseases such as schizophrenia, GWA studies appear to be better at detecting the small effects of single nucleotide polymorphisms (SNPs) (Manolio et al., 2008), despite their case-control structure introducing the possibility of stratification. GWLS can detect signals from a wider range of variants and have the added advantage that they can sometimes detect a pooled signal from closely linked variants, the components of which might contribute too small an effect to be identified individually. Linkage-based approaches may also have greater success in detecting rare, high impact variants (Cirulli and Goldstein, 2010) The primary difficulty with GWLS is that they require the recruitment of a large number of family samples with multiple affected members in order to be informative, as recruiting family samples is more time-consuming and difficult than recruiting individual cases and controls, well-powered GWLS are challenging to perform.

The latest Affymetrix chip for use in GWA studies (the Genome-Wide Human SNP Array 6.0) tests 1.8 million markers (SNPs and copy number variations, CNVs), offering a far greater density of coverage than previous versions. However, many of the studies performed to date have used earlier versions of the technology such as the Affymetrix 500k. Given that this chip

misses a great deal of the variation now established by the HapMap project, the results are far from comprehensive. Genome-wide testing enables researchers to search for possible disease-associated variants across the entire genome, rather than working in a hypothesis-led manner, and thereby restricting the analysis to a particular gene or region. This ability to scan widely is a major advantage when studying a disease where the causative factors are poorly understood. The limitations to genome-wide studies, however, include cost, level of detail in coverage, and the sheer number of tests involved. Testing 1.8 million markers generates a large possibility of inflating the false positive rate and stringent correction must be applied to compensate for this. The correction in turn necessitates the use of thousands of samples in order that true positives have sufficient statistical power to be detected. For highly polymorphic regions, such as the human leukocyte antigen (HLA) region, genome-wide approaches also lack the level of detail offered by more targeted analyses, even with the greater density of coverage now available.

Several large-scale collaborative GWA studies have recently been performed in the search for schizophrenia susceptibility loci, each employing thousands of samples. The International Schizophrenia Consortium (ISC) found a strong association at chromosome 22, located within the MYO18B gene ($P = 3.4 \times 10^{-7}$), while the best imputed SNP reaching genome-wide significance was on chromosome 6p, within the major histocompatibility complex (MHC) region (Purcell et al., 2009). The MHC finding was backed by the association of 450 SNPs spanning this region within the same study, and was echoed in another GWA study conducted by SGENE-plus (Stefansson et al., 2009), which also found MHC-located associations and confirmed them with their own follow-up study utilising a wider sample group (strongest P-value = 4.4×10^{-9}). The final study, performed by the Molecular Genetics of Schizophrenia (MGS) consortium, tested both European and African-American samples. No associations achieved genome-wide significance, however association was strongest at chromosome 2q in both ethnic groups, though at different loci (Shi et al., 2009). The paper by Shi *et al.* also reported a meta-analysis of the ISC and SGENE data sets (totalling 8,008 cases and 19,077

controls), identifying significant association for a region of LD on chromosome 6p22.1 ($p = 9.54 \times 10^{-9}$), re-affirming the potential of this MHC-containing locus.

Meta-analyses have succeeded in compiling multiple studies to effectively analyse them as a large, combined group, to some degree circumventing the difficulties facing individual studies with limited sample power. A recent example of this, (Ng et al., 2009) analysed 32 GWL studies, and found evidence suggestive of linkage on chromosomes 5q and 2q, in which the regions identified were consistent with those observed in a previous study by the same group (Lewis et al., 2003). When only European samples were considered, chromosome 8p also achieved suggestive genome-wide significance, although tests of differences between the European and Asian studies showed no statistically significant differences. Of the regions identified, both chromosomes 2q and 8p contain genes previously identified to be associated with schizophrenia.

Despite the great number of samples involved in these studies, the results are not all-encompassing. Regions harbouring many well-replicated gene associations, such as COMT, DISC1, AKT1 and DTNBP1, frequently fail to achieve significance, and the lack of association within GWAS does not automatically invalidate their link to schizophrenia. Alternatives to genome-wide methods include the use of association and linkage in traditional case control or familial studies, where smaller sample sizes are offset by a more targeted approach, testing fewer loci. Candidate gene studies are hypothesis-led and ask specific questions, and this specificity greatly reduces the extent of test-correction required. Conversely, the smaller sample sizes used in candidate gene studies reduce the statistical power available to detect associations. A major advantage of candidate gene studies is that they benefit from a greater flexibility in which loci are tested, allowing researchers to analyse a region in more detail. This depth of analysis is vitally important in highly polymorphic regions such as those containing the MCH genes. Candidate gene studies are also more amenable to incorporation of additional risk factors or biomarkers.

Given that the relative risk for individual variants identified by GWAS for schizophrenia are invariably small (<1.2), the ability to combine genetic analysis with other disease data is important.

Of the genes reported as associating with schizophrenia, many have a clear function within the brain, such as NRG1, COMT, RGS4, TAAR6, etc. Genetic predisposition to schizophrenia may, however, not be limited to genes operating within the brain. While genes predominantly associated with the brain are those most commonly associated with schizophrenia (a fact not independent of their being the most studied), they by no means account for all associations. With 1/3 of all genes being expressed to some degree in the brain, it would be flawed to assume that the relevance of any gene stems automatically from its neurological role, or conversely, that a gene associated with other functions cannot have effect there.

Recent GWA studies suggest that the strongest association out with recognized neurological genes is that of the MHC region (Stefansson et al., 2009, Purcell et al., 2009). How variation in this region contributes to schizophrenia has not yet been resolved, primarily due to the highly polymorphic nature of the MHC locus. Variability within the MHC is central to its function; the resulting diversity ensuring our immune system can cope with a myriad of pathogens (Traherne, 2008). This same diversity also makes analysis of the region notoriously difficult; genome-wide approaches particularly lack the level of detail required to home in on the origins of an association signal. Many of the SNP arrays used in GWAS have limited coverage in the MHC region, and this effect is especially pronounced for studies using earlier chip versions, such as Affymetrix 500 (2007).

Despite the challenges posed by its high degree of polymorphism, the MHC association locus offers a fascinating insight into the role of environmental factors in schizophrenia. Given the range of immunological processes that have been implicated in schizophrenia, it seems likely that this region might well harbour variants that mediate development of the illness, and

identifying these factors could be a promising method for understanding and intervening in the course of the disease.

The complex pathological mechanism of schizophrenia is one of the major reasons why identification of risk factors is so difficult. Few of the genes initially identified to be associated with the condition are fully confirmed and those that are tend to show a decrease in LOD (log of odds) score in successive reports (Pulver, 2000). Efforts to refine individual phenotypes are likely to be a key factor in successfully identifying the biological causes (Thaker and Carpenter, 2001)

1.1.4 The immunological hypothesis

The possible role of an autoimmune process in schizophrenia has been much-debated (Knight et al., 2007, Na and Kim, 2007, Potvin et al., 2008, Schwarz et al., 2001) and analysis of this relationship has been performed at molecular, cellular and population levels. Biological studies in this field tend to fall within the remit of one of the following theories: dysfunction of cytokines, autoimmune processes, viral infections or imbalance between T helper 1 (Th1) and T helper 2 (Th2) responses.

1.1.4.1 Cellular Immunity

Disturbances in cellular aspects of the immune system were first noted in 1903, when Bruce and Peebles (1903) reported an increase in leukocytes during the acute phase of schizophrenia. More recent studies designed to test T cell levels in patients with schizophrenia are not unanimous in their findings: both Nyland *et al.* (1980) and Muller *et al.* (1991) reported changes in T cell numbers, but in opposite directions. Interestingly, both noted that cell counts resolved to those of controls upon successful treatment with neuroleptics. This finding is echoed in a number of studies, and there also seems to be evidence that immune features may correlate with specific symptoms or prognoses. Maino *et al.* (2007) reported decreased CD3+ cells (T cells) and

increased CD19+ cells (B cells) during the acute phase of psychosis, with both cell counts leveling out following antipsychotic treatment. Amongst the patient group, patients with paranoid schizophrenia had the lowest CD3+ levels, and non-responders to therapy had the highest CD19+ levels.

There is debate over the reliability of immune cell studies, as counts of these cells are strongly influenced by infection, stress, and general illness. Over the years, differing methodologies have contributed to the confusion, for example, many studies have analysed peripheral blood mononuclear cells (PBMCs) as a single group (Bessler et al., 1995, O'Donnell et al., 1996); while others have taken into account the work showing that there is variability in how the different sub-populations react (Maino et al., 2007, Nyland et al., 1980). It is also worth identifying which data report on cellular changes observed during acute psychotic episodes (Maino et al., 2007), these studies offer insight into the biological processes accompanying that aspect of schizophrenia, but may not be representative of the condition as a whole. Finally, it has not been determined whether these alterations in cell counts truly reflect an underlying disease process, which drives schizophrenia, or whether they are merely reflective of the patient's general health in reaction to the psychotic episode.

1.1.4.2 Cytokines

Another widely studied aspect of immune function is the dysfunction of cytokines - the signaling proteins produced by immune-competent cells. As with cellular studies, there is a great deal of contradiction within the literature, although there are some findings which have been better replicated. The most commonly implicated cytokines are interleukin 2 (IL-2), interferon-gamma (IFN-γ) and interleukin 6 (IL-6). Increased levels of IL-2, a cytokine produced mainly by Th1 (CD4+) cells, have been observed in the serum of patients with schizophrenia (O'Donnell et al., 1996). However, other *in vivo* studies have not consistently replicated the finding (Na and Kim, 2007), and recent meta-analysis concluded that a significant result existed only for the *in*

vitro analyses, in which IL-2 secretion was decreased (Potvin et al., 2008). IFN-γ, a pro-inflammatory cytokine responsible for innate and adaptive immunity against viral and bacterial infections, as well as for tumour control and regulation of autoimmunity, has also been reported to be increased in patients with schizophrenia, with levels only descending to those of controls following successful treatment with neuroleptics (Kim et al., 2004, Na and Kim, 2007). As with the IL-2 findings, studies have reported contradictory results (Wilke et al., 1996), and again, meta-analysis (Potvin et al., 2008) concluded that there was no overall significant trend. The IL-6 cytokine is capable of performing both pro-inflammatory and anti-inflammatory roles, and responds to a range of environmental triggers, including trauma, infection and even exercise. Studies of IL-6 have not been entirely free from contradiction but they have produced the most consistent results (Shintani et al., 1991, Na and Kim, 2007) and, although there have been exceptions, meta-analysis supports a significant increase of plasma IL-6 in schizophrenia (Potvin et al., 2008). A functional role has also been suggested for IL-6, in line with the maternal infection hypothesis: administration of IL-6 to rats during gestation appeared to facilitate altered foetal brain development (Smith et al., 2007).

The patterns of alteration in cytokine levels, particularly the better-studied examples mentioned above, prompted the hypothesis that schizophrenia involves an imbalance between Th1 and Th2 cytokines. The Th1 process refers to the pattern of cytokines affecting cellular immunity, which is responsible for coordinating the defense against viruses and other intracellular pathogens. Th2 refers to humoral immunity and the response to extracellular pathogens via antibody production. The Th1/Th2 theory was based on a supposed shift towards Th2 dominance, with decreased Th1 cytokines such as IL-2 and IFN-γ and increased Th2 cytokines such as IL-4 and IL-6 (Kim et al., 2004, Na and Kim, 2007). While there has been evidence in support of this, including the confirmation of the increase in IL-6 and repeated findings of raised autoantibody levels in schizophrenia, extensive heterogeneity in studies of cytokine levels

have rather discredited the hypothesis. At this point, contradictory results have been obtained for all cytokines studied, and meta-analysis refutes the concept of a Th2 shift (Potvin et al., 2008). One answer to this is that Th2 dominance is relevant only for a sub-group of schizophrenia patients for whom both the pathophysiology of disease and psychotic symptoms are distinctly different (Schwarz et al., 2001). Although there is yet to be reliable definition of such a group, the concept of distinct aetiological pathways is popular, due to the extensive heterogeneity observed in schizophrenia.

Cytokine variation has also been studied in gestating mothers. Brown *et al.* (2004) found a significant increase in IL-8 during the second trimester in mothers whose offspring went on to develop schizophrenia. In animal studies, maternal infection also causes changes in cytokine levels, and results in offspring presenting with behavioural changes reminiscent of those observed in schizophrenia. IL-6 has been highlighted as particularly important; in rodents, maternal injection of IL-6 during pregnancy results in pre-pulse and latent inhibition in the resulting adult offspring, effects which appear to be counteracted by IL-6 gene knock-out or administration of anti-IL-6 antibodies (Smith et al., 2007).

When studying cytokines, there is considerable concern regarding the influence of confounding factors such as medication, obesity and stress, all of which are commonly present in schizophrenia, and all of which are known to impact on immune function. As with alterations in immune cell levels, the variance observed for cytokines has been correlated with acute episodes, and in some cases has been observed to resolve as patients respond to medication (Na and Kim, 2007). It is difficult to determine which changes are merely a side-effect of the disease state and which play a functional role in development of the disease. Certainly, while successful neuroleptic drug treatment can correlate with both symptom resolution and normalized immune cell counts, understanding of the mechanism behind the effect is poorly characterized.. There is evidence of anti-psychotic drugs affecting immune cells, particularly with regard to control of

metabolism and apoptosis (Fehsel et al., 2005, Nyland et al., 1980, Williams et al., 2000, Heiser et al., 2007). Clozapine in particular has been researched regarding this, due to its association with the uncommon but severe side-effect of agranulocytosis, which occurs in approximately 1% of patients prescribed clozapine and requires immediate withdrawal of the medication. The milder side effect of neutropenia occurs in around 3%.

There are some issues too regarding the derivation of the sample, with most being taken in the form of plasma samples rather than cerebrospinal fluid (CSF). Ellison *et al.* (2005) reported that there was no significant correlation in cytokine levels between plasma and CSF samples, and also showed that while cytokine levels in CSF might reflect an inflammatory process occurring within the brain, plasma levels could not be guaranteed to offer the same insights.

1.1.4.3 Infection

The study of gestational immune influence also includes illnesses experienced by the mother during pregnancy. The maternal infection hypothesis of schizophrenia postulates that maternal viral infection during pregnancy increases the risk of offspring developing schizophrenia (Brown et al., 2004, Smith et al., 2007). Originally based on epidemiological studies (McGrath et al., 1994), or longitudinal correlation of maternal test results with offspring illness in later life (Mortensen et al., 2010), animal models have since contributed physiological evidence of the effect of maternal infection on foetal brain development (Baharnoori et al., 2009). Promisingly, biomarkers defined for gestating mothers have now been analyzed in combination with susceptibility genes, with several promising results including implication of the MHC region on chromosome 6 (Brown and Derkits, 2010, Kim et al., 2007)

The viral hypothesis may be related to another theory, commonly referred to as the "winter hypothesis" of schizophrenia, which describes the phenomenon that children born in winter or

spring are at a slight, but statistically significant, increased risk of schizophrenia (Mino and Oshima, 2006). The winter hypothesis has been shown to relate not only to season, but also latitude (Saha et al., 2006a) and remains one of the best-reproduced environmental effects for schizophrenia (Davies et al., 2003). Explanations for the effect are not focused primarily on infection rates in mothers but, consistent with the findings for latitude, are thought to relate to other environmental factors such as decreased maternal vitamin D levels (as a result of reduced sunlight exposure) (Almeras et al., 2007, Bodnar et al., 2007). Other explanations include seasonal intake of folates, and even the effect of body temperature on foetal brain development (Schwartz, 2011).

The concept of viral activity is not limited to maternal infection; antibodies and viral loads have also been examined in patients themselves. The role of common viruses belonging to the Herpes family; Epstein Barr Virus (EBV) and Cytomegalovirus especially, have been studied via serum antibody levels and brain tissue analysis (Rimón et al., 1978, Delisi et al., 1986, Pandurangi et al., 1994, Gordon et al., 1996), following initial theories that latent infections might play a role in psychiatric illness. Despite some early interesting results, a study by Leweke *et al.* (2004) failed to find any difference in circulating levels of EBV antibodies between patients and controls, a negative finding mirrored in most of the recent studies assessing the hypothesis. It should be noted at this point, that the literature contains many conflicting results on this subject, for a variety of reasons including methodological changes, but influenced most heavily by small sample sizes (Rimón et al., 1978, Gordon et al., 1996).

Despite their negative results for EBV, Leweke *et al.* (2004) did, note increased IgG antibodies against cytomegalovirus and *Toxoplasma gondii,* which were significantly influenced by disease status, and decreasing in response to treatment. Cytomegalovirus and Herpes viruses, Herpes simplex 1 in particular, are the agents most consistently associated with schizophrenia (Torrey et al., 2006). The presence of these viruses has been linked specifically to symptoms of cognitive decline (Shirts et al., 2008, Schretlen et al., 2010, Yolken et al., 2011). Studies such as these,

where a mechanism or specific phenotype can be presented, are especially useful, as more general studies may be confounded by disease heterogeneity, and the impact of disease or medication on the immune system's normal functioning.

A possible mechanism by which viral infection may increase the risk of neurological damage is the cross-reactivity between virus-derived antigens and brain-expressed molecules. An early study performed by Heath *et al.* (1989) found that more than 95% of serum IgG fractions from un-medicated patients with schizophrenia were immunologically reactive against tissue homogenates from the septal region of rhesus monkey brain. These positive immunological reactions were detected in only 6% of fractions from patients currently taking neuroleptics and were not detected for any fractions derived from healthy controls. Despite such apparently clear delineation in results, a follow-up study by Knight *et al.* (1990) found positive reactions in all samples assessed, and questioned the reliability of the methodology (immunoelectrophoresis).

1.1.4.4 Epidemiology: Autoimmunity and Schizophrenia

A range of association studies have revealed links between autoimmune disorders and schizophrenia, examining both lifetime risk of disease and familial incidence of disease. One of the most extensive studies, which used the Danish population register, noted a 45% increase in the risk of schizophrenia for individuals who had a previous diagnosis of any autoimmune condition (Eaton et al., 2006). Within the literature, there are both positive and negative associations; some diseases, such as rheumatoid arthritis and type-1 diabetes, correlate with a decreased risk of schizophrenia (Mors et al., 1999), while others such as coeliac disease (CD), acquired haemolytic anaemia, interstitial cystitis, and Sjogren's syndrome (Eaton et al., 2004, Eaton et al., 2006) correlate with an increased risk of schizophrenia.

1.1.5 Coeliac disease and schizophrenia

Amongst the illnesses found to be associated with an increased the risk of schizophrenia, CD carries an increased relative risk of 3.2 ($p<0.0001$) for schizophrenia (Eaton et al., 2004). Although this report is relatively recent, studies describing a relationship between the two conditions stretch back as far as the 1950s (BENDER, 1953). Much work has been done to characterize the link between these illnesses, but although some common traits have been identified, the processes linking them remain elusive.

1.1.5.1 Coeliac Disease

CD is a chronic inflammatory condition, resulting in damage to the gut following ingestion of proteins called prolamins, which are found in plants from the grass family. First described around 1700 years ago, by a physician known as Aretaeus of Cappadocia, CD remains perhaps the best-characterised autoimmune condition, particularly in terms of understanding its genetic factors. Encoded by alleles at the major histocompatibility complex (MHC) locus, the HLA (human leukocyte antigen) serotypes associated with coeliac disease are extremely robust; ~95% of patients carrying the HLA-DQ2 variants and the remaining ~5% carrying the HLA-DQ8 (Sollid et al., 1989, Tighe et al., 1992). The allelic combinations that produce HLA-DQ2 and –DQ8, produce conformations which predispose them to bind prolamin proteins (Henderson et al., 2007). The CD-reactive components of prolamins include gliadin in wheat, hordein in barley, secalin in rye and, to a lesser extent, avenin in oats (Sollid, 2002).Of these grasses, wheat is the plant primarily associated with CD, and the term gluten (which in wheat is comprised of the immunogenic gliadin and also the glutenin fractions) is often used to describe prolamins in general.

When gluten proteins are ingested by an individual who has CD, the body mounts an immune response against them, and also against the enzyme responsible for their modification; tissue transglutaminase (transglutaminase 2, TGM2). TGM2 is central to the process of disease, as it is

modification of gluten (an otherwise highly digestion-resistant protein) by TGM2s that increases the affinity gluten for MHC class II receptors, thus increasing the likelihood of an immune response (Sjostrom et al., 1998). TGM2 is capable of both deamidating and transamidating gluten, with each process favored by specific conditions. Crucially, it is deamidation which converts glutamine residues in gluten to charged glutamic acid, thereby increasing its immunogenicity (Fesus and Piacentini, 2002). The TGM2 enzyme does not stimulate the immune system itself, but through complexing with gluten, is identified by the immune system as an additional target. The autoimmune response against TGM2 causes damage to the small intestine because the inflammatory response is turned against the body's own tissues.

Sufferers present primarily with abdominal symptoms and weight loss, although neurological, and other symptoms are also possible (Kaukinen et al., 2010). Initial investigations are performed using enzyme-linked immunosorbent assays (ELISA), which detect antibodies against specific wheat gluten epitopes, (the GAF-3X fragment of gliadin), against TGM2, and endomysial tissue (EMA). Diagnosis is ideally confirmed by taking a biopsy sample of the small intestine and examining it for villous atrophy, a flattening of the absorptive lining of the gut, which results from inflammatory damage (Excellence, 2009).

1.1.5.2 Relationship between Coeliac Disease and Schizophrenia

The link between gluten intolerance and schizophrenia has been documented since the 1960s (GRAFF and HANDFORD, 1961). One of the authors and main proponents of this theory, Dohan, had noted a low incidence of schizophrenia in non-wheat consuming societies, and the apparent decrease in prevalence of schizophrenia following grain shortages during World War 2 (Dohan, 1966a, Dohan, 1966b). Following such epidemiological data, Dohan's research with inpatients showed that remission of schizophrenic symptoms appeared to be achieved more rapidly if patients were placed on a gluten-free diet in addition to their standard treatment (Dohan and Grasberger, 1973). Since the initial case-control study was carried out (Dohan and

Grasberger, 1973), there have been a number of other such reports (De Santis et al., 1997, Singh and Kay, 1976), including those that incorporated an undisclosed wheat "challenge", and noted its correlation with a period of relapse (Singh and Kay, 1976, Vlissides et al., 1986). Although fascinating, all of these studies suffer from small sample size and, while some incorporated undisclosed gluten challenge, other studies were not so effectively blinded. There have been no recent attempts to replicate this work, partly perhaps on account of ethical considerations, but also because the focus has shifted towards more refined biological investigations of the theory.

While CD is primarily associated with gastrointestinal problems, there is no doubt that gluten intolerance can lead to neurological symptoms. Primarily observed in newly diagnosed patients who are not yet following a gluten-free diet, psychiatric problems such as gluten ataxia, depression, dysthymia, seizures and sometimes psychosis, have all been associated with gluten intolerance (Boscolo et al., 2007, Ciacci et al., 1998, Cicarelli et al., 2003). The mechanism through which these neurological effects occur is poorly understood, but in a case of progressive ataxia with likely (but untreated) CD, autopsy showed evidence of infiltration by cytotoxic CD8(+) T and NK cells, accompanied by gliosis and neuronal loss in the cerebellum (Mittelbronn et al., 2010). Another post mortem report, involving two individuals with diagnosed gluten ataxia, also observed morphological changes in the cerebellum, with evidence of lymphocyte infiltration (Hadjivassiliou et al., 1998). Such evidence is consistent with the theory that an inflammatory process is active within the brain, although the studies represent a relatively small sample group. Possibly, both cellular and humoral immune responses contribute to the pathophysiology of schizophrenia; circulating antibodies, although well established in their effects elsewhere, cannot easily pass through the blood brain barrier, therefore cellular immunity may play an important role in mediating an inflammatory process within the brain.

What is known is that clinical symptoms are generally resolved once patients diagnosed as having coeliac disease are placed on a gluten-free diet. This seems to be a feature that is in

common with the studies of schizophrenia patients treated in this way (Dohan and Grasberger, 1973, Vlissides et al., 1986, De Santis et al., 1997). While there is limited physiological evidence of the process underlying this, in a case report of CD where white matter lesions were observed by MRI, the lesions appeared to improve upon gluten withdrawal (Brown et al., 2009).

While it has been considered that impaired absorption of nutrients may simply leave patients malnourished, thereby leading to poor functioning of the central nervous system (CNS), it seems more plausible that an active disease process is occurring, perhaps akin to that underlying the neurological effects observed in CD. The biological studies mentioned earlier, in the broader context of schizophrenia and immunity, may also be applied to the relationship between gluten intolerance and schizophrenia. Research into this relationship (at the molecular level) has largely centered on the presence of antibodies against gliadin and TGM2 (serological markers for CD), and multiple reports exist that describe an increase in such antibodies in schizophrenia patients, compared to control subjects (Cascella et al., 2009, Dickerson et al., 2010, Jin et al., 2010, Reichelt and Landmark, 1995).

The most recent reports on gliadin antibodies in schizophrenia also represent the most substantial sample groups. The largest study tested 1401 patients with schizophrenia, of whom 23.1% had moderate to high levels of anti-gliadin IgA antibodies, compared with 3.1% of controls (Cascella et al., 2009). A smaller study performed in a Chinese sample group reported similar data for anti-gliadin IgA in patients (27.1%) but with a much higher rate amongst controls (17.8%). Such differences may be influenced by the detection methods used, although modern ELISA kits providing similarly high levels of both sensitivity and specificity for these antigens. The differing ethnicities of sample participants may be a more relevant factor, especially given the variance in diet.

Antibodies against TGM2 have also been reported to be increased in schizophrenia, although the prevalence of the anti-TGM2 antibodies in both cases and controls is much lower overall,

making it more difficult to achieve significance (Cascella et al., 2009, Jin et al., 2010). It should be noted that while antibodies against gliadin and TGM2 are more prevalent in schizophrenia, the third antibody traditionally associated with coeliac disease, anti-endomysial antibody (EMA), does not display such a relationship (Cascella et al., 2009, Peleg et al., 2004).

While anti-gliadin antibodies do appear to be substantially more common in patients with schizophrenia, the specific epitopes against which these antibodies are targeted seem specific to schizophrenia, and do not match the peptide conventionally associated with coeliac disease (Samaroo et al., 2009). The cause of this differing response may well be due to genetic variation in the MHC region, located on chromosome 6. Both candidate gene and GWA studies have repeatedly demonstrated association between the MHC region and schizophrenia (Shi et al., 2009, Stefansson et al., 2009, Purcell et al., 2009, Wright et al., 2001). As described previously, human leukocyte antigen (HLA) alleles, (encoded by the MHC) remain the primary genetic factor defined for CD, determining the propensity of an individual's immune system to bind gluten proteins. To date, there have been several HLA alleles showing weak positive association with schizophrenia (Li et al., 2001), but also reports of no association (Chao et al., 2008), or reduced transmission of alleles studied (Wright et al., 2001). Perhaps the most significant finding comes from Samaroo *et al.*'s (2009) study of gluten peptides in schizophrenia, in which schizophrenia patients showed no association with the CD alleles DQ2 and DQ8, even when divided according to gliadin sensitivity (2009).

As with the cytokine features discussed in section 1.1.4.2, it is uncertain whether these increased antibody levels represent a disease process which effects greater risk for schizophrenia, or merely dysregulation of the immune system due to the stress being experienced by an individual. Increased levels of anti-gliadin antibodies were found by Dohan *et al.* (1972) in psychiatric inpatients with a range of diagnoses, not just schizophrenia, suggesting that such immune features may merely be a side effect of acute psychiatric illness and the stress of hospitalization.

The possibility of cross-reactivity between antibodies and the brain (as described in section 1.1.4.3) is a potential mechanism for anti-gliadin antibodies, as for those directed at pathogens. A study of dietary and cerebellar antibodies in patients with autism showed substantial rates of binding between Purkinje cell-derived anti-cerebellar peptides and gliadin peptides, a result not mirrored for other dietary antigens (Vojdani et al., 2004). Purkinje cells are found in the cerebellum and localize alongside a proposed second dopaminergic region that is smaller, but similar in function to that of the striatum (Delis et al., 2007, Giompres and Delis, 2005). Aside from this link to the function of dopamine, the key neurotransmitter implicated in schizophrenia, Purkinje cells are also of interest because of reports that their size can be notably decreased in patients with schizophrenia (Mittelbronn et al., 2010, Tran et al., 1998). In patients experiencing gluten ataxia, the most common neurological manifestation seen in CD, there is evidence of direct antibody interaction, with both anti-gliadin and anti-TGM2 antibodies being found to cross-react with neural cells (Boscolo et al., 2007). Researchers have attempted to define the site of these antibodies within the brain, however the results lack consistency, alternately finding them co-localised to the cerebellum and brain stem (Hadjivassiliou et al., 2006) or not present in any neural tissues (Wiendl et al., 2003). Such studies may have been confounded by the specificity of antibody testing approaches; a follow-up study by Hadjivassiliou *et al* (2008) reported that neuronal gluten ataxia antibodies were specific to a distinct isozyme of transglutaminase, TGM6.

The transglutaminase family offer interesting candidates for studying the relationship between CD and neurological conditions, particularly the TGM2 gene. As already described, the TGM2 protein is the key enzyme involved in modification of gluten epitopes, and a target for autoantibodies in CD. Deamidation increases the immunogenicity of gliadin, while transamidation by TGM2 can lead to the cross-linking between extracellular proteins or peptides, a key factor not only in the generation of auto-antibodies but also a process thought to

play a role in neurological conditions such as Huntington's disease, where it may facilitate the formation of aggregates in the brain (Karpuj et al., 2002c). While no variations in the TGM2 gene have, as yet, been reported to be associated with either coeliac disease or schizophrenia, up-regulated TGM2 expression has been reported in the anterior cingulate of patients with schizophrenia, although this evidence must be viewed cautiously, coming as it does from a commercial patent rather than peer-reviewed publication (patent WO/2004/053157).

1.2 Hypothesis

The high heritability of schizophrenia makes it a promising disease for study through genetics. However, there is no doubt that environmental factors interact with genetics to bring about the condition, and any model of the illness should therefore involve both factors (Tsuang et al., 2004). The study of environmental interaction is additionally important, as it provides opportunities for intervention and therefore amelioration of risk. Studies show that the immune system, which responds to environmental factors in order to combat infection, may play an important role in the development of schizophrenia, a hypothesis encouraged by the repeated association of the MHC region with schizophrenia (Stefansson et al., 2009, Shi et al., 2009, Wright et al., 1996).

Of the immune disorders co-occurring with schizophrenia, CD is one of the most extensively studied. CD is also particularly well understood in genetic terms, with the primary genetic risk factor (HLA-DQ2 or –DQ8) having already been characterized (Sollid et al., 1989). As such, the relationship between CD and schizophrenia offers a base from which to explore the contribution of immune-relevant genetics to schizophrenia. If common factors can be identified for the two conditions at a genetic level, their relationship would be greatly clarified. Genetic risk factors discovered in this way are likely to both aid understanding of schizophrenic aetiology, and provide novel treatment pathways related to diet and/or immune regulation.

A number of genetic loci have already been identified for CD, and amongst these several make promising candidates for study in schizophrenia (Monsuur et al., 2005, van Heel et al., 2007, Adamovic et al., 2008). The selection of candidate genes enables a detailed analysis of the region under study, a factor especially important for analysis of the highly polymorphic MHC region on chromosome 6 (de Bakker et al., 2006). Also, this focused approach retains maximum statistical power for detecting genetic associations, an essential consideration in a sample group of reasonable, but limited size. The combination of genetic analysis with additional biomarkers (such as antibody or cytokine measurements) enables a further level of analysis; defining sub-groups with distinct phenotypes, and assessing the functionality of genetic variation identified.

1.3 Genes Selected For Research

Following on from the existing literature, a range of genes and loci were identified for further study, in the search for genetic variation which might explain the relationship between schizophrenia and coeliac disease.

1.3.1 The TGM2 gene

The TGM2 gene is located on chromosome 20q11, spanning 36,838 base pairs (bp) of DNA, its product, the TGM2 protein, is a member of the transglutaminase family and is extensively produced throughout the body and capable of performing a wide range of biological functions. Although located mainly in the cytoplasm, TGM2 is also present in the plasma membrane, extracellular matrix, and nucleus (Fesus and Piacentini, 2002). TGM2 is responsible for the post-translational modification of many proteins, and is best known for its ability to catalyse the deamidation and transamidation (cross-linking) of specific glutamine residues. Unusually for a transglutaminase, the transamidating activity of TGM2 is latent, requiring Ca^{2+} influx or a decrease in GTP to become active (Zhang et al., 1998). This requirement reflects the mutually

exclusive relationship between TGM2's transamidating activity, and its role as a GTPase (Monsonego et al., 1998), the latter of which is thought to enable it to function in several signalling pathways (Nakaoka et al., 1994, Iismaa et al., 2000). In addition to its signalling and enzymatic activities, TGM2 also regulates cell matrix adhesion and cell migration, acting via its binding to fibronectin and integrins (Akimov et al., 2000), through which TGM2 promotes wound healing. Other activities have also been proposed for this diverse enzyme, including protein kinase activity (Mishra et al., 2007) and a role as a protein disulphide isomerase (Hasegawa et al., 2003).

Mouse models in which TGM2 has been knocked out are phenotypically normal at birth but show a variety of symptoms later, including defective phagocytic clearing by macrophages (Szondy et al., 2003a), prompting questions of whether the protein might have a role in immune response. TGM2 is expressed on the surface of dendritic cells (Ráki et al., 2007) and its expression is markedly increased during differentiation into macrophages, with theories that it may enable monocyte migration during inflammation (Akimov and Belkin, 2001). Certainly, expression of TGM2 appears to be influenced by some cytokines; IFN-γ is capable of upregulating TGM2 expression, while transforming growth factor β (TGF-β), downregulates its expression (Kim et al., 2002). In addition to playing a role in the phagocytic clearance of apoptotic cells, some studies have also reported the involvement of TGM2 in apoptosis itself. TGM2 is capable of both promoting apoptosis (Fok and Mehta, 2007) and inhibiting it (Cao et al., 2008), with the nature of its effect apparently influenced by its cellular localisation, and the substrates it therefore interacts with (Milakovic et al., 2004).

TGM2 in coeliac disease

Highly expressed in the sub-epithelial cells of the gut, TGM2 exhibits strong affinity for gluten and is responsible for modifying this otherwise degradation-resistant protein, through deamidation of the glutamine residues within it. TGM2-mediated deamidation of gluten further enhances the affinity of gluten for the CD-related forms of MHC class II molecules (DQ2 and

DQ8), promoting the antigen's presentation to CD4+ cells and so driving the immune response observed in CD (Fleckenstein et al., 2004). Transamidation of gluten's glutamine residues by TGM2 can result in the creation of cross-linked gluten-TGM2 complexes. These complexes act as autoantigens, highly recognizable to the HLA-DQ2 and –DQ8 forms of the MHC molecules, and capable of triggering the HLA class II-restricted immune response for antibody production (Fleckenstein et al., 2004). The resulting antibodies produced by the immune system target not only gliadin itself, but also the TGM2 proteins cross-linked with it.

TGM2 in neurological and psychiatric disease

TGM2 is implicated in the pathogenesis of a number of neurological conditions, usually through its cross-linking activities in the brain, which are thought to accelerate an apoptotic process of neuronal cells. In Alzheimer's disease, TGM2 co-localizes with extracellular plaques and neurofibrillary tangles, as well as displaying increased activity and expression (Kim et al., 1999). In addition, an alternatively spliced TGM2 transcript has been found which is specific to the brains of AD patients (Citron et al., 2001). In Huntington's disease (HD), a similar relationship is observed, with increased TGM2 activity in the cortex and cerebellum, where it is thought to assist in the formation of Tau tangles (Karpuj et al., 2002c). Administration of the TGase inhibitor cystamine to HD mouse models slowed aggregate formation and improved survival (Karpuj et al., 2002a).

Despite common features of cognitive impairment, altered neurotransmission and behaviour, the aggregate formation seen in these neurological conditions is not observed in schizophrenia (Singer et al., 2002), and schizophrenia itself is notably episodic rather than progressive. However the assertion that TGM2 acts solely though assisting aggregate formation is contentious, with researchers questioning how functionally important aggregates are in diseases such as HD (Kuemmerle et al., 1999). Equally, while there has been little research into TGM2 in schizophrenia, a patent report records increased expression of TGM2 in the anterior cingulate

of patients with schizophrenia (patent WO/2004/053157). Although evidence specific to schizophrenia is sparse, the chromosomal location of the TGM2 gene is favorable, falling as it does within the region identified by linkage analysis for schizophrenia (Gurling et al., 2001). This region offers further promise, given its deletion in velo-cardio-facial syndrome (VCFS), a complex syndrome noted for its especially high rates of psychosis and schizophrenia (Murphy, 2002). Combined with its potential role in neurological disorders, and pivotal activity in CD, these factors make TGM2 a suitable candidate for further study.

1.3.2 MYO9B gene

The Myosin IXB (MYO9B) gene is located on 19p13.1 and encodes an unconventional myosin molecule with a GTPase activating domain. A Rho family protein with a role in the actin remodelling of epithelial enterocytes (and hence, tight junction assembly) (Post et al., 2002), it is hypothesized that MYO9B affects intestinal permeability, making it an attractive candidate for a functional entity in CD.

The first evidence of MYO9B's involvement in CD came from a study conducted by Monsuur *et al.* (Monsuur et al., 2005), who reported that a common variant located in intron 14 of the gene was associated with CD in two Dutch cohorts. In the combined population the rs2305767 SNP was highly associated with coeliac disease ($p = 1.16 \times 10^{-4}$). Attempts to confirm the result since then have been mixed, with studies finding association only in a sub-group (Wolters et al., 2007), or more commonly, finding no association between MYO9B and CD at all (van Heel et al., 2007, Núñez et al., 2006, Latiano et al., 2007, Giordano et al., 2006, Cirillo et al., 2007, Amundsen et al., Hunt et al., 2006).

Following the original study of MYO9B in CD, Jungerius *et al.* (2008) tested the hypothesis that the same variants within MYO9B were also a risk factor for schizophrenia. This study revealed

an association between schizophrenia and 2 SNPs in the MYO9B locus. Interestingly, this locus is within the 19p13 region earlier identified for schizoaffective disorders (Hamshere et al., 2005). The explanation for this shared genetic association is far from clear, but suggestions include a 3'-end MYO9B variant, leading to impaired interaction with RhoA and, therefore, altered function of tight junctions (Monsuur et al., 2005). An alternative idea suggests that MYO9B (again, acting via RhoA) might influence actin remodeling in the brain, although how this would fit with the original finding for CD remains unclear. As recommended for genetic associations, this study was replicated in our (separate) sample group, in an attempt to confirm the validity of the initial result.

1.3.3 IL-2 gene

As discussed earlier in this Chapter, Interleukin 2 (IL-2) is one of the most widely studied cytokines in schizophrenia. The first interleukin to be defined, IL-2 is part of the signaling process which enables the body to respond to infection. IL-2 is active in the selection of antigen-specific T-cells, and also T-regulatory cells, making it an important factor in maintaining tolerance (non-reactivity towards self-antigens) (Thornton et al., 2004). The IL-2 gene is located on the long arm of chromosome 4 and disruption of the gene in mice has been shown to result in an ulcerative colitis-like phenotype (Sadlack et al., 1993).

Despite reports of altered IL-2 *in vivo* (O'Donnell et al., 1996), meta-analysis does not define any statistically significant pattern (Potvin et al., 2008). Conversely, *in vitro* analysis of IL-2 production upon mitogen stimulation shows a clear pattern, with a decrease confirmed by meta-analysis (Potvin et al., 2008). This finding has led researchers to question whether IL-2 production is influenced by disease state, or whether it might be more fundamentally impaired in schizophrenia.

Levels of IL-2 receptors (Il-2R) and soluble IL2-R (sIL-2R) are altered in CD, with a marked increase observed in the active disease state (Blanco et al., 1992, Crabtree et al., 1989). Intestinal biopsy samples from coeliac patients have also shown that levels of IL-2 itself are increased (Westerholm-Ormio et al., 2002), with a recent study of intraepithelial cells recording the dominance of a subset of CD4+ cells, characterised by increased IL-2 production (Kolkowski et al., 2006). Interestingly, PBMCs from coeliac patients produce no more IL-2 in response to gliadin exposure than cells from control subjects. They are, however, far more likely to display apparently spontaneous IL-2 secretion (40% of patients compared with 0% of controls) (O'Keeffe et al., 1999), which could suggest a background of IL-2 dysregulation in CD.

The relevance of IL-2 to CD has been further suggested by data from several genetic studies. A GWA study by Van Heel *et al.* (2007) identified the region bordering IL-2/21 as the strongest CD-associated locus outside of the HLA. This locus was confirmed both with a separate sample group in the original study, and by two subsequent reports (Adamovic et al., 2008, Romanos et al., 2009), making it a robust association. The 4q27 locus identified includes four genes, TENR, IL-2, IL-21 and KIAA1109, a predicted gene with unknown function. Of these, IL-2 and IL-21 pose the greatest relevance for CD, both in terms of function and expression profiles. Although the strongest signal came from an SNP closest to IL-21 (rs13119723), the four genes identified around it exist within a region of strong LD, within which the locus of the disease-causing variant could not be resolved. The functional variant tagged by this SNP could theoretically be located in any one of these four genes, although the immunologically-relevant functions of the IL-2/21 genes make them more logical candidates. A study of the equivalent region in mice (Idd3) revealed alleles responsible for altered IL-2 expression and immune dysregulation (occurring via reduced function of CD4+CD25+ T regulatory cells) (Yamanouchi et al., 2007), supporting the view that the signal detected in CD represents an immunologically relevant variant.

On the basis of its repeated association with CD and its relevance to the cytokine disruption seen in both CD and schizophrenia, SNPs tagging this locus were selected as additional DNA markers for study.

1.3.4 HLA

Located in the short arm of chromosome 6 (6p21.3), the HLA locus is a superlocus comprised of immune-relevant genes (de Bakker et al., 2006). Many of the genes produce antigen-presenting proteins, which enable the immune system to react to invading pathogens. Due to their original discovery in the context of organ transplantation, where these proteins encoded within the HLA locus identify the organ as "foreign" within the recipient's body, proteins from this region are referred to as surface antigens. Within these surface antigens there are three classes, divided according to function. Class I antigens present fragments of pathogens from within a cell, class II present outside the cell, while class III are made up of components of the complement system. HLA proteins are located on the surface of cells where they perform a dual function; identifying the cell as self, and presenting pathogen-derived antigens, which are non-self, to T cells.

Within each class there are several types of HLA molecules; including A, B, C, E, F and G for class I, and DP, DM, DOA, DOB, DQ and DR for class II. If analysed at a molecular level, HLA molecules have several thousands of possible variations across the populations. This immense diversity enables the immune system to recognize, and respond to, the huge variety of pathogen-derived antigens or epitopes that may be encountered. Different HLA molecules are capable of recognizing different epitopes, and just as they can be specific in responding to pathogens, so can HLA types be linked to the reactions against self-molecules, when a breakdown of immunological tolerance occurs.

The association of HLA DQ2/DQ8 molecules with CD is perhaps the best defined genetic risk factor for an autoimmune condition. These HLA variants are formed by heterodimers of the HLA-DQA1 and –DQB1 variants, and there are four risk haplotypes for CD. The most common risk haplotype in CD is DQ2.5, followed by DQ8, in addition there are also the DQ2.2 and DQ7 haplotypes, although these constitute risk only in combination with one another, or with DQ2.5 (Sollid, 2002). Estimated to contribute around 36% of the genetic cause of coeliac disease, HLA type is considered to be the single greatest determining factor in the disease, with the ability of these HLA types to recognize and bind gliadin fragments being essential for the resulting autoimmune reaction (Henderson et al., 2007, Petronzelli et al., 1997). Despite the many links observed between autoimmune conditions and schizophrenia, and the repeated association of the HLA locus with schizophrenia in GWA studies, no HLA variants have yet been found to alter the risk of schizophrenia. The studies which have been performed in this area have selected only a specific subset of HLA (Chao et al., 2008). Such limitations are a result of the highly polymorphic nature of the region and the sheer number of HLA types (some of which are notoriously difficult to resolve), making analysis of this region difficult and expensive. All this highlights the need to apply an alternative approach to the study of an HLA-associated disease, such as the HLA-based tagging method devised by de Bakker *et al.* (2006).

In order to assess the relationship between schizophrenia and CD, we followed an approach that would enable us to analyse the association between the CD-associated DQ2 haplotypes and schizophrenia.

2 General Methodology

2.1 Subjects

2.1.1 Recruitment

Prior to recruitment, ethical approval was granted by the relevant local committee. In all cases participant wellbeing was considered the primary concern, in accordance with the principles laid out in the Declaration of Helsinki (2008 version). Given the nature of the disease under study, considerable care was taken to safeguard the wellbeing of case group volunteers; ensuring that the study would not in any way compromise their treatment or health, and that they were capable of giving informed consent.

British family trios or duos, consisting of fathers, mothers and affected offspring with schizophrenia, were recruited for genetic analysis. Samples were collected through the charity the Schizophrenia Association of Great Britain (SAGB), Bangor, UK, between 1990 and 2005.

Additional case/control structured samples were recruited in two separate collections. The first group were collected in Bangor. The second case/control group was comprised of patients recruited from New Craigs Hospital in Inverness, Scotland, and healthy control subjects recruited through the Highland Clinical Research Facility, Centre for Health Science, Inverness.

In order to keep ethnicity consistent for the genetic association studies, all volunteers were of Caucasian descent, identifying themselves as English, Welsh, Irish and Scottish. All subjects gave informed consent to giving blood samples either for use in a single study, or for retention and use in future studies as well. Recruitment and sample use in all cases was approved by the local research ethics committee.

2.1.2 Patient details

131 family trios were recruited. 92 of the patients recruited were male and 39 female; the mean age was 29.3±7.1 years. All patients were diagnosed as having schizophrenia by their treating psychiatrists, confirmed through a clinical interview. A positive diagnosis of schizophrenia was made according to either DSM-III-R or DSM-IV criteria. All patients were taking medication at the time of sampling. The antipsychotic drugs prescribed were chlorpromazine, clozapine, flupenthixol, fluphenazine, haloperidol, sulpiride, thioridazine, trifluoperazine and zuclopenthixol.

In the first case/control group, recruited in Bangor, there were 77 cases (49 males and 28 females, aged 35.7±6.1) and 52 healthy controls (26 males and 26 females, aged 39.2±19.5). Details for diagnosis and medication are as for the trios.

In the second case/control group, recruited in Inverness, there were 11 cases (7 males and 4 females, aged 37.8±10.5) and 20 controls (5 males and 15 females, aged 48.6±14.2). All but one of the patients was recruited from the hospital's clozapine-monitoring clinic and, as such, all patients were taking clozapine, at doses ranging from 250 to 600 mg/day. The non-clozapine patient was also medicated, taking risperidone at 3 mg/day. Due to the measures under scrutiny in this part of the study, both patients and controls in this group were asked to fill out a questionnaire confirming that they were negative for the following criteria; diagnosis of celiac disease, following a gluten-free diet, current or prescribed use of anti-inflammatory drugs such as corticosteroids.

2.2 Tissue culture

2.2.1 Culture of U937 cells

The following cell culture work was performed by Dr Aditi Mathur, a team member within the department.

U937 cells are a human cell line with monocytic characteristics, derived from the histiocytic lymphoma of a male patient. U937 cells were selected as a suitable model in which to study gene expression, in particular variation in gene expression following treatment with anti-psychotic drugs (Heiser et al., 2007). In order to assess the impact of anti-psychotic drug treatment of gene expression, cells were treated with clozapine concentrations of 1 and 2 μg/ml (with clozapine dissolved in DMSO). The concentrations were chosen to fall between the blood and brain concentrations determined for long-term clozapine treatment (Weigmann et al., 1999), with doses from the higher end of normal serum levels selected, to compensate for the relatively short duration of treatment.. Control samples were treated with equivalent volumes of DMSO alone.

Materials

Foetal calf serum was purchased from Fisher Scientific (Loughborough, UK), while penicillin and streptomycin were purchased from PAA Laboratories Inc. (Yeovil, Somerset, UK). All other chemicals were purchased from Sigma Aldrich (Dorset, UK). U937 cells were supplied by ECACC (European Collection of Cell Culture). Cell culture flasks, microtitre plates, tubes (Eppendorf® and Falcon) were purchased from Fisher Scientific (Loughborough, UK).

Cell culture protocol

U937 cells were incubated in t-flasks with RPMI 1640, to which 10% foetal calf serum, and 100 units/mL each of penicillin and streptomycin had been added. Cells were incubated for 48

hours at 37 °C, with 5% CO_2. Following incubation, cell counts were performed by staining 80µl of cell-containing medium with 20ul of the vital dye trypan blue (at 50 µL/mL), which identifies dead cells by selectively colouring them. 10µl of this medium/trypan solution was loaded onto the haemocytometer and live cell counts were assessed by microscope.

The remaining cells were centrifuged for 1 minute at 250 *g* and the supernatant discarded. The cell pellet was then used to seed cells at an optimum density of 3x 10^6 cells/10ml, in fresh media (Table 2-1).

Table 2-1 Media used to culture U937 cells

Components	Volume
RPMI 1640 + 2 mM glutamine	500 ml
5% Foetal calf serum	25 ml
0.5% Antibiotics (penicillin-streptomycin)	2.5 ml

To make a working stock solution of 5 mg/ml, 25 mg of powder form clozapine was dissolved in 5ml DMSO. The resulting solution was then filtered through Whatman® GD/X™ sterile syringe filters, with a pore size of 0.2 µm (Whatman, UK). The clozapine concentrations used to treat U937 cells are given Table 2-2.

Table 2-2 Clozapine concentrations used to treat U937 cells

	1 ug /ml treatment	2 ug /ml treatment	DMSO control
Volume clozapine added to 10 ml of medium	2.0 µl	4.0 µl	Equivalent volume DMSO only

For treatment with clozapine (or DMSO control), flasks were incubated at 37ºC and 5% CO_2 for 96 hours. Following incubation, cells were transferred to 10ml tubes and centrifuged for 1 minute at 250 *g*. The majority of the supernatant was discarded and pellets resuspended in the ~1.5 ml supernatant remaining. Cell solutions were transferred to 2 ml Eppendorf® tubes on ice, in preparation for mRNA extraction (as described in section 2.3.3).

2.3 Sample processing

2.3.1 Blood processing

Following participant consent, blood samples were taken by an assigned research nurse (for controls), or a psychiatric nurse, supervised by the overseeing consultant psychiatrist (for patients). Samples were collected in EDTA-coated tubes, to prevent coagulation occurring.

Blood samples collected through SAGB were either processed by Dr Jun Wei upon receipt, or stored unprocessed at -20ºC, then used for DNA extraction, as described in 4.3.2. For a subset of 80 cases and 59 controls the samples were split, with 1.5 ml being reserved for DNA extraction and 5-10 ml being used to obtain plasma. Plasma was separated from the blood sample through centrifugation (10 minutes at 350 *g*, 4ºC), following which it was aspirated, aliquoted into two 2 ml tubes and stored at -80 ºC.

For samples collected in Inverness, all samples were split; one 5 ml collection tube was divided into 3 aliquots, of which 1 ml was used immediately to extract total RNA and the remainder was stored at -20ºC until DNA extraction could be performed (within one month). The second 5 ml tube was used for plasma separation, as described above.

2.3.2 DNA extraction

Genomic DNA was extracted using either QIAamp® DNA Blood Mini kit (QIAGEN, Sussex, UK) or InstaGene™ Whole Blood kit (Bio-Rad Laboratories, Hemel Hempstead, UK) according to the manufacturer's instructions.

For the QIAmp® DNA kit, 20 μl of QIAGEN Proteinase was added to a 1.5 ml tube, followed by the addition of 200 μl of blood sample. Cell lysis was then achieved by the addition of 200 μl of Buffer AL, pulse-vortexing for 15 seconds, and finally incubation at 56ºC for 10 minutes. 200 μl of 96-100% ethanol was then added, the solution pulse-vortexed for 15 seconds, and then transferred to a QIAmp spin column, set within a 2 ml tube. The column was centrifuged at 6,700 *g* for 1 minute, following which the 2 ml collection tube was discarded and replaced. The column was then loaded with 500 μl of Buffer AW2, and centrifuged at 6,700 *g* for 3 minutes, after which the collection tube was discarded. The column was then loaded with 200 μl of Buffer AE, placed in a 1.5 ml tube, incubated for 1 minute at room temperature, and then centrifuged at 6,700 g for 1 minute. The resulting eluent was stored at -20ºC, with the manufacturer's documentation predicting a yield of 3-12 μg of DNA per sample.

Where the InstaGene™ kit was used, 6 μl of whole blood was diluted in 1 ml of nanopure water and left at room temperature for 15 minutes. The sample was then centrifuged at 6,700 *g* for 3 minutes, and the pellet resuspended in 200 μl of InstaGene Matrix solution. This solution was incubated at 56ºC for 30 minutes, then vortexed and incubated at 100ºC for 8 minutes, this "boiling" stage lyses the cells and absorbs their products into the matrix, thereby preventing their interference with PCR reactions. Finally, the sample was vortexed once more and then centrifuged at 6,700 *g* for 3 minutes to pellet the matrix-bound cells. The tube was then be stored at -20ºC and the supernatant within it used for PCR reactions, following the manufacturer's recommendation of adding 20 μl of sample per reaction.

2.3.3 RNA extraction

All tips and tubes for this work were treated overnight with 0.1% diethylpyrocarbonate (DEPC) supplied by Sigma Aldrich (Dorset, UK) to inactivate RNases, followed by autoclaving. RNaseZap® (Applied Biosystems, Warrington, UK) was used to clean all surfaces and equipment.

Extraction of RNA from blood samples

Total RNA was extracted from blood samples using QIAamp® RNA Blood Mini kit, supplied by QIAGEN (Sussex, UK), performed according to the manufacturer's instructions. Blood sample aliquots of 1.5 ml were used in each extraction, with the kit capable of extracting up to 100 μg nucleic acid per tube, within which larger RNAs (>200 nucleotides) were enriched for. In the first step, erythrocytes within the blood sample are selectively lysed, enabling the recovery of leukocytes. The leukocytes themselves were then lysed, and RNases inactivated to prevent the degradation of the target RNA. The RNA within the lysate was then bound to a silica membrane within the QIAmp spin column, through centrifugation. Contaminants were eluted via three wash stages, after which the RNA was eluted in 30 μl RNase-free water. The manufacturers suggest that each 1.5 ml blood sample yields an average of 4.5 μg of total RNA.

Extraction of RNA from cultured cells

Extraction of mRNA from cultured cells was performed using GE Healthcare's Illustra™ Quickprep Micro mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK). According to the manufacturer's instructions, a sample containing 1 x 10^7 cells (determined by haemocytometer) was pelleted by centrifugation at approximately 6700 *g*, then resuspended in 0.4 ml extraction buffer. Cells were lysed and transferred to the Oligo(dT)-Cellulose tube, which was then centrifuged for 1 minute at 6700 *g*, facilitating binding of the mRNA to the cellulose

resin. The resulting mRNA-containing resin was then purified by a series of five high and two low salt washes, rendering it essentially free of contaminating proteins, nucleic acids and carbohydrates Finally, the resin was transferred to manufacturer-provided mini columns, washed with low salt buffer, and eluted in 0.2 ml elution buffer. The manufacturer's guide estimates that a sample of 1 x 10^7 cells should produce approximately 6 μg of mRNA.

Quantification of RNA

All RNA extractions were performed in accordance with the manufacturer's instructions. Following extraction, RNA concentrations were measured using the Nanodrop spectrophotometer, selecting RNA-40 and λ=260 nm. Samples were stored at -20ºC for up to one month when in use, and at -80ºC for longer term storage.

2.3.4 Synthesis of cDNA

As for RNA extraction, all tips and tubes for this work were treated overnight with 0.1% DEPC, followed by autoclaving. RNaseZap was used to clean all surfaces and equipment.

cDNA conversions of RNA samples were performed using the High Capacity cDNA Reverse Transcription Kit, supplied by Applied Biosystems (Warrington, UK), according to the manufacturer's instructions.

Kits were thawed on ice and the Master Mix then made up (on ice) according to Table 2-3. 10 μl of the prepared 2X RT Master Mix was added to the wells of either a 96-well plate, or 8-well strips. 10 μl of sample RNA was then added to each well, bringing the mixture to a 1X concentration. Plates/tubes were centrifuged briefly, ensuring that no bubbles were present in the wells, then loaded into either a GeneAmp 2700 or 2720 thermal cycler (Applied Biosystems, UK). The program described in Table 2-4 was then run and the resulting cDNA was stored at -20ºC when in use, -80ºC for longer term storage.

Table 2-3 Master Mix components for cDNA reverse transcription

Component	Volume/Reaction (μL)
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H2O	3.2
Total per Reaction	10.0

Table 2-4 Reverse transcription thermal cycling program

	Stage 1	Stage 2	Stage 3	Hold
Temperature (°C)	25	37	85	4
Time (minutes)	10	120	5	∞

2.4 Genotyping

2.4.1 Analysis by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) protocol

For selected SNPs, genotype was determined through PCR amplification and subsequent RFLP analysis. Amplifications were performed in 15 μl reactions using DyNamzyme™ II Hot Start DNA polymerase

Primers

Primers were designed using Web Primer (Stanford University) and BEACON (Premier Biosoft) software, with the following criteria: length of 19-30 base pairs, Melting temperature (Tm) range of 50-65°C (optimum 60°C), amplicon size of 300 base pairs, GC content of 30-60% (optimum 45%). Primer sequences generated were checked by BLAST to search for any homologues within the human genome, and any sequences with non-specific matches of >70% were discarded. Primers were manufactured by Sigma Aldrich (Dorest, UK) and upon receipt were centrifuged, rehydrated to 100 μM with autoclaved, nanopure water, then vortexed before aliquoting. Aliquots were stored at -20°C and scaled to ensure that no tube underwent more than three freeze-thaw cycles in the course of use.

Amplification

The Master mix for PCR amplification was prepared based on overall volumes for any mix, calculated by multiplying the per-sample volume by the number of samples plus five percent. All reagents were supplied by Sigma Aldrich (Dorset, UK), with the exception of dNTP mix, which was supplied by Applied Biosystems (Cheshire, UK). The components of Master mix are given in Table 2-5. The standard conditions used for PCR amplification were as detailed in Table 2-6.

Table 2-5 The components of Master mix for PCR

Reagent	Volume (μl)	Final Concentration
Autoclaved water	9.95	-
DyNazyme™ II Hot Start 10x Reaction Buffer[1]	1.5	1X
Geneamp dNTP mix (2.5 mM each dNTP)	1.2	0.2 mM
primers (50 μM each)	0.15	0.50 μM
DyNazyme II polymerase (2 U/μl)	0.2	0.027 U/μl
DNA (2.5-10 ng/ μl)	2.0	5 – 20 ng/15μl
Final Volume	15	

[1] 1x buffer contains 15 mM Tris-HCl, 30 mM KCl, 5 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ and 0.02% bovine serum albumin.

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Table 2-6 The conditions used for PCR amplification

Stage	Temperature	Duration	Cycles
Activation	94°C	10 minutes	x1
Denaturation	94°C	45 seconds	
Annealing	60°C	1 minutes	X40
Extension	72°C	1 minutes	
Final Extension	72°C	10 minutes	x1
Hold Temperature	4°C	until removed	n/a

RFLP digest and product visualisation

Following amplification, the PCR products were completely digested using 8 units of restriction enzyme per sample. All enzymes were supplied by New England Biolabs (Hertfordshire, UK).

Reactions were performed using the buffer provided by the manufacturer, and incubated at the recommended temperature for three hours. Full details for individual enzymes and their corresponding buffers are given in the relevant experimental chapters.

The resulting DNA products were resolved on 3% agarose gels, made with 0.75 g agarose dissolved in 25 ml 1X Tris Borate EDTA (TBE) buffer. Prior to casting, gels were stained by addition of 3 μl 10 mg/ml ethidium bromide. Agarose, EDTA buffer and ethidium bromide were supplied by Sigma Aldrich (Dorset, UK). Gels were cast in 24-well moulds and left to set, before loading with 10 μl sample DNA, to which 1.5 μl of 6X loading dye had been added. 5 μl of PCR marker (300 μg/ml) was then added to the outside lane to enable estimation of fragment size, and the gel run at 80 volts for 20 minutes. Both PCR marker and loading dye were supplied by New England Biolabs (Herfordshire, UK). Gels were photographed using a Bio-Rad UV trans-illuminator and genotype calls made according to the bands present. Sample gel photographs are included in appendices 1 and 2.

2.4.2 Genotyping by real time polymerase chain reaction analysis – Taqman® method

Taqman® SNP Assays were ordered from Applied Biosystems™ (Warrington, UK). Reactions were performed according to the manufacturers guidelines, scaling up the reactions to 15 μl with the following reagents: Taqman® Genotyping 2X Master Mix (containing AmpliTaq Gold® DNA Polymerase, dNTPs without dUTP, Passive Reference Dye (ROX) and optimized mix components), primers (36 μM in a 40X mix) and probes (8 μM in a 40X mix) solution, genomic DNA at 2.5-10 μg/ml, and autoclaved water.

Protocol

SNPs were selected, either based on previously reported associations, or according to their location within the gene of interest and a minor allele frequency (MAF) of >5% in a Caucasian population. Individual kit details are provided in the relevant experimental chapters. PCR reactions were made up as detailed in Table 2-7, with 13 µl of reaction mix aliquoted to each tube before the addition of 2 µl cDNA. Plates were centrifuged to ensure that no air bubbles would disrupt the read stage of the PCR. The reactions were run on a Techne Quantica® Real Time Thermal Cycler supplied by Bibby Scientific Ltd. (Satffordshire, UK), conditions used for TaqMan-based PCR amplification are detailed in Table 2-8.

Table 2-7 The components for Taqman PCR reactions

Reagent	Volume (µl)	Final Concentration
Taqman® 2X Master Mix	7.5	1X
Water	5.125	-
Primers 36 µM (40X) Probes 8 µM (40X)	0.375	0.9 µM (1X) 0.2 µM (1X)
Final volume	13 µl	-
DNA (2.5-10ng/ µl)	2	5-20 ng/15 µl
Final volume reaction	15 µl	-

Table 2-8 PCR conditions used for TaqMan genotyping assay

Stage	Temperature	Duration	Cycles
Activation	95°C	10 minutes	X1
Denaturation	95°C	15 seconds	X40
Annealing, Extension and Read	60°C	1 minutes	
Hold Temperature	4°C	until removed	n/a

Filters included on the thermal cycler were VIC, FAM multiplex, FAM singleton and ROX. The VIC filter was assigned to allele 1 and the FAM multiplex to allele 2. Following preliminary analyses and the observation that the use of passive reference dye was not advantageous, no filter was assigned to act as passive reference dye. These settings were consistent for all analyses. Data from Taqman® reactions were analysed with Techne's own Quansoft software, using their Arithmetic 1 method for baseline determination (average of first 3 to 10 cycles fluorescence subtracted from all readings) followed by endpoint fluorescence for genotype call.

2.5 Gene expression analysis

This approach used a two-step PCR, first performing the synthesis of cDNA (as described in section 2.3.4) followed by real-time PCR amplification using SYBR Green technology (described in section 2.5.2). SYBR Green binds specifically to double-stranded DNA, thereby reporting the accumulation of PCR product as the reaction progresses. The point at which fluorescence rises above background is defined as the threshold, with the cycle number at threshold (Ct) reflecting the starting concentration of cDNA; lower Cts corresponding to higher initial cDNA concentrations.

2.5.1 Housekeeping genes

While equal volumes of mRNA samples were taken from each sample source and reaction conditions were kept consistent, gene expression analysis involve multiple stages at which variation may be introduced. To compensate for any differences in concentration of the mRNA samples, or for any variation in individual qRT-PCR reactions, housekeeping genes (HKGs, also called endogenous reference genes) were employed to normalise the results of all qPCR reactions.

While experiments such as these can be performed using only a single HKG, such an approach is extremely vulnerable to error; should the HKG itself be influenced by the test conditions, the analysis of the target gene is fundamentally flawed. HKGs are often somewhat arbitrarily chosen, based simply on their prior use as such. For the same reason they are frequently used without their stability of expression being assessed under the test conditions for which they are providing a baseline. One solution to the uncertainty of their stability is to perform experiments once with one HKG, then repeat the tests with another HKG, assuming that it would be unlikely for both HKGs to be similarly influenced by the conditions under investigation. Although an improvement on the use of a single HKG, this approach still relies on the assumption that most HKGs are inherently stable, and may introduce further room for confusion, by combining the results from two sets of experiments. The method selected for this work was more extensive; requiring selection of multiple, optimal HKGs for each experiment, and effective combination of these for analysis, with primer efficiency taken into account (Vandesompele et al., 2002, Pfaffl et al., 2002).

Primers for a panel of 6 HKGs were obtained from PrimerDesign Ltd (UK). The lyophilised primers were spun down, resuspended in 200µl autoclaved water and stored in aliquots at -20°C,

measured so that no aliquot undergoing more than three freeze/thaw cycles in the course of use. The genes included in the kit were:

- Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA.
- Homo sapiens ubiquitin C (UBC), mRNA.
- Homo sapiens beta-2-microglobulin (B2M), mRNA.
- Homo sapiens cytochrome c-1 (CYC1), mRNA.
- Homo sapiens topoisomerase (DNA) I (TOP1), mRNA.
- Homo sapiens ATP synthase, (ATP5B), mRNA

In order to identify the most suitable HKGs, the expression stability of each gene between and within sample groups was assessed. For each gene, five samples were amplified (in triplicate) for both treated and control groups, plus a duplicate negative control well containing no cDNA. The resulting data were analysed using two programs designed for this purpose; GeNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004). Both programs rank the HKG genes in order of their stability, and offer recommendations on the best combination for effective normalisation of genes of interest (GOIs), GeNorm also plots the stability values attributed to using different numbers of HKGs. While GeNorm is more widely cited in the literature and comes recommended by the supplier of the HKG panel, its pairwise approach to analysis can run the risk of mis-identifying genes as stable on the basis of their being co-regulated. To compensate for this, the results from Normfinder (which uses an alternative model-based approach) were also considered, and HKGs selected based on a combination of the results from each program (in many cases, the two are in close agreement).

All reactions were performed using the SYBR green method described for qRT-PCR in section 2.5.2.

2.5.2 qRT-PCR protocol

Following extraction of mRNA from blood or cell cultures (section 2.3.3) and conversion of the mRNA to cDNA (section 2.3.4), qRT-PCR was performed to quantify the relative levels of mRNA present in samples. Reactions were performed on a Rotor Gene 6000 Real Time Analyzer, supplied by Corbett Research (Cambridge, UK). Unlike most thermal cyclers, the Rotor Gene benefits from having no heating block, instead samples are arranged in a circle and spun while surrounded by heated air, removing the risk of temperature gradients skewing the results for individual wells. All reagents used were supplied by QIAGEN (Sussex, UK) and optimised for use with the Rotor Gene, with the exception of primers, which were supplied by Primerdesign Ltd. (Southampton, UK).

Protocol

cDNA samples (reverse transcribed from RNA as described in section 3.3.4) were defrosted at 4°C or on ice, and diluted 1/20 in water. Samples were vortexed briefly to ensure even mixing and placed back on ice. Master mix was prepared as detailed in Table 2-9, then aliquoted 8 µl to each reaction tube before the addition of 2 µl cDNA.

Table 2-9 Components of SYBR Green qPCR reactions

Reagent	Volume	Final Concentration
Rotor-Gene SYBR Green PCR Master Mix	5 µl	1X
Primers (conc)	0.7 µl	(not given by manufacturer)
Final volume mix	8 µl	
cDNA (diluted 1/20)	2 µl	Unknown
Final volume reaction	10 µl	

Complete tubes were loaded into the Rotor-Gene and run on the program described in Table 2-10. At the end of each run a melt curve was included so that products could be checked for specificity and the absence of primer dimers.

Table 2-10 The conditions for SYBR Green qRT-PCR amplification

Stage	Temperature	Duration	Cycles
Activation	95°C	10 minutes	x1
Denaturation	95°C	10 seconds	x40
Annealing, Extension and Read	6 °C	20 seconds	
Melt curve	45 –> 90°C (in 0.5°C increments)		x1

2.5.3 Primer efficiency calculation

While many calculations assume that all primers and all cDNA concentrations result in roughly equal (and maximal) PCR efficiencies, in reality this is not the case (Pfaffl et al., 2002). In order to account for any differences in primer efficiency, a standard curve was performed for each primer pair, over a range of cDNA dilutions. These standard curves also served to provide an appropriately calculated threshold which the Rotor Gene software could then import into each analysis.

Protocol

cDNA from six treated and six untreated samples were mixed to provide an homogenous stock cDNA. This stock was then diluted as detailed in Table 2-11. Master mix was prepared for each primer set (target genes and HKGs) and reactions were performed in triplicate for each dilution,

following the procedure described in section 3.5.2. Melt curves were checked to ensure the integrity of the reactions at each dilution, and results were analysed by plotting the Ct values against the dilution. The slope of the line was then used to calculate efficiency using the equation:

$$\text{Amp} = 10^{-1/\text{slope}}$$

Table 2-11 cDNA dilutions for standard curve titrations

Dilution number	Dilution in water
1	1:10
2	1:50
3	1:250
4	1:1250
5	1:6250

2.5.4 qRT-PCR data analysis

There are a variety of methods available for the analysis of qRT-PCR data, and a number of software applications capable of performing them. For this work the REST (Pfaffl et al., 2002) program was selected, based on its structured format (which reduces the risk of error) its ability to take individual PCR efficiencies into account and its capacity for normalising using multiple HKGs. The other major advantage of REST 2009 is that it not only provides estimates of the change in gene expression, but through randomization and bootstrapping techniques can also test the statistical significance of the results, even when results include outliers.

The data generated by each Rotor-Gene run were analysed by leaving the baseline at the automated setting and using the previously performed standard curves (section 2.5.4) to set the threshold for each gene independently. The average Ct values for each sample were copied into REST 2009 and the efficiencies set for each gene. The program was then used to analyse the data with 2000 randomizations.

2.6 Determination of IL2 levels

Serum levels of IL-2 were determined by Xu *et al.* in their paper (1994). Levels were determined for 80 cases and 59 controls, using radioimmunoassay, and expressed as means±SD (fmol/ml).

2.7 Enzyme linked immunosorbant assay (ELISA) analysis of antibodies

Levels of IgA and IgG serum antibodies against tissue transglutaminase, native-form gliadin and GAF-3X (the gliadin-analogue fusion peptide specific to CD) were determined for 11 patients and 20 controls, for which fresh plasma samples were available. The decision was made to test both native gliadin and the GAF-3X peptide based on the premise that gluten-reactivity in schizophrenia, might be triggered by a different antigen to that responsible for CD (Samaroo et al., 2009). IgA and IgG anti-tissue transglutaminase ELISA kits and IgA and IgG anti-GAF-3X ELISA kits were supplied by Euroimmun UK Ltd (Gwent, UK). IgA+IgG anti-gliadin (native form) ELISA kits were supplied by Genesis Diagnostics (Cambridge, UK).

Protocol

Blood samples were processed as described in section 2.3.1 and the plasma stored at -20ºC. Once recruitment was completed, plasma samples were defrosted at 4ºC and diluted according to the ELISA manufacturer's instructions. All kits and reagents were brought to room temperature before use. Each sample was tested in duplicate, with positive and negative controls on each plate. All tests were performed quantitatively with 5 or 6 calibrator samples being used to define a standard curve, excepting the test for IgG anti-tissue transglutaminase antibodies, the kit for which contained only one calibrator.

All reactions were performed according to the manufacturer's instructions, the stages of ELISA are visualised in Figure 2-1. Briefly, following serum dilution samples were added to wells which had been pre-coated with target antigen (gliadin, GAF-3X or tissue transglutaminase). After incubation, wash steps were performed to remove unbound serum components. Secondary antibody; Rabbit anti-human IgA or IgG, conjugated to horseradish peroxidase was then added for a second incubation. Wash steps removed unbound secondary and conjugate, before addition of 3,3',5,5'-tetramethylbenzidine (TMB), a substrate for the peroxidase enzyme. Peroxidase catalysis of TMB then produced a colour change, which was terminated after 10 minutes by addition of Stop Solution. The optical density in each well was read at 450 nm on a Varioskan Flash spectral scanning multimode reader (Fisher Scientific, Loughborough, UK). Measurement of optical density enabled determination of the level of target antibody present in the original sample.

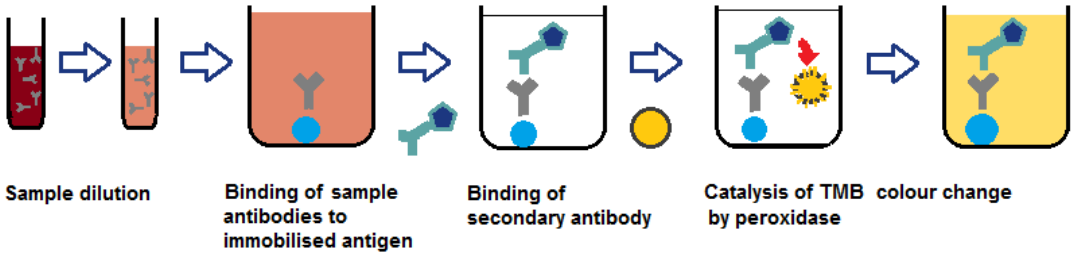


Figure 2-1 Stages of ELISA

2.8 Data analysis methods

2.8.1 General statistic methods

A number of standard statistical approaches were used in the course of this research; including Chi-squared, ANOVA, t-test, Kruskal-Wallis and Mann-Whitney. Brief definitions of each of these are given below, while specific justifications for their use are described in the relevant chapters.

Pearson's Chi-squared test

This test can be used to define goodness-or-fit for data distribution, or to determine the independence of two variables. The calculation for the Chi-squared statistic is shown below, where O and E refer to observed and expected, respectively.

$$\mathrm{X}^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i},$$

The Chi-squared statistic generated can then be used to determine a p-value for the significance of the result.

Student's t-test

This test compares the means of two, normally distributed groups. The test can be adapted to allow for repeated measures of a single factor (such as before and after measurements), or to analyse two groups of unequal sample size.

ANOVA

Analysis of Variance (ANOVA) assesses the equality of means across multiple groups, rather than being limited to a maximum of two, as with a t-test. A parametric test, ANOVA relies on normal distribution in all groups, equality of variances, and the independence of all groups tested.

Levene's equality of variances

Levene's test is used to establish equality of variances between sample groups. Where variance is equal, parametric tests such as t-test and ANOVA can be employed, where variance is not equal, non-parametric tests must be used instead.

Mann-Whitney U test

A non-parametric test, Mann-Whitney can be used where a Student's t-test might otherwise be, but where the test needs to be robust to outliers, or involves ordinal data.

Kruskal-Wallis

An extension of the Mann-Whitney (in that it can be used to test multiple groups), Kruskal-Wallis is essentially a one-way ANOVA, but one which allows for non-normal distribution by using ranks rather than the original data.

2.8.2 UNPHASED

UNPHASED is an application designed for the analysis of genetic association (Dudbridge, 2008). UNPHASED was used to assess the relationships between genetic variation and disease, as well as between genetic variation and quantitative traits. For the genetic association studies performed, two versions were employed; version 2.4 was used to perform the transmission disequilibrium test (TDT) in family trios, while version 3.1 was used to perform likelihood-based association analysis for nuclear families and unrelated subjects with missing parental genotype data to test an overall association in a combined sample consisting of family trios, duos and unrelated case and control subjects

When analysing family samples, the alleles transmitted by parents were treated as cases and those untransmitted as controls. In the TDT, only heterogeneous parental genotypes were available for analysis. When the TDT failed to show disease association for a target gene, additional samples (family duos and unrelated case and control samples) were then genotyped. More recent versions of the program (such as version 3.1) are capable of factoring in combined samples to maximise the samples available and hence, the sample power. The UNPHASED program (version 3.1) uses the expectation-maximization (EM) algorithm to estimate the untransmitted allele frequency of missing parents in family duos.. When performing haplotype analyses, only those haplotypes with relative frequencies greater than 1% were considered. To circumvent the problem of multipoint testing, UNPHASED can apply permutation testing to the global null hypotheses, with the assumption that all the odds ratios were equal. Permutation testing gives a significance level corrected for the number of markers and haplotypes used within a given test. In all cases 10,000 permutations were run for this correction.

2.8.3 Haploview

Version 4.0 of Haploview (Barrett et al., 2005) analyses allele frequencies in order to generate a map of linkage disequilibrium (LD) across a specified region of the genome, which can be done using either individual study data or the HapMap data available online (http://www.broadinstitute.org/mpg/haploview). The pair-wise LD between SNPs was calculated using the Haploview program, according to the confidence intervals method (Gabriel et al., 2002) and reported as both D' and r^2 values. Values range from 0 (no disequilibrium) to 1 (complete disequilibrium), with a value smaller than 1 indicating that historical recombination has occurred between DNA markers in question.

Genotyping data were also analysed using the Haploview program in order to confirm that the results of all trios conformed to Mendelian inheritance. Lastly, Haploview was used to test whether SNPs were in Hardy-Weinberg equilibrium, using the chi-square (goodness of fit test) for the genotypic distributions of SNPs.

3 Association of the TGM2 gene with schizophrenia

The work presented in this chapter includes data published by the author in 2009 (Bradford et al., 2009).

3.1 Introduction

As described in section 1.2.1, the TGM2 gene (MIM: 190196) represents a potential source of genetic overlap between coeliac disease (CD) and schizophrenia. Located on chromosome 20q11, the TGM2 gene codes for the tissue transglutaminase 2 protein which is expressed throughout the body, and which plays a central role in the process of gluten reactivity. TGM2 is capable of deamidating and transamidating a variety of proteins, most notably targeting the glutamine residues within gluten. The deamidation of gluten facilitates its identification by the immune systems, and enhances its presentation to CD4+ cells. The transamidation (cross-linking) of gluten with TGM2 itself, results in the creation of auto-antigens, resulting in an immune response against the body's own tissues, as well as the gluten protein itself.

In addition to its role in CD, TGM2 is also implicated in the pathogenesis of a number of neurological conditions, including Alzheimer's disease, Parkinson's disease and Huntington's disease. While the exact mechanism for this activity has yet to be confirmed, it is clear that TGM2 is both present and active within the brain. The TGM2 gene also falls within a highly promising region of the genome; one identified by linkage analysis for schizophrenia (Gurling et al., 2001).

As such, TGM2 was selected as the first candidate gene to be assessed through an association study. Single nucleotide polymorphisms (SNPs) across the gene were selected and genotyped in the family trio samples available, in order that the transmission of alleles between unaffected

parents and offspring with schizophrenia could be analysed with the transmission disequilibrium test (TDT). Following the association test, relevant regions of the TGM2 gene were probed through the application of sequence analysis software, to determine whether associated regions contained any functional variants or elements which might impact on the expression or activity of TGM2.

3.2 Methods

3.2.1 Association genotyping

3.2.1.1 Subjects

Of the samples available (detailed in section 2.1), only complete trios were used in the association study; 131 British family trios, consisting of fathers, mothers and affected offspring with schizophrenia. The families included 131 patients (92 males and 39 females), aged 29.3±7.1 years.

For the functional variant analysis, 148 patient samples were used; 122 from the trios group, plus an additional 26 from an extended family group. This patient group included 104 males and 44 females, aged 30.17±6.46.

3.2.1.2 Selection of SNPs

Eight SNPs present in the gene were selected based on the following criteria:

1) Distribution across the TGM2 gene

2) MAF of greater than 0.1 in a Caucasian population

3) Presence of an RFLP site

The location of these 8 SNPs within the gene is given in Figure 3-1.

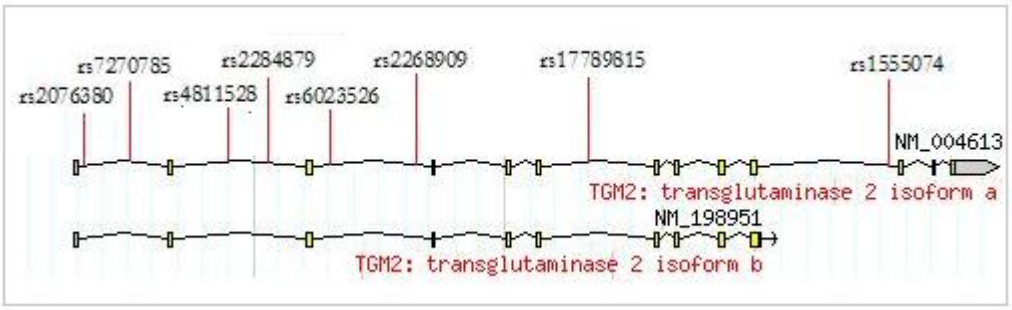


Figure 3-1 Location of SNPs within the TGM2 gene

3.2.1.3 Genotyping of SNPs

The genomic DNA used for PCR amplification was extracted from whole blood samples as described in 2.2.2, and stored at -20ºC. Genotyping was performed as described in section 2.3.1 using PCR-based RFLP analysis. An exception to the method was made for rs2076380, for which more stringent conditions were required. It appeared that there was a greater degree of homology in these primer sequences, and as such, multiple products were initially observed. Altering the conditions to start with a higher initial annealing temperature removed the non-specific binding, while latter cycles performed with lower annealing temperatures ensured sufficient amplification of the desired fragment. PCR cycle conditions for rs2076380 are given in Table 3-1. The primers used for PCR amplification, details of the restriction enzymes employed for each SNP and the corresponding buffers are listed in Tables 3-2, 3-3 and 3-4, respectively. Genotyping for four of the SNPs was performed by another researcher, Mr Andrew Stewart.

Genotyping was repeated for 10% of samples, randomly selected, to ensure the accuracy of the genotype calls. Sample gel photographs for genotype calling can be seen in appendix 1.

Table 3-1 PCR conditions for genotyping rs2076380

Stage	Temperature	Duration	Cycles
Activation	94°C	5 minutes	x1
Denaturation	94°C	45 seconds	x40
Annealing[1]	65°C	1 minutes	
	64°C		
	63°C		
	62°C		
	61°C		
	60°C		
Extension	72°C	1 minutes	
Final Extension	72°C	10 minutes	x1

[1] Each temperature stage was repeated for 5 cycles, with the exception of the final 60ºC stage, which was repeated for the final 15 cycles

Table 3-2 Primer sequences for genotyping TGM2 SNPs

SNP ID	Primer (Fwd)	Primer (Rvs)
rs2076380	CTGATCCAGTGACGCAAGGGT	CTATAAGTTAGCGCCGCTCTC
rs7270785	GGACAGAAGCTTGATACGTGAAAGT	TCTCCCACTGCAAGGACCAACT
rs4811528	CCTGGAAGTTCTTGGTTGCTTG	AATTGTCTGGGCAGCGTAGTGT
rs2284879	TTTGAATGCTCCCTGACTTCCAG	GCCTGTCCTCTTTAACCTTCAC
rs6023526	AACACGTTGCAGTGAGCTGAGATT	AGCAGTATGATATGCCCAATTCC
rs2268909	TTTGGAAACGAAAGCCCTGA	TAAATGCCCTGCATTTTCCAGC
rs17789815	CCAAGTTTTCCCTGGCTCC	CCGTGACCTCTGAATTCCTTTT
rs1555074	CTGGAGAGTGGACACTGAGC	GATGTCGGCTCTGATCCTCC

Table 3-3 Restriction endonucleases and digestive conditions for genotyping TGM2 SNPs

SNP	major allele	minor allele	R.E.	allele Cut	digestion conditions
rs2076380	G	A	*Msp*I	G	3 hours - 37°C
rs7270785	T	G	*Fok*I	G	3 hours - 37°C
rs4811528	A	G	*Msp*I	G	3 hours - 37°C
rs2284879	A	G	*Hinf*I	A	3 hours - 37°C
rs6023526	T	C	*Msp*I	C	3 hours - 37°C
rs2268909	G	A	*Sac*I	A	3 hours - 37°C
rs17789815	A	G	*Fok*I	G	3 hours - 37°C
rs1555074	G	A	*Taq*I	G	3 hours - 50°C

Table 3-4 Restriction enzymes and corresponding buffers

RE	Buffer	Buffer components
SacI	NEBuffer 1	10 mM Bis-Tris-Propane-HCl 10 mM $MgCl_2$ 1 mM Dithiothreitol
MspI FokI HinfI TaqI	NEBuffer 4	50 mM potassium acetate 20 mM Tris-acetate 10 mM Magnesium Acetate 1 mM Dithiothreitol

3.2.2 Association data analysis

Tests for deviations from Hardy-Weinberg Equilibrium for each SNPs were performed using Haploview version 4.0 (Barrett et al., 2005), as described in Section 2.9.2. The UNPHASED program, version 2.4 (Dudbridge, 2008) was used to analyse allelic and haplotypic associations with schizophrenia, as described in Section 2.9.1. Permutation testing (10,000 permutations) was performed to correct for a false positive rate due to multiple testing for allelic association and 2-SNP haplotypic association and the Bonferroni correction was applied to correct individual p-values of the one degree of freedom (1-df) test in haplotype analysis

The pair-wise LD between SNPs was calculated using the Haploview program and was reported as the measures of both D' and r^2 values.

3.2.3 Analysis of potential functional variants

3.2.3.1 Coding SNPs

Following the association study, the region surrounding disease-associated SNPs was screened for possible functional variants which might be in LD with the intronic SNPs. Examination of the schizophrenia-associated region revealed only one validated coding SNP, rs1062735. Located in exon 2 (Figure 3-2), rs1062735 conventionally codes for a glutamic acid residue at position 51, however non-synonymous substitution with a glutamine residue has been reported (HapMap data, heterozygosity unknown). Analysis of this SNP was performed for a pilot group of 148 patients, using standard protocols for PCR-based RFLP genotyping analysis, and the primers used are listed below. For RFLP analysis the BstNI restriction endonuclease was used with its manufacturer recommended incubation of 3 hours at 50ºC.

Primers for rs1062735

Forward 5'-CTCATGCGTCTCCTTCTGT –3'

Reverse 5'-GGTTCTGGGGCATGTCGG-3'

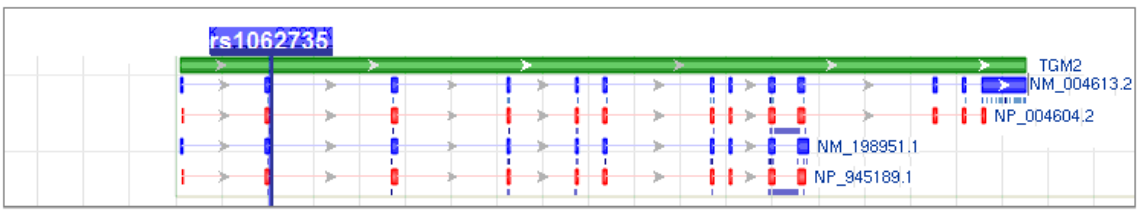


Figure 3-2 Location of coding SNP rs1062735 within the TGM2 gene

3.2.3.2 Non-coding sequence conservation

While nearby coding SNPs provide an obvious source of functional variation, genetic associations are also likely to stem from variation within the intronic regions themselves, where there can be extensive regulatory elements (Cobb et al., 2008). To assess the likelihood that disease-associated regions contain such elements, the UCSC genome browser (version number GRCh37; (Kent et al., 2002) was employed to search for signs of high conservation of this region among vertebrates. If important regulatory sequences are located within a region, a high degree of conservation would be expected. Conserved regions, once identified, were then correlated with the LD blocks indicated by the HapMap database, in order to assess the probability of their being in LD with disease-associated SNPs and so relevant to any genetic association observed.

3.2.3.3 Functional elements

In order to screen for possible functional elements across the wider region, the UCSC genome browser was also used to search the 5'-end 20kb regions of the TGM2 gene plus an additional 20kb upstream, for predicted transcription factor binding sites (TFBs) conserved across human, rat and mouse.

At the associated SNPs themselves, sequences were analysed software designed to identify the following regulatory elements;

- The Alibaba2.1 (www.gene-regulation.com, accessed 2009)program was used to screen for TFBs, with parameters set to 80% minimum conservation.
- The ESEFinder program (Cartegni et al., 2003) was used to search for exonic splice enhancers.

SNPs were also assessed manually for the presence of intronic splice sites, using the sequences given in Table 3-5.

Table 3-5 Splice site recognition sequences

Splice site type	Recognition sequence
5' splice site	**AGguragu**
3' splice site	**yyyyyyy-nagGU**
branch point site	**ynyuray**

3.3 Results

3.3.1 Association analysis

Analysis by UNPHASED (version 2.4) using the TDT revealed allelic association of four of the eight SNPs with schizophrenia; rs2076380, rs7270785, rs4811528 and rs6023526 (Table 3-6). The global p-value for allelic association was 0.029 following 10,000 permutations.

Four 2-SNP haplotype systems (each involving at least one of the associated SNPs) were also associated with schizophrenia; rs2076380–rs7270785, rs7270785–rs4811528, rs2284879–rs6023527, and rs6023526–rs2268909 (Table 3-7). The UNPHASED test showed a global p-value of 0.008 following 10,000 permutations, while individual tests showed greatest significance for the T-A haplotype of rs7270785-rs4811528 ($p = 0.001$).

The 8-SNP haplotype system consists of 19 individual haplotypes with a frequency of 1% or over. UNPHASED was used to test haplotypic association and the global test of significance indicated there were significant differences in haplotype frequencies between groups (χ^2=44.98, df=18, p=0.0004). When the 1-df χ^2 tests were performed to obtain individual p-values for

each of the haplotypes, the A-T-A-A-T-G-A-G haplotype showed excessively transmitted (χ^2=16.98, corrected p=0.0007), with UNPHASED ascribing a relative risk of 2.13, no other haplotypes showed significance (Table 3-8).

Table 3-6 TDT analysis for allelic association of TGM2 SNPs with schizophrenia

SNPs	Distance (bp)	Non-transmitted Alleles		Transmitted Alleles		χ2	p-value
		Major	Minor	Major	Minor		
rs2076380	0	G=60	A=37	G=37	A=60	5.51	0.019
rs7270785	1911	T=59	G=32	T=32	G=59	8.13	0.004
rs4811528	4191	G=47	A=26	G=26	A=47	6.13	0.013
rs2284879	1496	A=24	G=24	A=24	G=24	0.00	1
rs6023527	2353	T=26	C=47	T=47	C=26	6.13	0.013
rs2268909	3470	G=16	A=28	G=28	A=16	3.32	0.069
rs17789815	6759	A=22	G=25	A=25	G=22	0.19	0.662
rs1555074	12154	G=25	A=32	G=32	A=25	0.08	0.353
Global p-value following 10,000 permutations							**0.029**

Table 3-7 Analysis of haplotypic association of TGM2 SNPs with schizophrenia

SNPs	Haplotype	Transmitted	Not Transmitted	Global χ^2 test (df=2 or 3)		1-df χ^2 test	
				X^2	p-value	χ^2	p-value
rs2076380 - rs7270785	G-G	29	57	8.16	0.043	7.18	0.029
	G-T	2	3			0.2	NS
	A-G	8	10			0.2	NS
	A-T	63	32			8.02	0.018
rs7270785 - rs4811528	G-G	23	35	16.18	0.001	2.5	NS
	G-A	19	36			5.34	NS
	T-G	1	7			5.06	NS
	T-A	64	29			13.5	0.001
rs4811528 – rs2284879	G-A	20	30	5.63	0.131	2.01	NS
	G-G	12	19			1.59	NS
	A-A	47	34			2.1	NS
	A-G	8	4			1.36	NS
rs2284879 – rs6023526	A-C	14	32	7.92	0.048	7.24	0.029
	A-T	46	26			5.63	NS
	G-C	19	21			0.1	NS
	G-T	1	1			0	NS

SNPs	Haplotype	Transmitted	Not Transmitted	Global χ^2 test (df=2 or 3)		1-df χ^2 test	
				X^2	p-value	χ^2	p-value
rs6023526 – rs2268909	C-A	5	18	9.23	0.026	7.8	0.021
	C-G	28	32			0.27	NS
	T-A	7	8			0.07	NS
	T-G	48	30			4.19	NS
rs2268909 – rs17789815	A-A	16	24	1.92	0.383	1.61	NS
	G-A	45	34			1.54	NS
	G-G	22	25			0.19	NS
rs17789815 – rs1555074	A-G	31	24	1.24	0.743	0.89	NS
	A-A	7	8			0.07	NS
	G-G	2	4			0.48	NS
	G-A	17	21			0.42	NS

Global p-value = 0.008 after 10,000 permutations.

Table 3-8 Testing for association of 8-SNP haplotypes with schizophrenia

Haplotype	Transmitted	Non-transmitted	χ^2	p-value[1]
G-G-G-A-T-A-A-G	2	3	0.20	NS
G-G-G-A-T-G-A-G	7	11	0.90	NS
G-G-G-A-T-G-A-A	1	2	0.34	NS
G-G-G-G-C-G-G-A	2	9	4.82	NS
G-G-A-A-C-A-A-G	1	10	8.55	NS
G-G-A-A-C-G-A-G	2	8	3.86	NS
G-G-A-A-T-G-A-G	6	6	0	NS
A-G-G-A-T-A-A-G	2	0	2.77	NS
A-G-G-A-T-G-A-G	2	1	0.34	NS
A-G-G-G-C-G-G-A	0	2	2.77	NS
A-G-A-A-T-G-A-G	0	2	2.77	NS
A-T-G-A-T-G-A-G	0	4	5.55	NS
A-T-A-A-C-A-A-G	2	3	0.20	NS
A-T-A-A-C-G-A-G	4	2	0.68	NS
A-T-A-A-T-G-A-G	**55**	**20**	**16.98**	**0.0007**
A-T-A-A-T-G-A-A	0	2	2.77	NS
A-T-A-A-T-G-G-G	0	2	2.77	NS
A-T-A-A-T-G-G-A	2	3	0.20	NS
A-T-A-A-C-G-G-A	4	2	0.68	NS

[1] P-values were corrected by Bonferroni method, all values >0.05 were recorded as non-significant (NS).

3.3.2 Linkage disequilibrium and Hardy-Weinberg equilibrium

LD measures estimated by the Haploview™ program are given in Figure 3-3. The results demonstrated that rs2284879 and rs6023526 were in the same haplotype block (D'=0.95, r^2=0.39), as were rs2076380 and rs7270785 (D'=0.93, r^2=0.74).

Assessment of Hardy-Weinberg equilibrium confirmed that all SNPs were in HWE for both patient and parent groups, with the exception of rs4811528, which appeared to deviate from HWE in the patient group (p=0.013).

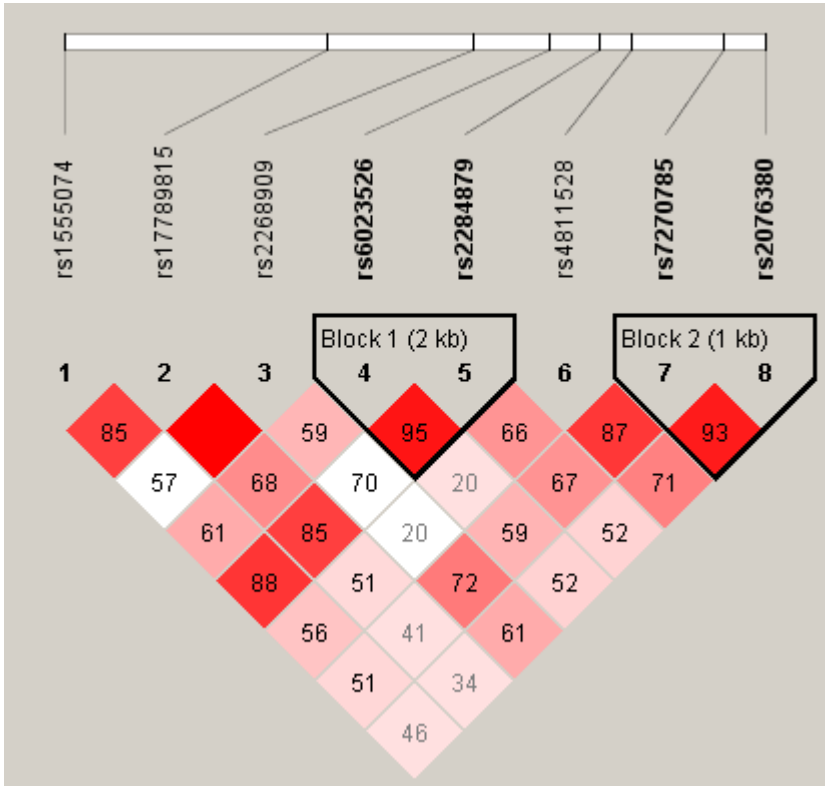


Figure 3-3 LD map constructed with 8 TGM2 SNPs in studied samples, D' values shown

(SNP order is shown 3' - 5', in chromosomal orientation)

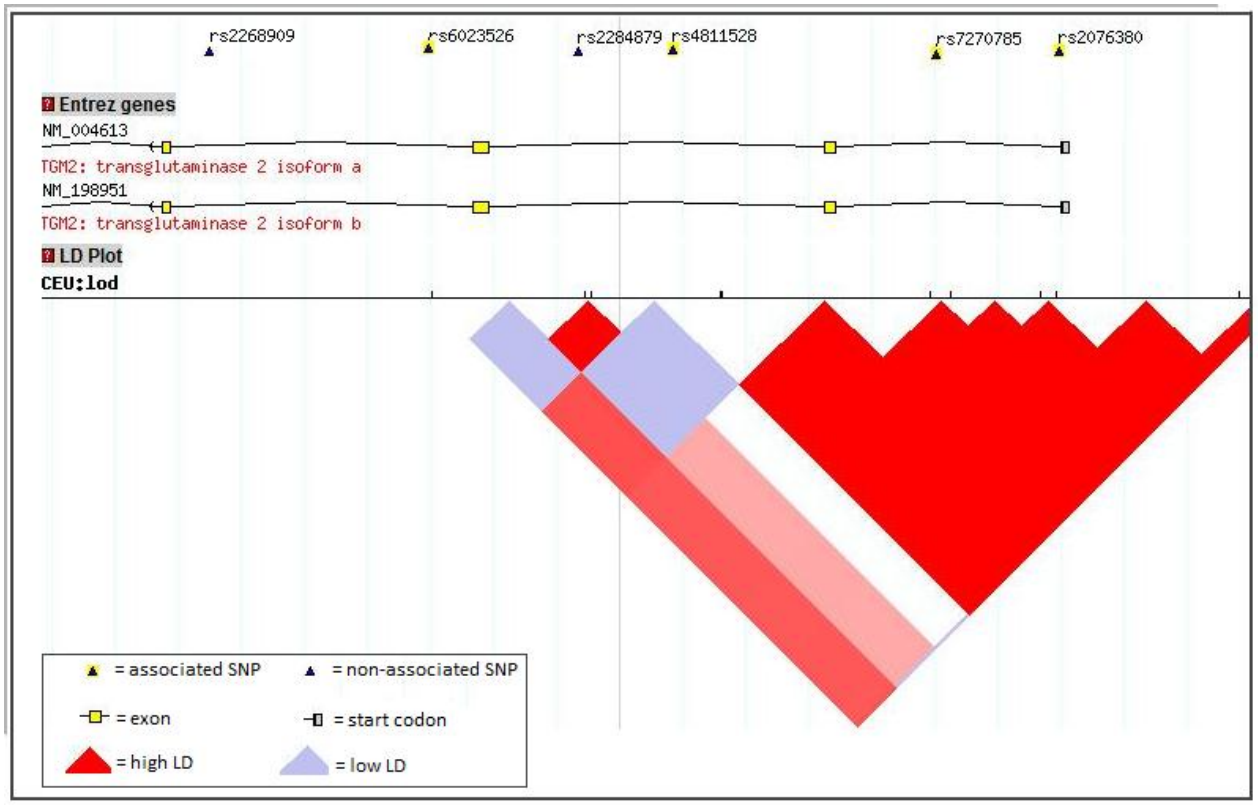


Figure 3-4 LD blocks predicted by Haploview in a European population, corresponding to TGM2 SNPs genotyped

3.3.3 Coding SNP and sequence conservation

Of a pilot group of 148 patients, none showed polymorphism of the coding SNP rs1062735, effectively ruling out this locus as a functional variant for schizophrenia. A sample gel photograph for this genotyping can be seen in Figure 3-5.

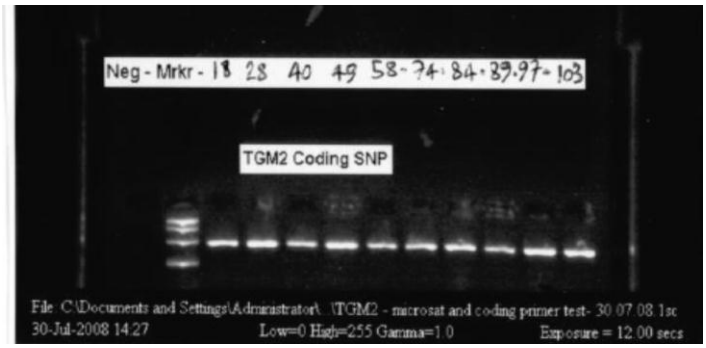


Figure 3-5 Results of genotyping at rs1062735 locus

When assessed using the UCSC genome browser, the only conservation observed in the 5'-end region of the TGM2 locus fell within the gene's exons (Figure 3-6), making functional intronic elements within this region less likely. While several sites of conservation are present upstream of the TGM2 promoter region, they are closer to the KIAA1755 gene, and ~20kb out with the most 5'-end LD blocks of the TGM2 gene, making it unlikely that these sites would be in LD with the TGM2 SNPs found to associate with schizophrenia.

Figure 3-6 Sequence conservation within the 5'-end region of TGM2

3.3.4 Identification of functional elements

Screening of the 5'-end region of TGM2 and the 20kb region upstream of it identified two potential human TFBs. The first, located in intron 1 was indentified as a match for Myocyte-specific enhancer factor 2A (MEF2A), a binding site normally involved in regulation of muscle gene expression. The second, located in intron 2, was identified as a match for MRF-2, a binding site thought to be relevant in the process of fat metabolism.

Analysis of SNP loci revealed no intronic or exonic splice elements. The most strongly associated SNP, rs7270785, did identify as a possible TFB. When the over-transmitted G allele was present, it created a potential binding site for the transcription factor nuclear factor-kappaB (NF-κB).

3.4 Discussion

3.4.1 Genetic association

The results show both allelic and haplotypic associations with schizophrenia. Allelic associations were observed for rs2076380, rs7270785, rs4811528 and rs6023526, peaking at rs7270785 ($p=0.004$). The 8-SNP haplotype system assessed consisted of 19 individual haplotypes with a frequency of 1% or over, of which the A-T-A-A-T-G-A-G haplotype was significantly over-transmitted (corrected $p=0.0007$), with transmission in 55 cases versus non-transmission in 20 controls. The association signal extended across 2.3kb of DNA, and was not confined to a single haplotype block when assessed using either our own genotyping data, or according to the blocks predicted by the HapMap project. The LD structure within this region can be determined to be at least partly in agreement with the LD structures predicted for the region by Haploview (Figure 3-4),

with rs2076380 and rs7270785 in LD, while low values for D' are observed at rs4811528. Moving away from the 5'-end region the information is, however, incomplete, making comparison difficult. It is possible that the locus responsible for the disease association could generate a LD signal which extends beyond its own immediate block, or, the extension of association could indicate the presence of multiple rare variants occurring within the gene. Lastly, association signals may be either diluted within, or duplicated across multiple haplotypes, where subsequent mutations have occurred close to an original functional mutation.

3.4.2 Functional Variants

Analysis of the SNPs themselves revealed only one potential functional element; a possible binding site for NF-κB. NF-κB is involved in the regulation of cytokines, performing an established role in immune regulation (Liou, 2002). A study of medication-naïve schizophrenia patients, showed PBMCs drawn from those patients to have increased NF-κB expression and activation levels, compared to controls. This increase was mirrored by increased levels of the cytokines tested; IL-1β and TNF-α (Song et al., 2009).The involvement of NF-κB in immune regulation, its reported upregulation in schizophrenia make the detection of a possible binding site in the region an interesting finding. That considered, the regulation of the TGM2 gene by NF-κB is unreported; rather, reports of interaction centre on the TGM2 protein regulating NF-κB, maintaining NF-κB's activation by cross-linking its inhibitory subunit (Mann et al., 2006). It is possible that NF-κB could in turn influence TGM2 expression, acting as a feedback loop and thereby altering TGM2 production, however this has not been reported in the literature to-date. The downstream effects of

such a process are difficult to predict, the regulation of TGM2 by NF-κB (itself activated by factors including inflammatory cytokines and bacterial or viral components) could be a mechanism through which TGM2 activity relates to immune assault. However, this binding site is based solely on sequence-based predictions and should therefore be considered with caution until interaction can be functionally tested, by techniques such as a reporter gene assay.

The associated SNPs were all located towards the 5'-end region of the TGM2 gene, suggesting they might be in LD with variants located in the promoter region. Screening of this area for conservation (out with exons) revealed no conserved sites within ~20kb of the associated SNPs; those areas where conservation was observed were distant from TGM2 both physically and in terms of predicted LD structure. Any functional variation within the 5'-end region is therefore likely to be within exons, or to result from a mutation which generates a novel site capable of influencing gene function. Elements that influence TGM2 transcription have been described in the 5' region of the gene before; including a retinoid binding site (Nagy et al., 1997)and a region of hypermethylation (Dyer et al., 2011). However, neither of these studies were concerned with the neurological activity of TGM2.

Within the 5' TGM2 exons, two potential TFBs were identified. Though located close to the associated SNPs, in intron 2, the role of MRF-2 in fat metabolism makes it an unlikely candidate for this study. Located in intron 1, the function of MEF-2A in muscle gene expression has some overlap with TGM2 (which can play a role in regulating smooth muscle cell proliferation) but in a manner that is unlikely to be relevant to schizophrenia. More interestingly, variants within MEF-2A have been associated with Alzheimer's disease (González et al., 2007). The authors of this study hypothesised that the anti-apoptotic activity of the MEF-2 protein group might explain this association, with MEF-2 transcription factor activity having previously been reported to facilitate

neuronal survival (Mao et al., 1999). TGM2 is known to be an important factor in determining apoptosis itself (Cao et al., 2008, Falasca et al., 2005, Fok and Mehta, 2007, Szondy et al., 2003b), and it is possible that MEF-2A is relevant to TGM2 in this capacity. To date, however, there have been no published reports describing interaction between MEF-2A and TGM2, so such a concept is purely speculative at this stage.

3.4.3 Hardy-Weinberg Equilibrium

Of 8 SNPs tested, rs4811528 alone was not in Hardy-Weinberg equilibrium, however this was observed only in the patient group. Had results from the parent group deviated from HWE there would have been questions regarding the validity of the associations observed (Trikalinos et al., 2006). Such deviations within a patient group however, can add credibility to an association, with HWE testing having been suggested as an alternate method for locating disease-associated markers (Nielsen et al., 1998).

3.4.4 Quality control

Throughout the 10% repeat calls performed, no conflicting results were observed for genotyping. The frequency of Mendelian errors detected by Haploview was 0.001% (three errors out of 3,144 genotypes), making the data set essentially free from error. The genotypes for these trios were genotyped again and corrected in the data set.

3.4.5 Significance of finding

Without evidence of a functional variant in the gene region, it is only possible to speculate about the mechanism through which TGM2 might be involved in schizophrenia. The change to the gene could come in the form of altered activity, expression or affinity for the immune system.

In line with the CD hypothesis, variation in activity might occur through the resulting TGM2 protein exhibiting a greater affinity for substrates such as gluten, thus increasing the risk of an immune response. While this dovetails well with the search for overlap between the two conditions, it is worth noting that no mutations have, as yet, been detected for TGM2 in CD (van Belzen et al., 2001), meaning that any such mutation would be unlikely to play a major role in the majority of coeliac disease cases.

It is equally, if not more possible, that changes to the coding sequence exert a negative effect, rendering TGM2 less capable of its biological activity. Certainly the novel mutations observed for TGM2 in early-onset type 2 diabetes appear to be of this nature, with both the variants identified occurring within the catalytic site of TGM2 (Porzio et al., 2007) and resulting in impaired transamidating activity *in vitro*.

If the variant does lie within the promoter or another regulatory region, it could alter the expression of TGM2 under specific circumstances, disrupting levels of TGM2 either in the gut, or elsewhere in the body. The potential NF-κB TFB site identified for the associated allele at the rs7270785 locus, could present a link with either apoptotic or immunological pathways. Based as it is on only a homology-based prediction however, such a hypothesis remains speculative. While the influence of TGM2 on NF-κB is well-defined (Mann et al., 2006, Cao et al., 2008), evidence for a relationship in the opposing direction has not yet been reported. NF-κB has itself been implicated in the process

of immune dysregulation in schizophrenia (Song et al., 2009), and while the authors had no cause to include TGM2 in their analysis, the result was consistent with the expected influence of TGM2; showing increased NF-κB expression and activation.

3.4.6 Future experiments

In order to confirm the association of TGM2 with schizophrenia, a follow up study is ideally required in a separate sample group. Such confirmation was out with the immediate capabilities of this project, work was instead carried out to elucidate the mechanism behind the association. The following chapter describes several lines of investigation, designed for this purpose.

4 Functionality of the TGM2 genetic association

In light of the association of the TGM2 gene with schizophrenia (uncovered in chapter 3), follow-up studies were designed to analyse the functionality of the observed association. Data within this chapter has been published by the author (Bradford et al., 2011).

4.1 Introduction

Immunological changes in schizophrenia were first reported by Bruce and Peebles in 1903, and key features of the literature since then are summarized in section 1.1.4. As described in 1.1.4.2, research into the immunological features of schizophrenia has yielded a variety of hypotheses, concerning different aspects of the immune system. Cytokines, particularly IL-2 and its soluble receptor (sIL-2R), have been widely studied, with alterations in serum levels often observed (Crabtree et al., 1989, O'Donnell et al., 1996). Serum IL-2 levels had previously been determined for a subset of the samples available to this project, and so these measurements were selected to act as markers for altered immune function. Analyzed in combination with genotyping data, comparison of the IL-2 levels between patients and controls was used to replicate previous analyses, and also to examine the correlation between serum IL-2, and the disease-associated alleles identified in Chapter 3.

As described in Chapter 3, the SNPs associated with schizophrenia were located towards the 5' region of the TGM2 gene. As such, altered TGM2 expression was considered a promising mechanism for impact on disease. To assess this, a gene expression study was designed to determine whether patient status and/or TGM2 genotype correlated with TGM2 expression level. A potential confounding factor is the influence of medication on gene expression; the drug clozapine was prescribed to all but one of the patients in this study of TGM2 expression. To assess the potential

impact of clozapine on TGM2 expression, an additional experiment was designed in which cells were treated *in vitro* with clozapine, and their gene expression compared to untreated cells.

In response to reports of increased antibody production against TGM2 and gliadin in schizophrenia (Reichelt and Landmark, 1995, Dickerson et al., 2010, Jin et al., 2010) another line of investigation focused on this hypothesized aetiological link between CD and schizophrenia. It is notable that while increased anti-gliadin antibody levels are observed in schizophrenia, they are considerably less frequent than for CD, prompting the question of why some individuals with schizophrenia display this phenotype, while others do not. It seems plausible that variation within the TGM2 gene might explain this variation in antibody response. A study of autoantigen gene sequences revealed that SNPs were over seven times more common in these genes compared to those coding for other proteins (Stadler et al., 2005). Specific mutations within genes may effect an increase in antigen affinity through mechanisms such as increased homology to pathogens or sequence alteration which disrupts normal cellular localization, a mechanism observed for the La protein in a patient with Sjogren's syndrome (Bachmann et al., 1996). To investigate this hypothesis, plasma levels of antibodies against both TGM2 and gliadin (IgA and IgG forms) were assessed in patients with schizophrenia and controls. Antibody production was then correlated with both patient status and TGM2 genotyping data.

Lastly, following a report of novel gliadin-antibody response in schizophrenia, antibodies against two forms of gliadin were tested for; the deamidated gliadin-derived GAF-3X peptide, specific to CD, and the native form of the gliadin protein. Comparison of the results for these two tests was designed to confirm whether the gliadin antibody response observed in the patient group was equivalent to that seen in CD, or whether it was specific to schizophrenia itself.

Several hypotheses were tested in the course of these experiments:

i. The presence of schizophrenia-associated alleles, within TGM2, correlates with altered TGM2 gene expression in PBMCs.

ii. The presence of schizophrenia-associated alleles, within TGM2, correlates with an alteration in serum IL-2 levels.

iii. The presence of schizophrenia-associated alleles, within TGM2, correlates with an increase in CD-associated antibodies.

Antibodies from schizophrenia patients show reactivity towards native gliadin peptides, but are not specific to the deamidated gliadin peptide defined for CD.

4.2 Methods

4.2.1 Subjects

Samples used for serum IL-2 analysis

Subjects were drawn from archived case/control samples collected by Dr Jun Wei (in Bangor, Wales, UK). The origins of these samples are described fully in Section 2.1.2. A total of 77 cases (49 males and 28 females, aged 35.7±6.1) and 52 healthy controls (26 males and 26 females, aged 39.2±19.5) were available, for which serum IL-2 levels had previously been determined.

Samples used for gene expression and antibody analysis

These subjects were from the case/control group collected in Inverness between 2009 and 2010, as described in Section 2.1.1. The group included 12 cases (7 males and 4 females, aged 37.8±10.5) and 20 controls (5 males and 15 females, aged 48.6±14.2).

4.2.2 Influence of TGM2 allelic variation on serum IL-2

Genotyping

The four schizophrenia-associated SNPs; rs2076380, rs7270785, rs4811528 and rs6023526, were genotyped as described in Section 3.2.1. The results were used to group samples in order to assess the influence of disease-associated alleles on phenotypic effects.

Determination of plasma IL-2 levels

Plasma IL-2 cytokine levels were determined by radioimmunoassay in 1994, as described in the resulting paper by Xu et al. (1994). Briefly, a 20-ml fasting blood sample was drawn from the antecubital vein between 8am and 9am in the morning; 5 ml blood was put into a K^+EDTA tube and centrifuged immediately to separate plasma; aliquoted plasma samples were stored at –45 ^oC and were assayed using radioimmunoassay within 10 weeks. The IL-2 levels were expressed as means±SD (fmol/ml).

Data analysis

Distributions of data were assessed using Levene's test for equality of variances. In accordance with data distribution ($F > 0.05$), the correlation between TGM2 genotypes and plasma IL-2 levels was analysed using non-parametric statistical methods.

For each SNP, subjects were grouped according to TGM2 genotype. Kruskal-Wallis analysis was then applied to assess the differences in plasma IL-2 levels between the 3 genotype groups.

For statistically significant results, a further test (binary logistic regression) was performed in order to determine allelic effect, and to correct for potential confounding factors (age and gender). For logistic regression analysis, all genotypes containing the minor allele were treated as one group, and

homozygous genotypes composed of 2 major alleles as the other. All the statistical tests were performed with the SPSS software for Windows 14.0 (2005).

4.2.3 The effect of clozapine treatment on TGM2 gene expression in U937 cells

Cell culture and generation of cDNA

The U937 cell line has been established as a model for a range of in vitro work, and was selected for this study based on its monocytic characteristics, which made it suitable for use as a model for blood monocytes. Additionally, the U937 cell line has been used before for research into TGM2 activity (Behrendt et al., 1993, Oliverio et al., 1997, Amendola et al., 2002) and as a model for studying the impact of clozapine treatment (Heiser et al., 2007).

Cells were cultured and treated by a team member (Dr Aditi Mathur) as described in Section 2.2.1. The effect of clozapine treatment was assessed at two doses, 1 μg/ml and 2 μg/ml clozapine. Plasma concentrations are generally slightly lower than this, nearer 500 ng/ml, however these levels build up over the course of treatment (Weigmann et al., 2001). As the cells could not be cultured for several weeks duration, higher therapeutic doses were selected to compensate. Six biological replicates were cultured for both clozapine-treated and –untreated cells for each experiment. As described in Sections 2.3.3 and 2.3.4, mRNA was extracted following cultured and then converted to cDNA. The resulting cDNA was then used for quantitative analysis of gene expression.

Gene expression analysis

Gene expression was compared between clozapine-treated and untreated cells. TGM2 mRNA levels, considered representative of gene expression, were analysed using qRT-PCR, with all reactions performed as described in Section 2.5.2. Primers used for amplification of TGM2 cDNA were custom designed by Primerdesign Ltd, and all other reagents were described in Section 2.5.2.

Before gene expression could be assessed, standard curves were performed using the cDNA samples, in order to determine the optimum dilutions in light of limits of detection, reproducibility and reaction efficiency. To achieve this, qRT-PCR was performed with pooled sample cDNA, titrated over a series of five, ten-fold dilutions. Each dilution was amplified in triplicate, with reactions run for both a highly-expressed house-keeping gene (HKG), GAPDH, and the less-expressed target gene, TGM2. The optimal concentration was defined as the dilution at which variance, expressed as SD, between replicates was minimal, and at which Ct values for GAPDH and TGM2 were as close as possible, both falling within 15-30 Ct. For cell culture cDNA, the optimal dilution was determined to be 1:20, diluted in deionised water.

The optimum number and combination of HKGs were determined using the GeNorm and Normfinder programs in combination, as described in Section 2.5.1. Figure 4-1 shows GeNorm's graphical representation of the number of HKGs required for correction of expression, to achieve the recommended V value of ≤ 0.15 in this experiment. In this case three HKGs were required, of which ATP5B, GAPDH and B2M were ranked highest by both GeNorm and Normfinder.

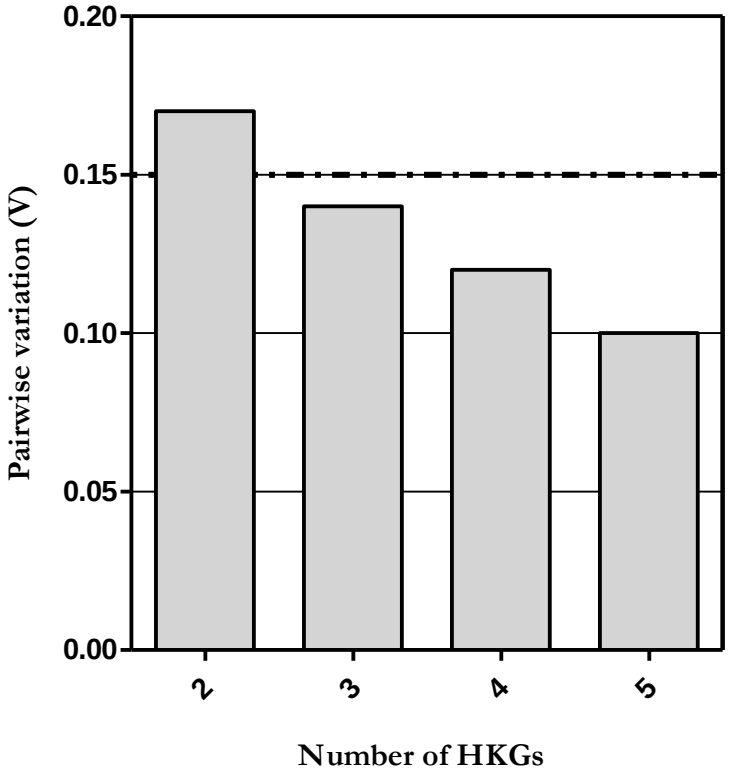


Figure 4-1. The pairwise variation determined by GeNorm according to the number of HKGs employed.[1]

[1]The dotted line indicates the V = 0.15 cut-off beneath which pairwise variation should ideally fall. In this case three HKGs are required to meet this criterion.

Primer efficiencies and amplification standard curves were calculated for both the HKG primers and the TGM2 primers, as detailed in Section 2.5.3.

qRT-PCR reactions were performed in triplicate for each cDNA sample; six case and six control replicates, with a duplicate no template control (NTC) on each plate. The sample maximisation approach described by Vandesompele et al. (2002) was used for all experiments, with each sample

for a given gene being performed of the same plate, thus removing the need for inter-run calibrators.

Data analysis

Analysis of gene expression in case and control groups was performed as described in section 2.5.4, using the REST 2009 program to generate final estimates of altered expression along with p-values for significance.

4.2.4 Comparison of TGM2 gene expression in patients and controls

Blood sampling and generation of cDNA

Blood samples from the Inverness case/control cohort were obtained and processed as described in Section 2.3, following which mRNA was extracted and converted to cDNA as described in Sections 2.3.3 and 2.3.4 respectively. At the time of experimentation, only 11 cases were available, as such analyses were performed with 11 cases and 11 controls.

Gene expression analysis

Comparison of gene expression between the patient and control groups was conducted using the same protocol that was applied to the cell culture samples (4.2.3). Specific details for this experiment are given below.

Optimal cDNA dilutions for this experiment were determined as 1:10.

GeNorm analysis revealed that due to lower variation amongst samples, only two HKGs were required in this instance (V = 0.085). The two most suitable HKGs were identified as GAPDH and ATP5B.

qRT-PCR reactions were performed in triplicate for each sample; 11 patient samples and 11 control samples, with a duplicate no template control (NTC) on each plate. The sample maximisation approach described by Vandesompele et al. (2002) was used as before.

Data analysis

Analysis of gene expression in case and control groups was performed as for cell culture samples.

4.2.5 Correlation of TGM2 gene expression with TGM2 genotype

Due to the low number of samples available for cases and the potential bias introduced by medication, the influence of TGM2 variation on expression was analysed using samples from the control group only.

Blood sampling and generation of cDNA

Performed as in Section 4.2.4, only control samples were used.

Analysis of genotypic association with gene expression

Analysis of the original genotyping study revealed four SNPs that were associated with schizophrenia. Of these, rs7270785 was selected as the most suitable SNP to define samples as case or control (i.e. those carrying a risk-associated variant, and those not). This SNP was selected for several reasons:

- Most significant individual association with schizophrenia ($p=0.001$)
- In LD with at least one other, strongly associated SNP, rs2076380.
- Sufficient heterozygosity in cohort; all genotypes were present in the control group.

Individuals who were homozygous for the major allele (genotype TT) were considered as controls. Individuals in whom the minor G allele was detected were considered as cases (genotypes GG and GT). This method of division resulted in groups of seven controls and 12 cases (of which eight were heterozygotes).

Comparison of gene expression between the designated case and control groups was conducted as much as for the case/control analysis (Section 4.2.4). Exceptions are detailed below.

GeNorm analysis revealed that, as for the case/control analysis, only two HKGs were required in this instance (V=0.071). The two most suitable HKGs were identified as GAPDH and CYC1.

qRT-PCR reactions were performed in triplicate for each cDNA sample; with a duplicate of no template control (NTC) on each plate. The sample maximisation approach described by Vandesompele et al. (2002) was used as before.

Data analysis

Analysis of differences in gene expression between case and control groups was performed as for cell culture samples.

4.2.6 Correlation of TGM2 genotype with antibody levels

Genotyping and ELISA

Following the genotyping of patient and control samples (Section 4.2.1), ELISAs were performed to assess the levels of antibodies against TGM2 and gliadin, as described in Section 2.7. Both IgA and IgG forms were tested in each case. All samples were tested in duplicate and scored according to

cut-offs defined by the manufacturer, positive and negative controls were supplied and were run with each plate.

Data analysis

For each test (excepting anti-TGM2 IgG, for which only a single calibrator was supplied) standard curves were graphed according to the mean values for each calibrator, using Graphpad Prism 5. The slope of the line was then calculated and the equation for this used to determine the antibody quantities (in RU/ml or U/ml) for each sample. Samples were determined as positive or negative according to the cut-offs specified for each kit.

4.3 Results

4.3.1 Correlation of TGM2 allelic variation with serum IL-2 levels

There were no significant differences in plasma IL-2 levels between the patient group (71.3±22.0 fmol/ml) and the control group (69.5±19.4 fmol/ml, t= 0.473, df=127, p=0.637).

Kruskal-Wallis analysis revealed a correlation between alleles at the rs4811528 SNP and plasma IL-2 levels in the patient group (χ^2=7.60, df=2, p=0.022) (Table 4-1). Presence of the major allele A at rs4811528 was correlated with an increases in plasma IL-2 levels (Cohen's d = 0.86, effect size r = 0.39). The relationship between re4811528 and IL-2 levels was not mirrored in the control group (χ^2=0.096, df=1, p=0.756). Binary logistic regression analysis was performed by dividing the patients into two groups; minor allele homozygotes and heterozygotes (GG and AG genotypes) as one group, major allele homozygotes (GG genotypes) as the other. This analysis confirmed the association of rs4811528 with plasma IL-2 levels in the patient group, with results remaining significant after adjustment for age and sex (χ^2=8.191, df=3, p=0.042). When considered in the context of the within-group variation seen for each genotype, the scale of the differences between groups is modest, however the calculated effect size is large (SD of >0.8, as defined by Cohen's conventions). The box and whisker plot format of Figure 4-2 displays the mean levels of plasma IL-2 for each genotype group, showing the spread of data within those groups once the outlying individual has been defined as such.

The other 3 SNPs tested showed no genotypic association with plasma IL-2 levels in either the patient group or the control group (Table 4-1).

Table 4-1 Kruskal-Wallis analysis of genotypes and plasma IL-2 levels in patient and control groups.

SNP	Genotype	Mean IL-2	S.D.	N	Mean rank	Chi-squared	D.F.	Asymptomatic significance
Control group								
	AA	57.15	11.41	3	15.33			
rs2076380	AG	63.76	19.94	23	21.98	3.728	2	0.155
	GG	74.64	17.76	22	28.39			
	GG	73.98	14.42	4	29.75			
rs7270785	GT	63.67	19.69	25	22.18	2.613	2	0.271
	TT	75.17	17.98	21	28.64			
	AA	-	-	0	-			
rs4811528	AG	67.19	22.34	20	25.68	0.096	1	0.756
	GG	71	17.57	32	27.02			
	CC	-	-	0	-			
rs6023526	CT	71.8	18.5	16	28.72	0.495	1	0.482
	TT	68.53	19.98	36	25.51			
Patient group								
	AA	69.16	10.21	6	40.92			
rs2076380	AG	73.63	19.07	27	42.39	1.59	2	0.452
	GG	70.44	25.18	43	35.72			

	GG	75.56	14.67	9	46.67			
rs7270785	GT	72.43	18.25	28	40.2	3.534	2	0.171
	TT	69.62	26.63	37	33.23			
	AA	78	13.49	4	49.88			
rs4811528	AG	80.79	31.89	24	48.02	7.604	2	**0.022**
	GG	66.22	13.94	49	33.69			
	CC	81.5	14.32	6	55.92			
rs6023526	CT	71.45	19.52	22	40.73	4.356	2	0.113
	TT	70.1	23.78	49	36.15			

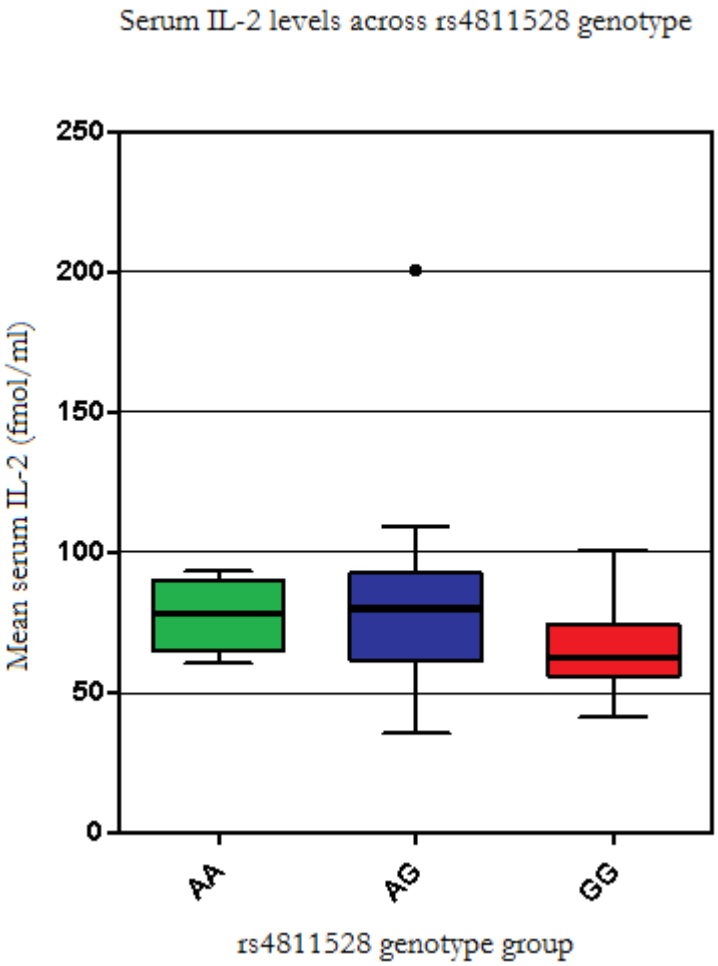


Figure 4-2 Variance of serum IL-2 according to rs4811528 genotype. [1]

[1]A box and whiskers graph in which the middle line of each floating column represents the median of IL-2 levels for that genotype group. Whiskers are determined by Tukey measurements (the shorter of 1.5 times the interquartile distance to either the higher the highest or lowest point), dot points represent the IL-2 level of individuals out with this range.

4.3.2 The effect of clozapine on TGM2 expression in U937 cells

Analysis of cells treated with 1 μg/ml clozapine revealed that TGM2 expression was significantly different in treated versus untreated cells (Table 4-2). According to analysis by REST, expression of

TGM2 in the treated cell group was down-regulated (p=0.013) relative to expression in the untreated group.

A similar effect was not observed in the 2 μg/ml treatment group; TGM2 expression did not significantly differ between treated and untreated cells (p=0.880, Table 4-3). It is notable that in this analysis, both standard error and confidence intervals were much wider than for the 1 μg/ml test.

Table 4-2 TGM2 expression in cells treated with 1 μg/ml clozapine versus controls

Gene	Status	Efficiency	Expression	S.E.[1]	95% C.I.	p-value[2]	Regulation
TGM2	Target	0.83	0.085	0.014	0.008 - 0.685 - 1.855	0.013	DOWN
GAPDH	HKG	0.99	0.961				
ATP5B	HKG	0.85	1.009				
B2M	HKG	0.79	1.031				

[1]Standard error

[2]2000 permutations

Table 4-3 TGM2 expression in cells treated with 2 μg/ml clozapine versus controls

Gene	Status	Efficiency	Expression	S. E	95% C.I.	p-value[1]	Regulation
TGM2	Target	0.83	0.849	0.050 - 7.145	0.013 - 43.259	0.88	none
GAPDH	HKG	0.99	1.113				
ATP5B	HKG	0.85	0.711				
B2M	HKG	0.79	1.264				

[1]2000 permutations

4.3.3 TGM2 gene expression in patients versus controls

Analysis of TGM2 gene expression showed no significant difference between patients with schizophrenia and control subjects (Table 4-4). The level of variation between individual samples was notably low.

Table 4-4 TGM2 gene expression in patients versus controls

Gene	Status	Efficiency	Expression	S. E	95% C.I.	p-value[1]	Regulation
TGM2	Target	0.95	0.669	0.280 - 1.426	0.128 - 2.572	0.143	none
GAPDH	HKG	1	1.109				
ATP5B	HKG	0.85	0.902				

[1]2000 permutations

4.3.4 Relationship between TGM2 genotype and TGM2 gene expression

When analysed in the control group, genotype at the rs7270785 locus showed no relationship with TGM2 gene expression (Table 4-5). The level of gene expression was also highly consistent between individuals, regardless of genotype.

Table 4-5 Influence of rs7270785 genotype on TGM2 gene expression

Gene	Status	Efficiency	Expression	S. E	95% C.I.	p-value[1]	Regulation
TGM2	Target	0.95	1.005	0.574 - 1.657	0.372 - 3.309	0.982	none
GAPDH	HKG	1	0.986				
CYC1	HKG	0.76	1.015				

[1]2000 permutations

4.3.5 Gliadin and TGM2 antibody levels in schizophrenia patients versus controls

There was no correlation observed between antibody levels in patients, for either anti-gliadin or anti-TGM2 antibodies, with data instead confirming the null hypothesis. Table 4-6 shows the mean antibody level determined for each sample in these tests. One sample, from the control group, was removed from the data set following results highly suggestive of undiagnosed CD. The individual tested strongly positive for antibodies in five out of six tests and a consultant gastroenterologist confirmed that these results were indicative of CD. While the consistently strongly positive results obtained for five out of six antibodies emphasised the effectiveness of these tests, the result also

required that this sample be excluded from all antibody analyses, as an individual with suspected CD could not be considered a suitable control subject.

The remaining samples showed only three incidences of a positive test result; two of which came from the control group, one from the patient group. In all three cases the values were relatively weak positives, only slightly in excess of the test cut-off and far below the levels recorded for the removed sample from the suspected CD case. It became apparent that there were too few positive results for a meaningful qualitative analysis, and insufficient (significant) variation to compare antibody levels between genotype groups. As such, histograms were constructed for the mean antibody levels of each sample, and a Student's *t*-test (where Levine's test showed equality of variances) or a Mann-Whitney (where Levine's test revealed inequality of variances) were performed to compare antibody levels in the patient and control groups.

Figures 4-3 to 4-8 show the results of each antibody test across samples and groups. Tables 4-7 to 4-12 summarise the statistical analyses performed for each data set, designed to test whether there was any significant sub-clinical (i.e. below cut-off) variation in antibody levels. When comparing antibody levels between patients and controls, the Student's *t*-test was employed, except in conditions where Levine's test indicated inequality of variances, in which case the non-parametric Mann-Whitney test was substituted. In each case the analysis confirmed that there were no statistically significant differences between the two groups.

Table 4-6 Antibody test results in patient and control groups

Antibody tested	GAF IgA	GAF IgG	tTG IgA	tTG IgG	Gliadin IgA	Gliadin IgG
Units of measurement	RU/ml	RU/ml	RU/ml	Sample / Calibrator	U/ml	U/ml
Positive cut-off	25	25	20	1[a]	4	10
Control samples						
1	3.79	1.41	1.83	0.26	0.60	6.06
2	6.71	3.40	1.82	0.29	0.16	6.00
3	2.46	2.06	1.39	0.31	1.06	1.30
4	7.17	-0.04	2.85	0.28	0.10	3.21
5	2.73	0.66	4.17	0.22	0.91	3.11
6	1.10	0.06	1.33	0.23	0.16	1.68
7	1.01	-0.36	1.52	0.46	1.28	1.41
8	2.56	0.11	1.78	0.25	1.76	**11.28** [b]
9	1.07	0.76	2.80	0.28	0.28	0.90
10	0.26	4.34	1.11	0.21	0.76	0.70
11	1.62	8.43	1.97	0.29	-0.14	2.48
12	9.68	16.80	9.51	0.36	0.62	4.22
13	4.16	4.10	1.07	0.35	**4.32**	2.33
14	4.20	0.98	3.99	0.27	0.82	4.82
15	2.10	0.69	2.70	0.34	0.01	0.93
16	9.89	0.25	5.94	0.41	0.42	1.29
17	2.23	-0.52	4.49	0.24	0.96	3.48
18	1.66	0.74	0.27	0.71	1.53	2.87
19	8.23	1.33	4.30	0.29	0.47	4.25
20	**2320.14**	**> 200**	**> 200**	0.28	**20.45**	**71.75**

Antibody tested	GAF IgA	GAF IgG	tTG IgA	tTG IgG	Gliadin IgA	Gliadin IgG
Units of measurement	RU/ml	RU/ml	RU/ml	Sample / Calibrator	U/ml	U/ml
Positive cut-off	25	25	20	1[a]	4	10
Patient samples						
101	7.62	5.96	1.18	0.32	1.07	0.66
102	5.26	1.41	2.93	0.47	2.86	2.84
103	0.95	1.68	1.35	0.26	0.59	6.80
104	2.28	1.11	2.90	0.28	0.50	0.89
105	1.05	0.21	5.17	0.46	0.26	0.15
106	2.19	1.32	5.85	0.31	2.00	4.12
107	0.09	1.77	7.69	0.69	1.52	0.74
108	-0.23	3.24	17.51	0.29	0.18	7.42
109	**28.21**	3.81	9.65	0.24	0.18	3.88
110	7.96	1.93	2.24	0.66	1.01	0.80
111	17.91	1.13	1.01	0.64	1.21	1.78
Expected non-CD positives	0 (2%)	0 (2%)	0 (<1%)	0 (0%)	1 (5%)	1 (4%)
Actual positives (- sample 20)	1 (3%)	0 (0%)	0 (0%)	0 (0%)	1 (3%)	1 (3%)

[a] < 1 = Neg, 1-2 = Weak positive, 2-5 = Weak positive, > 5 = Strong positive

[b] **Bold** text indicates a positive result.

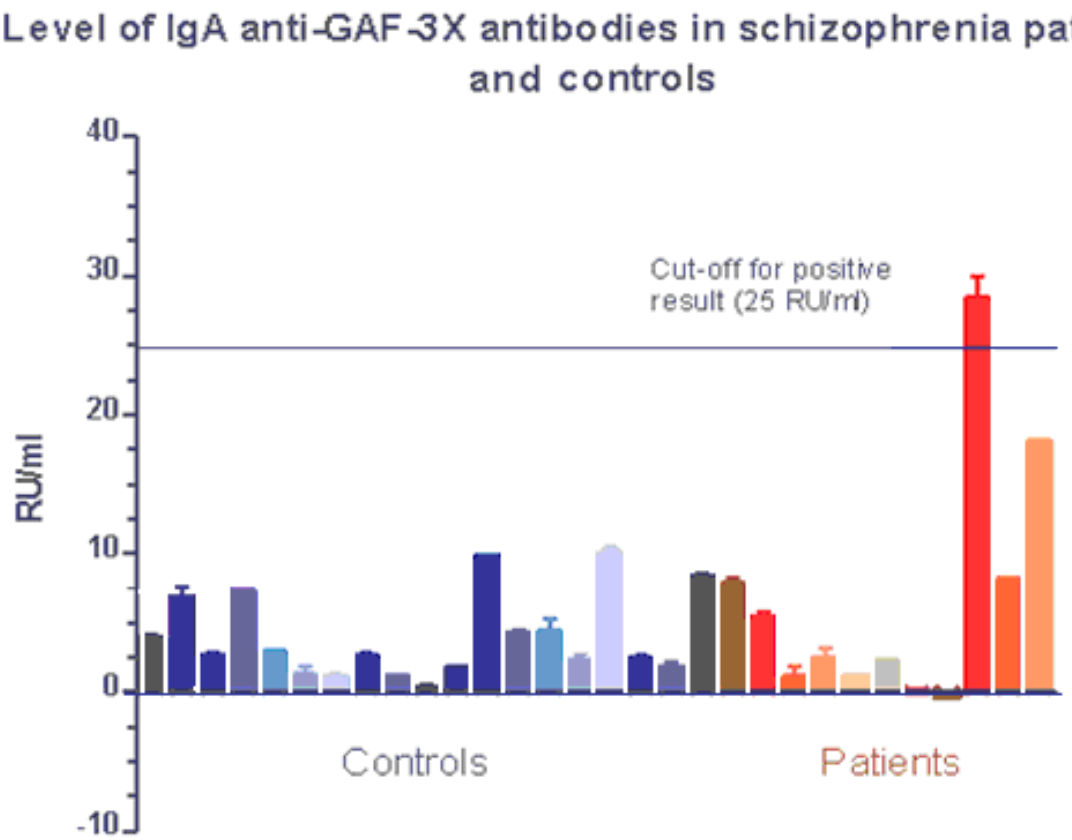


Figure 4-3 Mean levels of IgA anti-GAF-3X antibodies in patient and control samples, as determined by ELISA

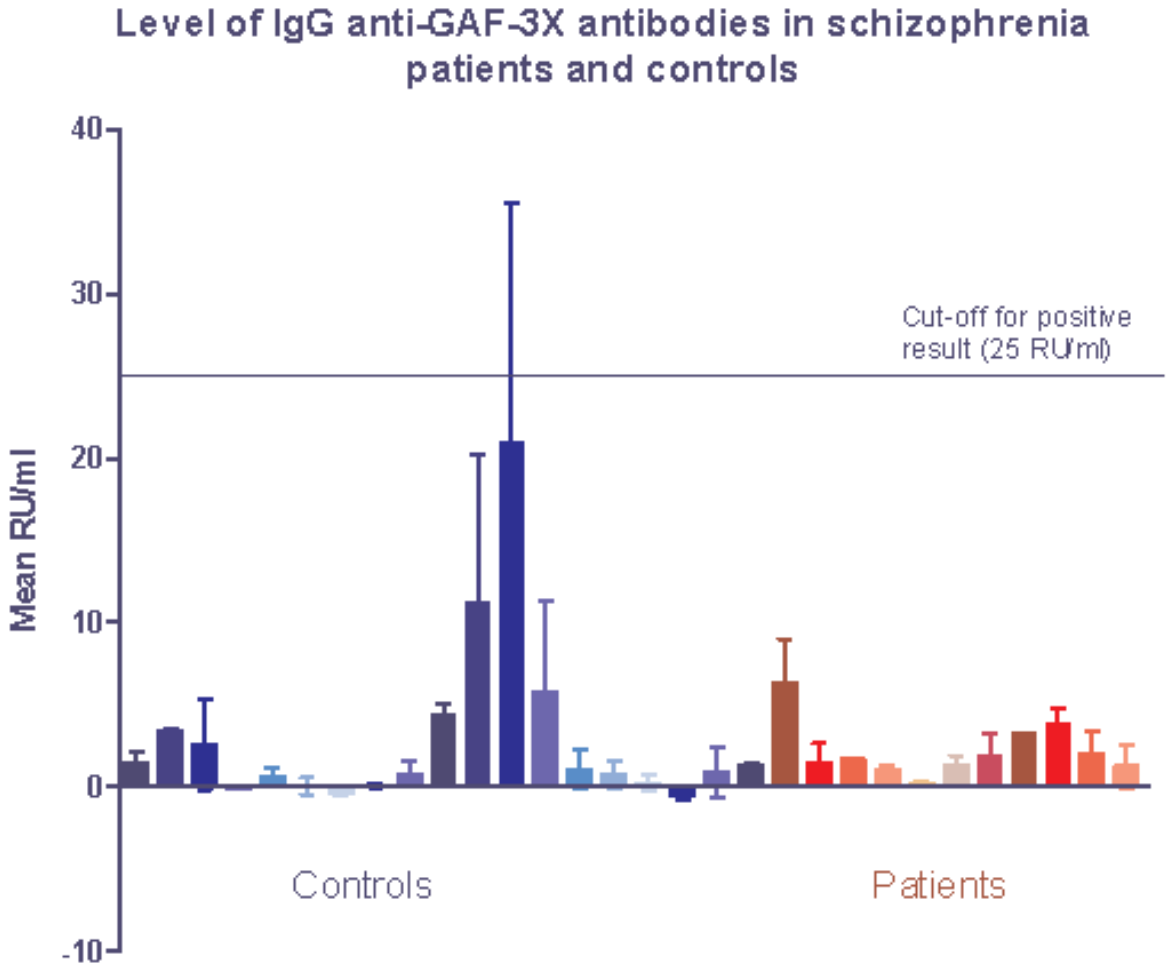


Figure 4-4 Mean levels of IgG anti-GAF-3X antibodies in patient and control samples, as determined by ELISA

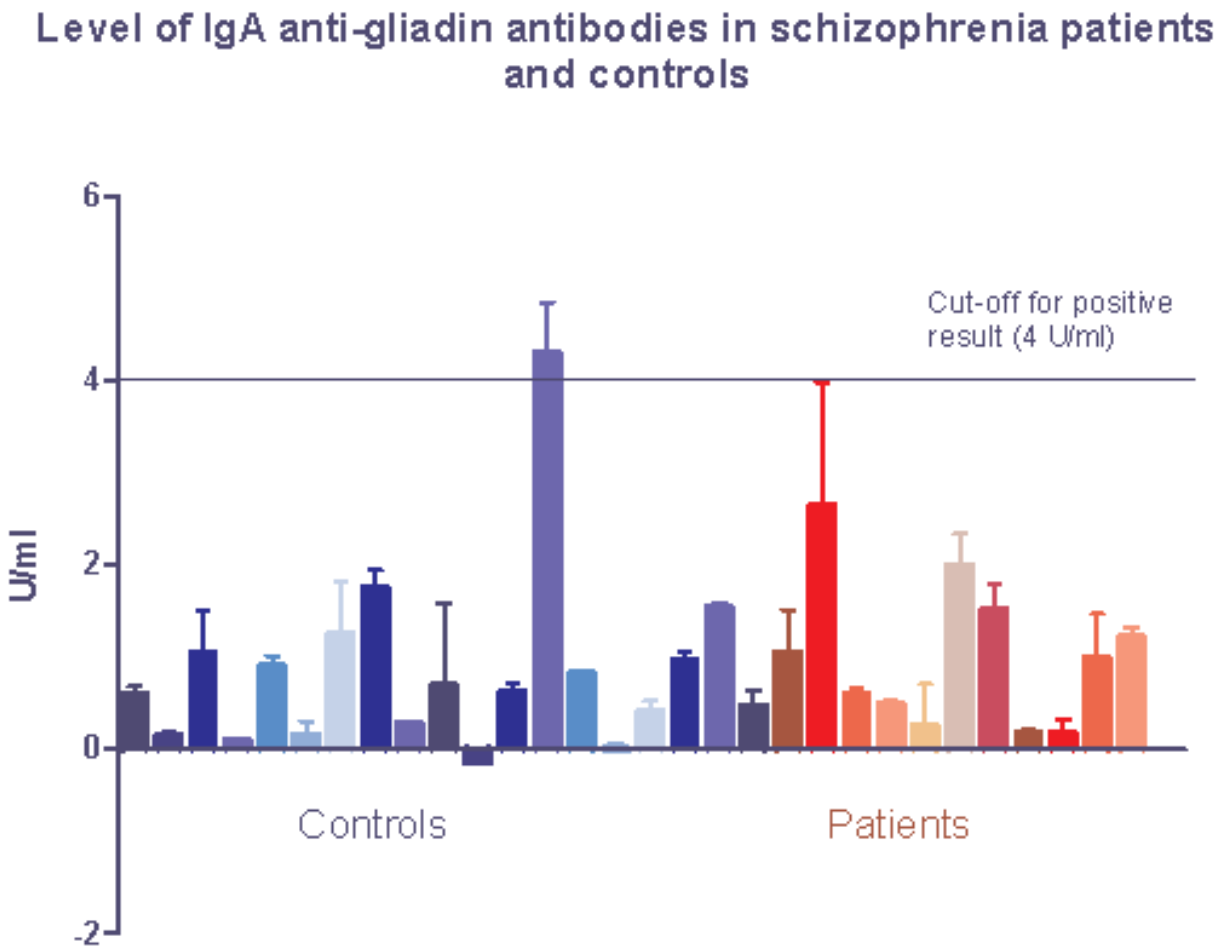


Figure 4-5 Mean levels of IgA anti-gliadin antibodies in patient and control samples, as determined by ELISA

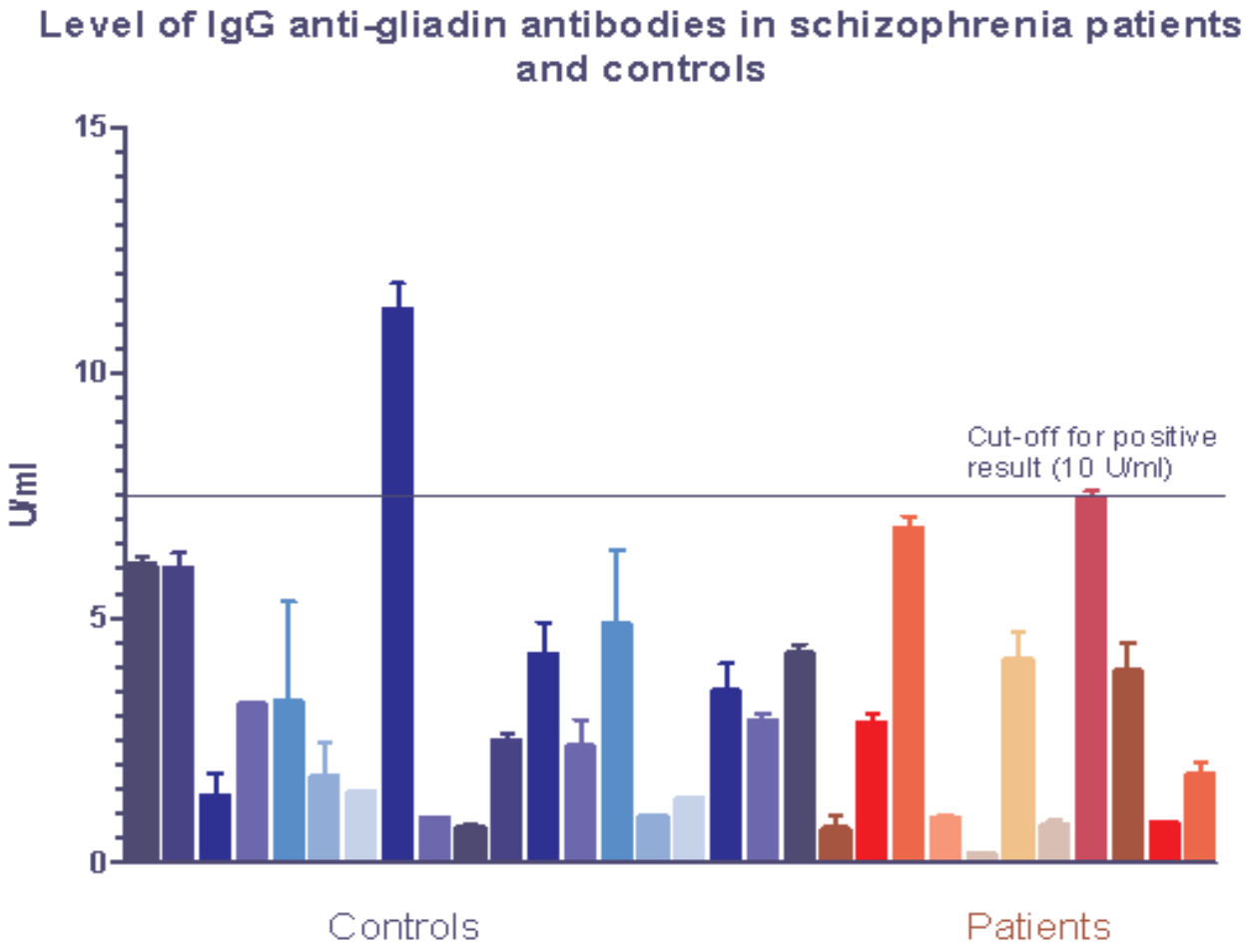


Figure 4-6 Mean levels of IgG anti-gliadin antibodies in patient and control samples, as determined by ELISA

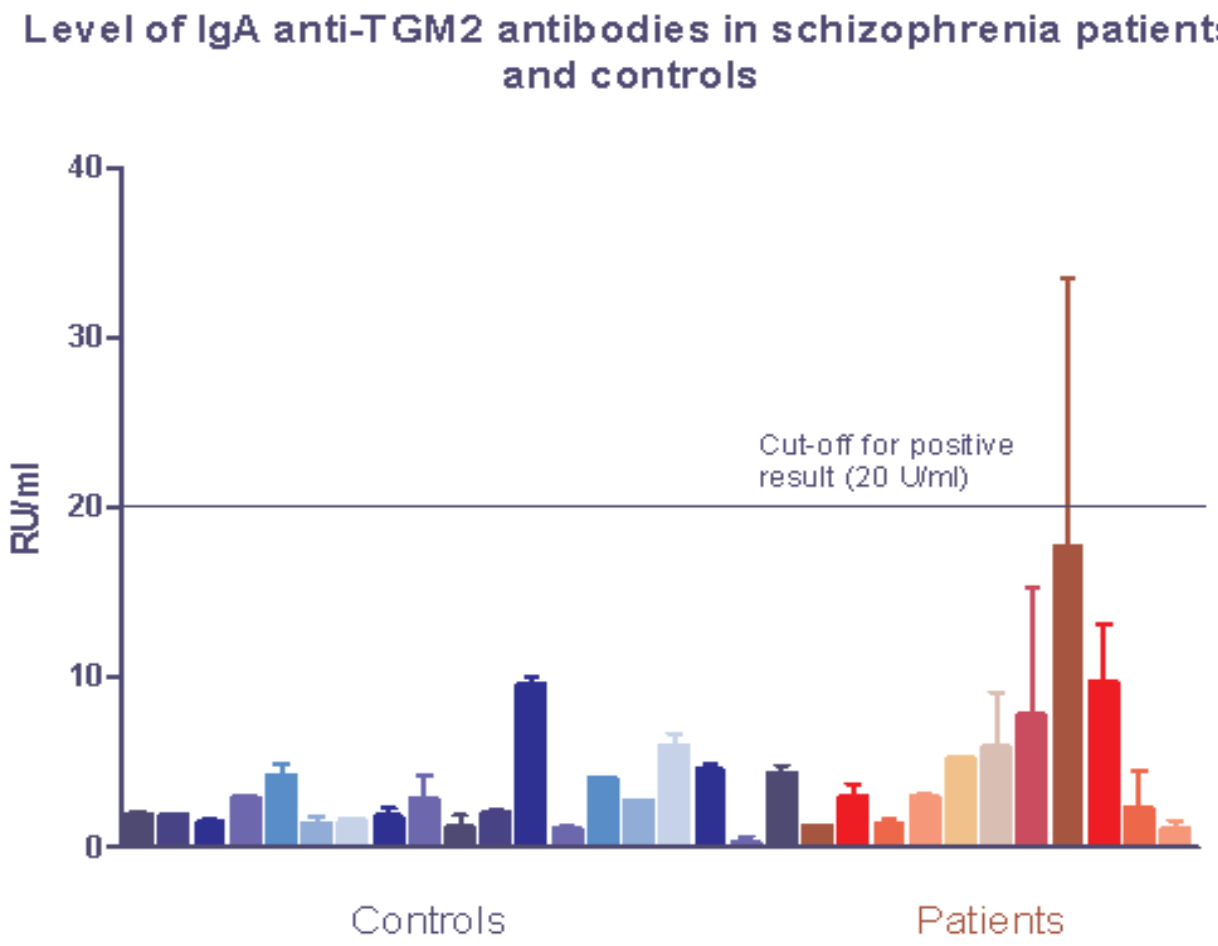


Figure 4-7 Mean levels of IgA anti-TGM2 antibodies in patient and control samples, as determined by ELISA

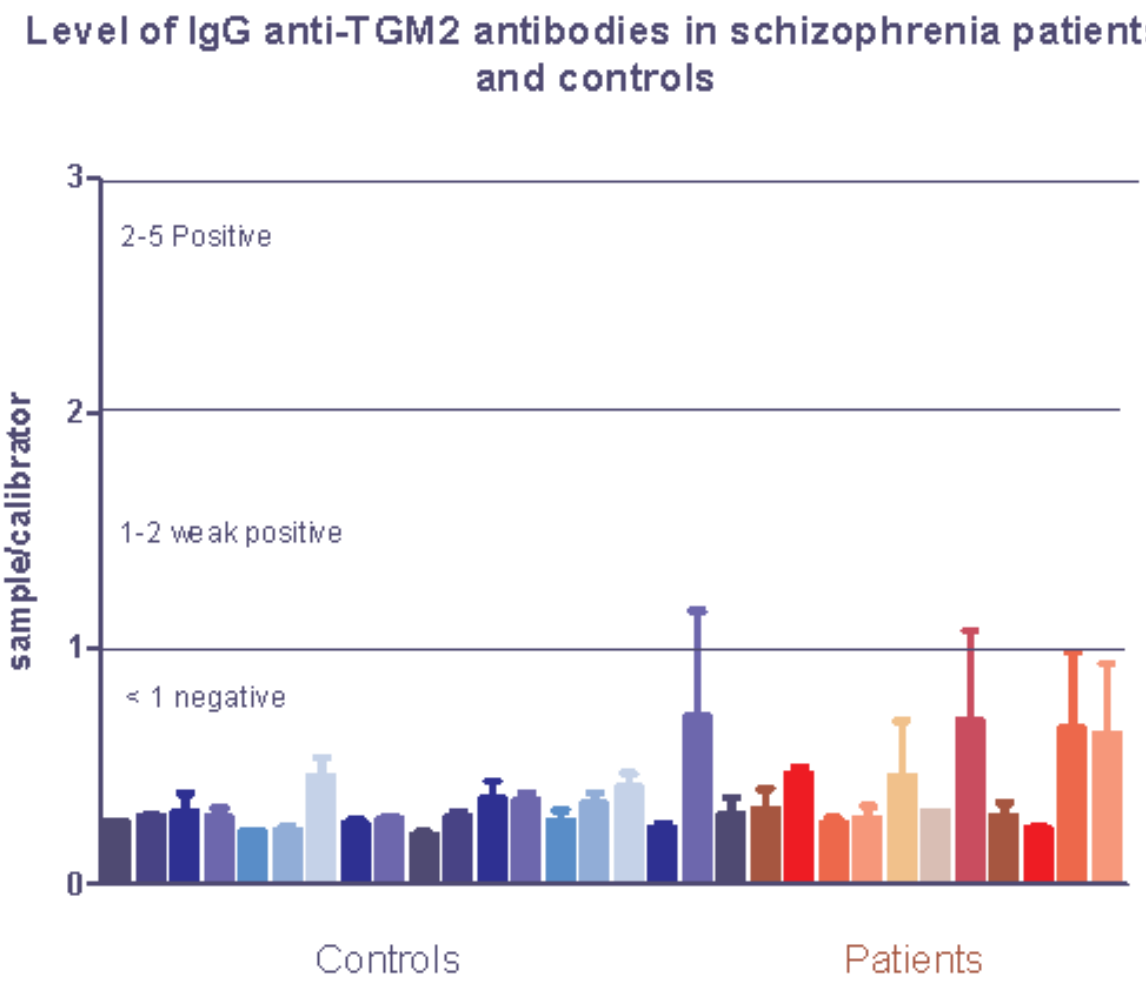


Figure 4-8 Mean levels of IgG anti-TGM2 antibodies in patient and control samples, as determined by ELISA

Table 4-7 Comparison of mean anti-gliadin IgA antibody levels between patient and control groups

Anti-gliadin (IgA) antibody analysis	
Unpaired t-test, two-tailed	
p- value (significant <0.05)	0.601
t	0.529
df	28
Mean ± SEM of control group	0.85 ± 0.23
Mean ± SEM of patient group	1.04 ± 0.25
95% confidence interval	-0.92 to 0.54
F-test, are variances significantly different?	No (p = 0.612)

Table 4-8 Comparison of mean anti-gliadin IgG antibody levels between patient and control groups

Anti-gliadin (IgG) antibody analysis	
Unpaired t test, two-tailed	
p-value (significant <0.05)	0.577
t	0.565
df	28
Mean ± SEM of control group	3.28 ± 0.59
Mean ± SEM of patient group	2.74 ± 0.77
95% confidence interval	-1.43 to 2.52
F-test, are variances significantly different?	No (p = 1.037)

Table 4-9 Comparison of mean anti-GAF-3X IgA antibody levels between patient and control groups

Anti-GAF-3X (IgA) antibody analysis	
Mann Whitney test, two-tailed	
p value (significant <0.05)	0.931
Exact or approximate P value?	Gaussian Approximation
Sum of ranks in control , patient	297 , 168
Mann-Whitney U	102.0
F-test, are variances significantly different?	Yes (p = 0.0001)

Table 4-10 Comparison of mean anti-GAF-3X IgG antibody levels between patient and control groups

Anti-GAF-3X (IgG) antibody analysis	
Mann Whitney test, two-tailed	
p value (significant <0.05)	0.182
Exact or approximate P value?	Gaussian Approximation
Sum of ranks in control , patient	263 , 202
Mann-Whitney U	73.00
F-test, are variances significantly different?	Yes (p = 0.0045)

Table 4-11 Comparison of mean anti-TGM2 IgA antibody levels between patient and control groups

Anti-TGM2 (IgA) antibody analysis	
Mann Whitney test, two-tailed	
p value (significant <0.05)	0.2120
Exact or approximate P value?	Gaussian Approximation
Sum of ranks in control , patient	265 , 200
Mann-Whitney U	75.00
F-test, are variances significantly different?	Yes (p = 0.0024)

Table 4-12 Comparison of mean anti-TGM2 IgG antibody levels between patient and control groups

Anti-TGM2 (IgG) antibody analysis	
Unpaired t test, two-tailed	
p- value (significant <0.05)	0.0702
t	1.883
df	28
Mean ± SEM of control group	0.32 ± 0.026
Mean ± SEM of patient group	0.42 ± 0.053
95% confidence interval	0.42 ± 0.053
F-test, are variances significantly different?	No (p = 0.1163)

4.3.6 Influence of TGM2 genotype on production of antibodies against TGM2 and gliadin

The initial intention of correlating TGM2 genotype with antibody production (against TGM2 and gliadin) was frustrated by two factors. Firstly, a smaller than intended sample group reduced the numbers of samples overall, and secondly, within that sample group, fewer patients exhibited the immune response expected based on previous studies (Reichelt and Landmark, 1995, Samaroo et al., 2009, Dickerson et al., 2010, Jin et al., 2010). The control sample group showed equally few positive results; conforming to the results expected for healthy controls (Jin et al., 2010) but meaning that the analysis could not be performed in this group either. As a result, there were insufficient individuals with raised antibody levels for a meaningful analysis of the relationship between this trait and TGM2 genotype.

4.4 Discussion

4.4.1 Relationship between TGM2 and serum IL-2 levels

The lack of a significant difference in serum IL-2 levels between patient and control groups is consistent with a meta-analysis of previous studies (Potvin et al., 2008). Despite this lack of an overall association of IL-2 with schizophrenia, there was evidence of an association between IL-2 levels and one of the schizophrenia-associated SNPs within the TGM2 gene. SNP rs4811528 showed genotypic association with serum IL-2 levels only in the patient group. As shown in Figure 4-2, presence of the A allele at rs4811528 appears to correlate with elevated IL-2 levels in patients, although the trend is skewed slightly upwards in the heterozygote group, due to a single outlier. Further analysis with binary logistic regression allowed the analysis of the IL-2 association to be adjusted for age and sex. The association between rs4811528 and serum IL-2 levels remained significant following these adjustments.

SNP rs4811528 is intronic and unlikely to play a role as a risk factor in mediating immune function in schizophrenia. The association between rs4811528 and serum IL-2 in schizophrenia suggests that the major allele, rs4811528-A, could tag a functional variant existing within the TGM2 locus. Attempts to search for functional domains adjacent to rs4811528 have, as yet, revealed no such sites; as described in Chapter 3, a nearby coding SNP was investigated, but displayed no polymorphism when tested in a sub-group of our patient samples. It is probable that the disease-relevant site is located elsewhere within the 5' region of the gene.

A concern with analysis of cytokines is that their measurements can be altered by a wide range of confounding factors. Age and sex were corrected for when tested with binary logistic regression, but serum cytokine levels can also be affected by an individual's current health status, including their

medication (Xu et al., 1994, Dohan et al., 1972). While the influence of patients' medication was not corrected for, a previous study using subjects from the same sample bank, showed no difference in serum IL-2 concentrations between patients with and without neuroleptic treatment, increasing confidence that neuroleptic medication has not substantially influenced these IL-2 measurements (Xu et al., 1994).

Following the evidence of association between TGM2 and schizophrenia (Bradford et al., 2009), the apparent connection between rs4811528 and IL-2 levels offers an intriguing insight into the possible role of TGM2 in the pathophysiology of schizophrenia. While further work is needed to confirm if the IL-2 association reflects a change in TGM2 function, and to clarify the mechanism through which TGM2 variation correlates with IL-2 levels, this result raises the possibility that TGM2 variants may confer a risk for schizophrenia, one likely acting via an immune pathway.

4.4.2 Decreased TGM2 expression in cells treated with clozapine

Cells treated with clozapine gave notably different results dependent on the concentration of clozapine used. Cells treated with 1 μg/ml concentration showed a statistically significant down-regulation of TGM2 expression ($p=0.013$), while those treated with 2 μg/ml displayed no significant change in expression ($p=0.880$). A repeat of the cDNA conversion and qRT-PCR stages of the 1 μg/ml experiment yielded a consistent result (TGM2 down regulated, $p=0.003$), confirming that these latter stages were introducing minimal variance and so were unlikely to have substantially skewed results.

The differing results for 1 and 2 μg/ml may represent a concentration-dependent effect. Of the two results the 1 μg/ml experiment yielded the most consistent results, while the standard error

observed for cells cultured with 2 μg/ml was noticeably larger, with distinct variation between the replicate samples of each group.

It is possible that 2 μg/ml treatment constitutes too high a dose, and is exerting a cytotoxic effect that disrupts the regulation of gene expression. Clozapine itself is described as toxic at supratherapeutic concentrations (100 - 300 μM, equivalent to 33 - 98 μg/ml, based on its molecular weight of 326.83). However its intermediary metabolites can accelerate cell death at as little as 1 – 3 μM (Williams et al., 2000). Sub-toxic doses as low as 12.5 μg/ml have also been reported to impact on cell metabolic activity (Heiser et al., 2007), which may be a more likely explanation for the effects observed in these experiments. The 2 μg/ml concentration used in this experiment is equivalent to 6.1 μM, slightly in excess of the upper limit of normal serum levels (~4.6 μM (Williams et al., 2000), but still below the recognized toxic range of 100 – 300 μM. It seems unlikely that the concentration of clozapine used in this study would be toxic, however, for future work it would be interesting to examine the concentration-response effect over a wider range, including the lower limits of therapeutic dosing.

The patent described previously (Patent: WO/2004/053157) reports that TGM2 expression is up-regulated in the anterior cingulate cortex of patients with schizophrenia. As such, it is tempting to consider that clozapine-induced down-regulation of TGM2 expression might reflect a pharmacological role in treatment of the disease, restoring balance to a dysregulated pathway.

The anterior cingulate cortex (ACC), shown in red in Figure 4-9, has received considerable attention in schizophrenia research. In terms of morphology, the ACC shows a decrease in both neuron density and glial cells in patients versus controls (Benes et al., 2001, Stark et al., 2004). Activated by arousal and stress, the ACC also shows high levels of dopamine innervation, the highest, in fact, within the cerebral cortex (Paus, 2001). In schizophrenia, however, this feature is distorted, with

patients showing ~12.5% fewer dopamine D2 receptors than control subjects. Furthermore, the decreased numbers of D2 receptors was found to correlate with patient scores for positive symptoms (Suhara et al., 2002).

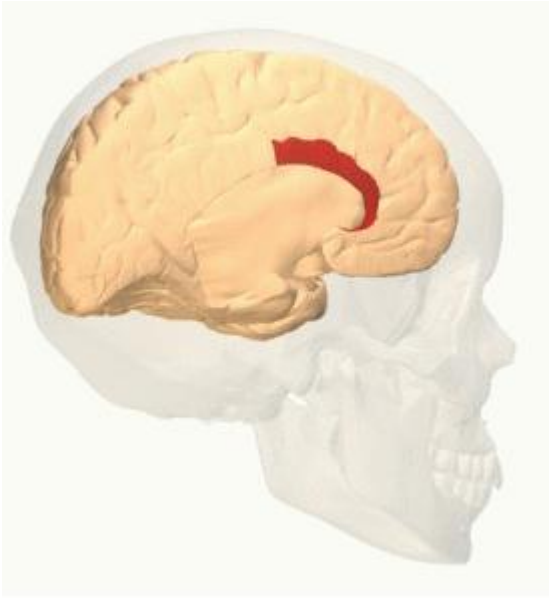


Figure 4-9 Anterior cingulate gyrus (left cerebral hemisphere)[2]

In terms of symptomatology, a review of 49 studies found that hypoactivity of the ACC region was common during scored tasks (Adams and David, 2007). The authors of this review also noted that both hypoactivity of the ACC and task performance were improved in medicated versus non-medicated patients, although it should be noted that while 47 of these studies included medicated patients, only 11 included non-medicated individuals. This restoration of normal activation was

[2] Image derived from Ver 2.0 Ministry of Education, Culture, Sports, and Technology(MEXT) Integreated Database Project, with permission granted under the under the Creative Commons Attribution 2.1 Japan license.

observed for Clozapine in particular, which has been reported to normalize ACC activity levels, in a study where subjects were engaged in an auditory discrimination task (Lahti et al., 2004).

A possible connection between ACC functioning and TGM2, comes from reports that apoptotic regulation may be dysregulated in this region in schizophrenia (Benes et al., 2006, Jarskog et al., 2005). TGM2 is well known as a regulator of apoptosis, capable of both promoting and inhibiting the process (Milakovic et al., 2004). Interestingly, while the trend is for upregulation of pro-apoptotic genes in bipolar disorder, the reverse appears to be true for schizophrenia, despite the loss of neurons observed in both conditions (Benes et al., 2006). In an interesting echo of the results for TGM2 expression in U937 cells, Benes *et al.* reported that the pattern of gene expression was sensitive to the dose of neuroleptics taken by patients, with a higher dose favoring pro-apoptotic gene expression. Although performed using frozen tissue, this study involved a robust two-stage methodology (expression analysis by chip, followed by qRT-PCR of specific genes of interest), making the complex results worthy of consideration.

Despite originating from a patent, and therefore lacking peer review or full disclosure of methodology, the gene expression data available for TGM2, in the context of the ACC, remains interesting, especially given the significance of apoptotic processes in this region. The effect of increased TGM2 expression is difficult to determine, given the protein's paradoxical roles as both a promoter and inhibitor of apoptosis, but it seems probable that this aspect of its activity may explain its relevance to schizophrenia. Among the apoptotic pathway genes reported as downregulated in schizophrenia, was the pro-apoptotic BAX gene (Benes et al., 2006), the protein of which TGM2 is thought to bind, in the process of promoting apoptosis (Ai et al., 2008). In combination with the overall trend for gene expression patterns, which suggest the dominance of a pro-survival pathway in this brain region in schizophrenia, this suggests that TGM2 may not be as

anenhancer of apoptosis, but rather in conferring resistance to it. If this were the case then upregulation of the TGM2 gene would increase the pro-survival bias noted by Benes *et al.*, while treatment with antipsychotics could in turn reduce it.

4.4.3 TGM2 gene expression remains consistent across patients and controls

The expression of TGM2 in cells derived from patient and control blood samples was highly consistent, with no significant differences between the two groups ($p = 0.143$) and low levels of variation between individual samples. While the patient group was small ($n = 11$) and any results should therefore be interpreted with caution, the risk alleles identified within the TGM2 gene were identified as present for several of this group without any obvious effect.

The fact that all patients were medicated is another potentially confounding factor, especially given the suggestion that clozapine might influence TGM2 expression (Section 1.4.2). However, the expression level determined by REST for patients (compared with controls) was 0.669, a non-significant decrease in TGM2 expression. If the down-regulation observed in cells treated with 1 μg/ml clozapine were representative of the *in vivo* effect, then medication would only serve to increase the trend observed for the patient group, not mask it.

4.4.4 No association observed between TGM2 genotype at the rs4811528 locus and TGM2 gene expression

Due to the potential confounding effect introduced by clozapine medication, the correlation of TGM2 expression with TGM2 genotype was assessed in the control group. Unsurprisingly, given

the minimal variation detected in general, there was no evidence of a relationship between these two variables. Altering the grouping structure did not affect the result, which in each case gave a robust p-value > 0.89.

Although the sample group was small, each genotype group was represented, and it seems unlikely that this schizophrenia-associated SNP exerts any direct effect on TGM2 expression. That considered, these results come from an immortalized tumour-derived cell line, while they provide a practical vehicle for initial analysis, it cannot be assumed that they are representative of the response to clozapine *in vivo*. The possibility remains that there is a functional change affecting TGM2 expression, but that it is observable only under certain conditions. For example, if genetic variation within TGM2 involved the creation or deletion of a transcription factor binding site, TGM2 expression could be significantly altered when that particular pathway is activated.

4.4.5 No evidence of increased anti-gliadin or TGM2 antibody levels in schizophrenia patients

Contrary to the positive reports which led to this work on gluten-related antibodies in schizophrenia (Samaroo et al., 2009, Reichelt and Landmark, 1995, Cascella et al., 2009), no evidence was observed of increased antibody levels in cases versus controls. For both groups the incidences of positive test results were as predicted by the manufacturers for any non-coeliac sample. When results were further analyzed to check for sub-diagnostic variation in antibody levels, again, no significant differences were seen between cases and controls.

While this result does not support the hypothesis of increased antibodies in schizophrenia patients, neither does it refute it. Due to a challenging recruitment process, the sample group in this

experiment was small (11 patients), representing only 35% sample power if compared to the study by Cascella *et al* (2009). The finding of increased gluten-related antibodies in schizophrenia is not unanimously supported in the literature, with some studies reporting no significance differences between cases and controls. The disparity in results may be explained by the specific tests used, Peleg *et al*., for example, tested for anti-EMA IgA antibody only, not for gliadin itself, and did so in a relatively small sample of 50 patients (2004).

Recent work suggests a specific epitope within gliadin is responsible for the reaction in schizophrenia (Dickerson et al., 2010, Samaroo et al., 2010), highlighting the sensitivity of results to the form of protein selected; the deamidated gliadin peptide identified for CD, for example, is unlikely to be useful in detecting antibodies in schizophrenia, based on these reports. In evidence perhaps related to the immune features observed during acute psychotic episodes, the stage or subtype of illness may also introduce variation in results. In a well-powered study, Dickerson et al. tested antibody levels in patients with both multi-episode schizophrenia and recent-onset psychosis. Their results showed significant increases in IgG antibodies against gliadin in both groups, but with increased IgA only in the psychosis group (2010). This finding of increased antibodies in a non-schizophrenic psychiatric illness is reminiscent of Dohan *et al*.'s 1972 paper, in which it was shown that increased antibody levels may be related as much to the stress of psychiatric illness and resulting hospitalization, as to the pathology of the illness itself. Despite this, the majority or recent reports, in which testing methods were more consistent, support an increase in gliadin antibodies in schizophrenia (Cascella et al., 2009, Dickerson et al., 2010, Jin et al., 2010, Samaroo et al., 2010). What remains is for the status of these antibodies; as either pathological mechanism or simply as a biomarker, to be clarified.

Due to the lack of patients testing positive for increased antibody levels, certain downstream analyses could not be performed. In particular, it was not possible to analyze the impact of TGM2

genotype on antibody production. Given that the schizophrenia-associated alleles were present and almost no antibody tests were positive, it is tempting to conclude that TGM2 genotype does not influence antibody levels. A larger sample group (with a greater number of positive antibody tests) would be required to be certain of this, but certainly, it makes a direct link unlikely.

The lack of patients testing positive for the anti-gliadin antibody tests also ruled out any comparison of reactions against native gliadin and GAF-3X (coeliac-specific) peptides. Had there been a greater number of samples testing positive for native gliadin antibodies, it would have been interesting to determine whether this was equivalent to the response against GAF-3X, or whether the schizophrenia sample were responding to different peptides than those defined for coeliac disease. The difficulties in sample recruitment within the region (Scottish Highlands) limited the testing of these certain hypotheses, however the methodology has shown to be sound and could easily be applied to a larger sample group in future.

5 Analysis of coeliac-associated genes in schizophrenia

Data presented within this chapter has been accepted for publication.

5.1 Introduction

While the primary genetic influence in coeliac disease, that of HLA serotype, was established in 1974 (Mulder, 1974), this single factor accounts for only 36% of the genetic contribution to the disease (Petronzelli et al., 1997). Presence of the HLA-DQ2 or –DQ8 haplotypes is not just associated with CD, but is essential for the disease to occur; the conformation of these MHCII proteins facilitates their binding to gluten proteins, thereby instigating an immune response against these dietary antigens. Researchers have continued to search for the remaining genetic factors that drive development of the condition. In recent years, two genetic regions were identified that offered both plausible functional roles and evidence of genetic association with disease: the MYO9B gene and the region containing the IL-2 and IL-21 locus (a full review of the evidence for these regions is included in Chapter 1).

This Chapter describes work undertaken to test the hypothesis that the CD-associated loci; HLA-DQ2, MYO9B and IL-2/21, are also associated with schizophrenia. It was considered that if CD and schizophrenia truly share a genetic basis, the clearest evidence of this would be an increased frequency of the HLA- DQ2 variants, which are carried by >90% patients with CD. In addition, two of the most promising candidates for additional genetic factors in CD, the IL-2/21 region and the MYO9B gene, were also assessed, to investigate the possibility of additional/alternative shared genetic factors.

5.1.1 The MYO9B and IL-2/21 genes in schizophrenia

Multiple studies have investigated the involvement of the MYO9B gene in CD (Amundsen et al., Cirillo et al., 2007, Giordano et al., 2006, Hunt et al., 2006, Núñez et al., 2006, Wolters et al., 2007), albeit with different outcomes. More directly relevant to the aims of this study, is the report by Jungerius et al (2008), which described the testing of the SNPs identified in CD, in a schizophrenia patient cohort. This study found that the association of MYO9B with CD was mirrored in schizophrenia ($p = 1.16 \times 10^{-04}$). The most highly associated SNPs from this study were identified and tested in our British sample group, to investigate whether this association could be replicated. The null hypothesis was that these SNPs would be no more common in schizophrenia patients than in control subjects.

As described in Chapter 1, the IL-2 cytokine remains one of the most intensely studied signs of immune dysregulation in schizophrenia, although a firm conclusion regarding the nature of this remains elusive (O'Donnell et al., 1996, Potvin et al., 2008). So it was with interest that reports of association between the IL-2/21 locus and CD were noted (Adamovic et al., 2008, van Heel et al., 2007). Although evidence for this locus was available for CD alone at this stage, it was felt that the result warranted investigation in a schizophrenia sample group, as the region represented a promising target for genetic overlap between CD and schizophrenia.

5.1.2 The association of HLA with schizophrenia

The genes responsible for HLA class II molecules are established as the best-defined genetic contribution to CD, with all sufferers having either HLA-DQ2 or HLA-DQ8 variants. While GWAS have identified the HLA region on chromosome 6 as a candidate locus for schizophrenia

(Shi et al., 2009, Stefansson et al., 2009, Purcell et al., 2009), few allelic associations have yet to be confirmed. Earlier studies in this field were often frustrated by the methodology, and the move from serotyping to PCR-based technologies has improved reproducibility. For most studies, sample sizes were also small (< 100 cases), a likely limiting factor in analysing a complex disease such as schizophrenia.

A negative association was reported for HLA-DQB1*0602 in an African American sample group (Nimgaonkar et al., 1993), and was later replicated in participants of Chinese ethnicity (Nimgaonkar et al., 1995); the association does not, however, appear to hold for Caucasians (Jönsson et al., 1998). Testing of the CD-associated HLA-DQ2 and HLA-DQ8 markers has now been performed in a subset of schizophrenia patients, who had already tested positive for anti-gliadin antibodies. In this group, 38.5% of patients were positive for either DQ2 or DQ8 (Samaroo et al., 2010), falling within the 20-40% range seen in the general population, and well below the rates for CD. Due to its testing only a sub-group of patients, this study had especially limited numbers, including only 13 cases in this analysis. As such, this result cannot easily be generalised to schizophrenia patients, although the result adds weight to the hypothesis that gluten reaction in schizophrenia is not equivalent to gluten response in CD.

The fact that there have been multiple MHC region associations but relatively few distinct loci identified for schizophrenia, reflects the limitations of using GWAS to analyse the HLA region. This area of chromosome 6 is highly polymorphic, exhibiting several thousand variants, often located in close proximity to one another. This close-set variance makes resolution of disease-associated variants difficult, not least because the sheer proximity of the variants can inhibit reliable probe binding in microarray set-ups. Further to this, many HLA-tagging SNPs have only been defined relatively recently, meaning that earlier arrays (such as the Affymetrix® Mapping 500K array) did not include them.

Hypothesis-driven research into HLA variation now tends to utilise PCR-based technology rather than serological techniques. The use of tag-SNP approaches has further helped to minimise the extensive tests otherwise required to define HLA haplotypes (de Bakker et al., 2006, Klitz et al., 2003, Reinton et al., 2006, Monsuur et al., 2008). With a view to adopting such techniques in this study, the various approaches and tag SNPs for DQ2 were assessed on a number of criteria: the extent of testing previously performed, the ethnic group used in development, matching between predicted and obtained allele frequencies, as well as false positive/false negative rates. Following this assessment , we elected to test the frequency of the coeliac-associated HLA-DQ2 variants in schizophrenia, using a tag-SNP genotyping approach and employing SNPs identified by Monsuur *et al.* (2008). This approach requires only four SNPs to determine the presence of the DQ2.2 and DQ2.5 haplotypes, and the authors reported sensitivity of >0.991, specificity >0.996.

This section of work was part of a wider project analysing a variety of HLA types in schizophrenia, however only testing of HLA-DQ 2.2, 2.5 and 4 variants were completed as part of this thesis.

5.2 Methods

5.2.1 Genotyping of the IL-2/IL-21 locus

Following the reports about association between the IL-2/21 locus and CD (Adamovic et al., 2008, van Heel et al., 2007), key SNPs were identified, located on chromosome 4. Initially four SNPs were selected for genotyping; rs13151961, rs13119723, rs6840978 and rs6822844, and Figure 5-1 shows the location of these SNPs in relation to the two genes of interest.

To maximise sample power, a mixed database of samples was used, including 331 patients (221 male and 110 female) aged 38.7±12.0, 120 unrelated controls (56 males and 64 female) aged 42.1±12.8 and 360 first degree relatives of probands (mothers, fathers and full siblings). All samples were drawn from the groups detailed in section 2.1.

Genotyping was performed by two methods: Taqman™ protocol where genotyping reagent kits were available for the locus in question, and RFLP analysis for the RFLP-containing SNPs for which Taqman™ kits were not available. Details of these methodologies can be found in Sections 2.4.2 and 2.4.1, respectively. Table 5.1 lists the specific reaction conditions (RFLP) and kit details (Taqman™) for each SNP.

Four SNPs reported to be associated with CD were initially selected for this study; rs13151961, rs13119723, rs6840978 and rs6822844. Of these four SNPs, only two were tested in the full complement of samples; rs13119723 was discarded because it failed to amplify and its sequence did not map to the genome as expected, rs13151961 mapped and amplified successfully, but was discarded following evidence of no association in the initial samples tested (129 family trios) (p-value = 0.81) and evidence of low heterozygosity. The 2 remaining SNPs, rs6840978 and rs6822844, were genotyped using TaqMan™ real-time PCR assays, as described in Table 5.1. Samples of gel electrophoresis results can be found in Appendix 2.

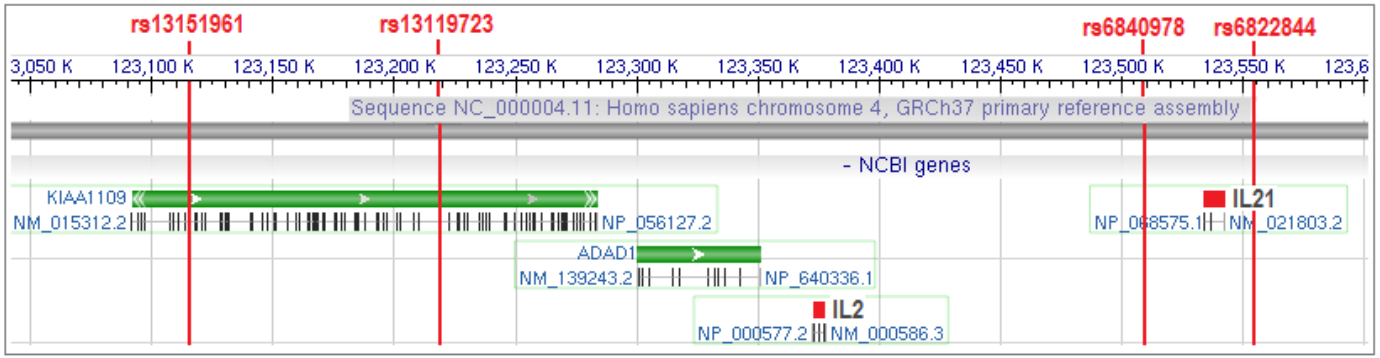


Figure 5-1 Locations of the IL-2/IL-21 -proximal SNPs tested

Table 5-1 RFLP conditions and Taqman™ kit references for genotyping of IL-2/IL-21 SNPs

SNP I.D.	Minor Allele Freq[a]	Method	Kit reference[b] / Primers (5'-3')	R.E.; digest
rs13151961	0.19 (G)	RFLP	**F**:ATTCTTGCATTATGCTAAAACAATACTTCCTT **R**: TAGGAAAATCTTACCAGAAGAGG	AflII; 3 hours at 37 °C
rs13119723	0.15 (G)	RFLP	**F**: AGGACATAGGCACCAGCAAAGAT **R**: ATACGTTTGTTGGCCACGTGAA	DraI; 3 hours at 37 °C
rs6840978	0.18 (T)	Taqman	C__1597502_10	n/a
rs6822844	0.20 (T)	Taqman	C__28983601_10	n/a

[a] As defined by HapMap-CEU data

[b] Kits supplied by Applied Biosystems

The resulting genotyping data were analyzed using the Haploview program (see Section 2.8.2) to check for any inconsistencies with Mendelian inheritance in family samples, and to estimate linkage disequilibrium (LD) between these 2 SNPs. The Hardy-Weinberg equilibrium for the genotypic distributions of all the SNPs detected was also tested by the Haploview program. The UNPHASED program v3.1.3 (see Section 2.8.1) was used to analyse the genotyping data for both allelic and haplotypic associations.

5.2.2 Genotyping of the MYO9B gene

The two tag SNPs reported to be associated with schizophrenia by Jungerius et al. (2008); rs2305767 and rs1545620, were selected for this replication study. In addition, rs8113494, a SNP located 5' to the most strongly associated SNP in Jungerius' report (rs2305767), was also selected. The chromosomal locations of the three SNPs are shown in Figure 5-2.

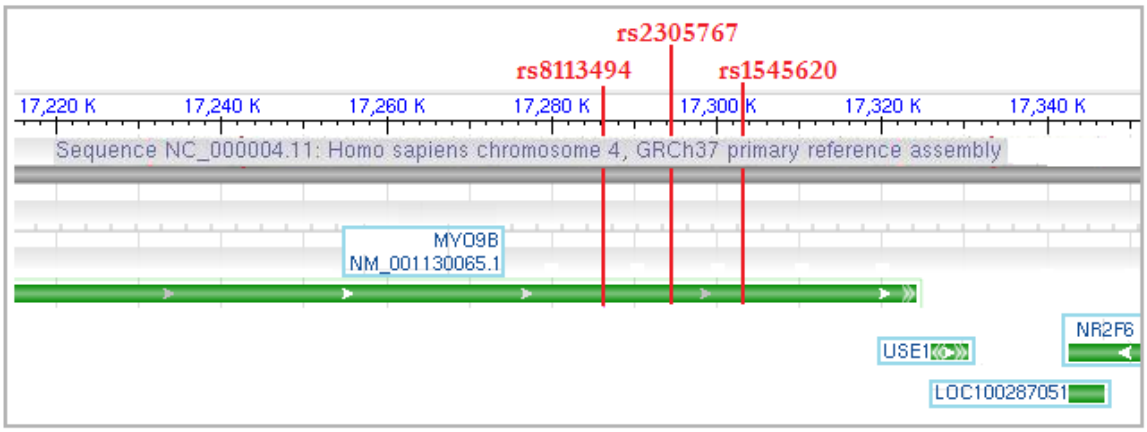


Figure 5-2 Location of SNPs genotyped to assess association between the MYO9B gene and schizophrenia

The sample set was composed of 129 family trios, 86 family duos, and 36 unrelated patients without parents, all drawn from the sample sets described in Section 2.1. All three SNPs were genotyped using Taqman Assays, performed as detailed in Section 2.4.2, using the reagent kits listed in Table 5.2.

Table 5-3 Details of SNPs genotyped within the MYO9B gene

SNP ID	Association in Jungerius	Minor allele frequency [a]	Kit ref.[b]
rs2305767	1.16 E^{-04}	0.39 (C)	C___1654927_20
rs1545620	**2.36 E^{-03}**	0.43 (G)	C___1654896_10
rs8113494	**N/A [C]**	0.22 (T)	C__30348012_10

[a] As defined by HapMap-CEU data [b] Kits supplied by Applied Biosystems

[c] Additional SNP selected for this study

The SPSS sample power 2.0 program was used to calculate power for our own sample, using the minor allele frequency of each tag SNP and a small effect size (OR=1.5) as based on Cohen's convention. In our sample group, power for each tag SNP was >80%. Analysis of genotyping data was performed as for IL-2/IL-21 data, described in Section 5.2.1.

5.2.3 Genotyping of HLA-DQ 2.2 and 2.4 variants

As part of a broader investigation into HLA haplotypes in schizophrenia, an SNP–based tagging approach was applied to genotype HLA-DQ2.2, DQ2.5 and DQ4 variants (de Bakker et al., 2006). While DQ4 was not of great interest in itself, samples positive for this can appear as false positives in the DQ2.2 test, hence the tagging SNP for DQ4 was tested to identify these samples and remove their apparent positive result from the DQ2.2 test. In all cases the SNPs were genotyped using Taqman assays and methodology was as described in Section 2.4.2, details of individual kits are given in Table 5.3.

For this analysis, 129 trios, 86 duos, 128 unrelated patients and 121 unrelated controls were utilised, of which 11 unrelated case and 20 unrelated control samples were newly collected from the New Craigs hospital in Inverness, Scotland. A total of 809 samples in total were available for genetic analysis.

Table 5-2 Details of Tag SNPs genotyped and corresponding HLA haplotypes

SNP ID	Haplotype	DQA1*	DQB1	Minor allele frequency [a]	Kit ref.[b]
rs2395182	HLA-DQ 2.2	0201	0202	0.29 (G)	C__11409965_10
rs7775228				0.10 (C)	C__29315313_10
rs2187668	HLA-DQ 2.5	0501	0201	0.09 (A)	C__58662585_10
rs4713586	HLA-DQ 4	03/0401	0402	0.03 (G)	C__27950246_10

[a] As defined by HapMap-CEU data

[b] Kits supplied by Applied Biosystems

The UNPHASED program was used to predict (from individual allele results) the number of individuals carrying the HLA-DQ2.2 and DQ2.5 variants. Following this, the relative haplotype numbers were analysed using Pearson's chi-squared test, to assess the association between schizophrenia and DQ2.2 and DQ2.5 variants.

5.3 Results

5.3.1 The IL-2/IL-21 association in schizophrenia

Analysis of the two coeliac-associated SNPs revealed no evidence of allelic association between this IL-2/IL-21 locus and schizophrenia. As shown in Table 5-4, allele frequencies were equivelant in cases and controls, (p-values >0.5 for both SNPs). Table 5-5 summarises the results of the haplotype association test. Again, frequencies were equivelant and the resulting p-value ($p = 0.846$) strongly indicates that there is no association between this locus and schizophrenia.

Table 5-3 Test of allelic association between the IL-2/21 locus and schizophrenia

SNP	Allele	Case	Control	Case Freq.	Control Freq.	Odds Ratio	χ^2	95%Low	95%High	p-value
rs6840978	T	107	100	0.214	0.201	1	0.305	1	1	0.581
	C	393	398	0.786	0.799	0.903		0.626	1.3	
rs6822844	T	93	85	0.185	0.17	1	0.413	1	1	0.52
	G	409	415	0.815	0.83	0.883		0.603	1.293	

Table 5-4 Test of haplotypic association between the IL-2/21 locus and schizophrenia

Haplotype	Case	Control	Case Freq.	Control Freq.	Odds Ratio	χ^2	95%Low	95%High	p-value
T-T	92	82	0.182	0.164	1		1	1	
T-G	17	17	0.034	0.034	0.891		0.373	2.13	
C-T	2	3	0.004	0.007	0.531	0.816	0.072	3.906	0.846
C-G	393	399	0.78	0.795	0.862		0.584	1.27	

5.3.2 Replication of the MYO9B association with schizophrenia

Analysis of the previously reported MYO9B SNPs showed no evidence to suggest their association with schizophrenia (Table 5-6). Analysis of an additional SNP (rs8113494), located 5' to the strongest original association signal (Jungerius *et al.* (2008), also showed no significance ($p = 0.903$). In addition, the SNP that came closest to significance in this study (rs154620; $p = 0.103$), was not the most significant locus in the original study by Jungerius *et al.* (2008).

The three-SNP haplotype test gave similar results, with none of the haplotypes showing any association with schizophrenia (Table 5-7). When haplotype analysis was performed using only those SNPs located within the same haplotype block (rs2305767 and rs1545620) (Figure 5-3), the A-A haplotype did initially achieve statistical significance ($p = 0.021$), however this association did not withstand correction by permutation testing (corrected $p = 0.084$, following 10,000 permutations).

Comparison of allele frequencies between the case and control groups showed that the two SNPs tested in both studies showed no significant variation between the two populations, although it should be noted that control subjects were unrelated healthy individuals in Jungerius' study, whereas in our study the control samples included non-transmitted alleles from parents (Figure 5-4).

Table 5-5 Test of allelic association between the MYO9B gene and schizophrenia

SNP	Allele	Transmitted (case)	Un-transmitted (control)	χ^2	p-value
rs8113494	A	329	330	0.015	0.903
	T	161	158		
rs2305767	A	292	288	0.017	0.896
	G	204	206		
rs1545620	A	306	276	2.653	0.103
	C	188	216		

Table 5-6 Test of haplotypic association between the MYO9B gene and schizophrenia

Haplotype	Transmitted (case)	Un-transmitted (control)	χ^2	p-value
3-SNP haplotypes [a]				
AAA	16	8	2.181	0.14
AAC	141	159	1.24	0.265
AGA	175	168	0.099	0.754
TAA	92	66	3.79	0.052
TAC	43	55	1.552	0.213
TGA	26	36	1.478	0.224
2-SNP haplotypes [b]				
AA	107	75	5.292	0.021
AC	185	214	2.582	0.108
GA	201	205	0.065	0.799
GC	4	3	0.115	0.734
Adjusted p-value from 10,000 permutations				**0.084**

[a] SNPs ordered: rs8113494 – rs2305767 – rs1545620

[b] SNPs: rs2305767 – rs1545620

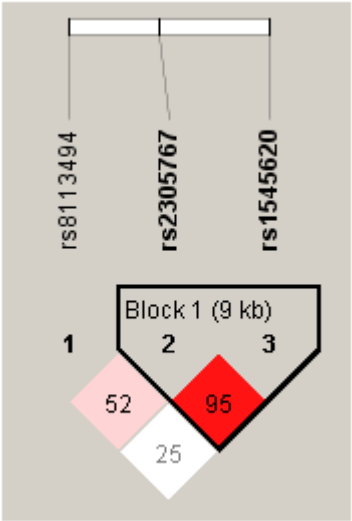


Figure 5-3 Linkage disequilibrium (D') between the MYO9B SNPs analysed.

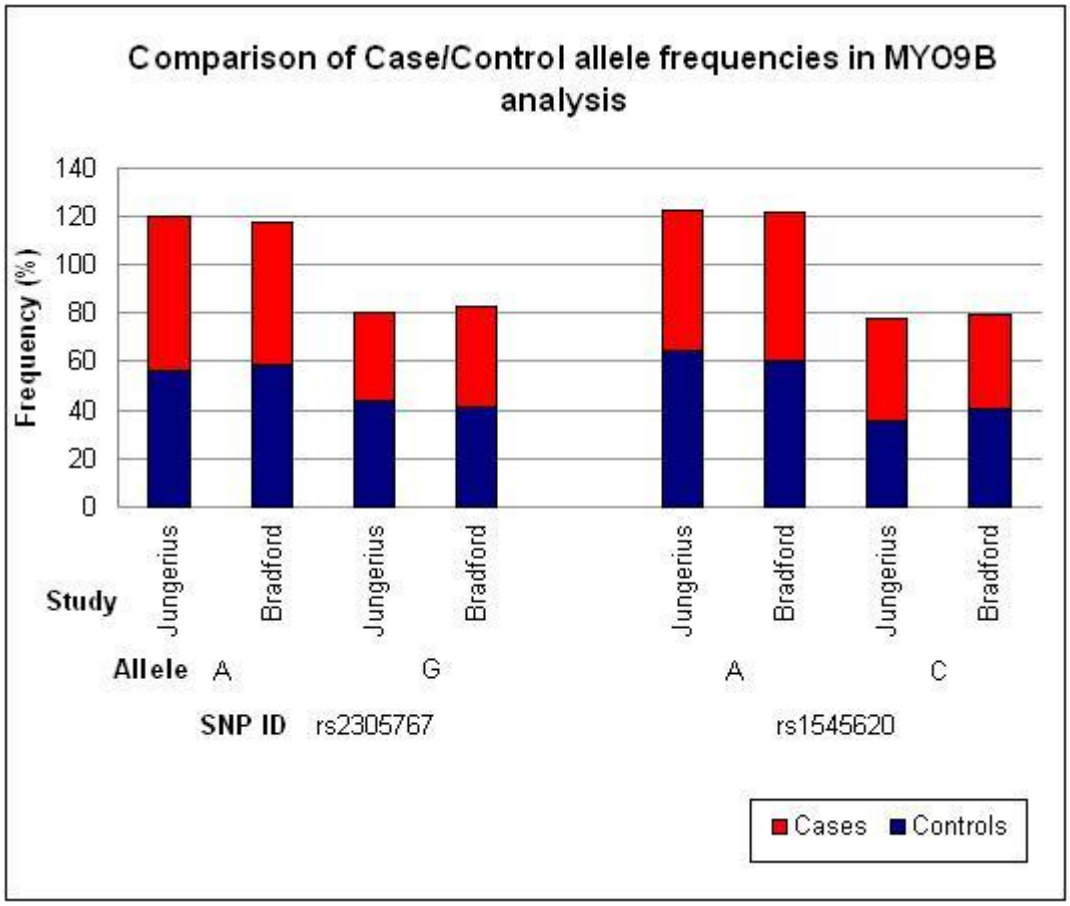


Figure 5-4 Minimal variation is apparent in the case/control allele frequencies across the two studies (Jungerius *et al.* 2008 and the data presented here)

5.3.3 Analysis of the HLA variants in schizophrenia

Chi-squared analysis of the HLA haplotype frequencies (as predicted using the UNPHASED expectation-maximization algorithm) showed no association between schizophrenia and the HLA-DQ2.2 or HLA-DQ2.5 variants (Table 5-8). Figure 5-5 shows the consistency in frequencies of these 2 variants across the patient and control groups, with frequencies of these haplotypes not being significantly different in cases compared to controls.

Table 5-7 X^2 Analysis of HLA-DQ2.2 and –DQ2.5 association with schizophrenia

Haplotype *SNPs*	**DQA1***	**DQB1**	**Haplotype Freq. (N)** ***Case***	***Control***	**$X^{2\,a}$**	**P [b]**	**OR (95% CI)**
DQ2.2 *rs2395182 rs7775228 rs4713586*	0201	0202	59 (650)	82 (686)	2.69	0.1	0.741 (0.521-1.055)
DQ2.5 *rs2187668*	0501	0201	80 (650)	98 (684)	0.95	0.329	0.841 (0.612-1.154)
DQ4 *rs4713586*	03/0401	0402	18(650)	16(686)	0.26	0.61	1.179 (0.592-2.347)

[a] chi-squared result with 1 d.f.

[b] two-tailed p-value.

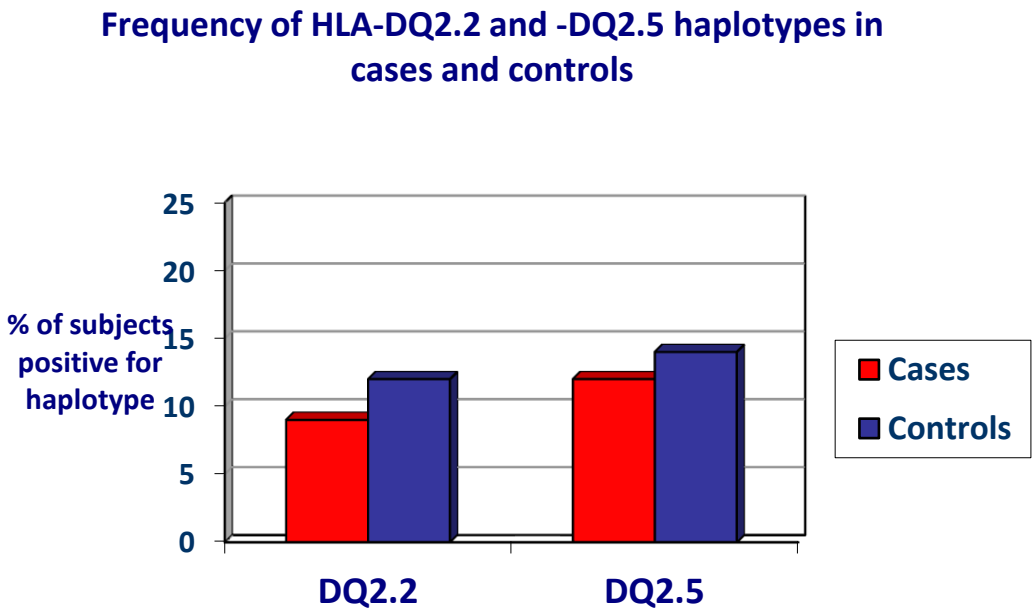


Figure 5-5 Frequency of HLA-DQ2 and -DQ8 haplotypes are closely matched in the patient and control groups.

5.4 Discussion

5.4.1 Coeliac association for the IL-2/21 locus is not replicated in schizophrenia

Other genes in the coeliac-associated regions include KIAA1109, within which both rs13119723 and rs13151961 are located, a gene hypothesized to regulate epithelial growth and tumor development. The gene coding for adenosine deaminase domain containing 1 (ADAD1), also known as Tenr, is located between the two sets of SNPs tested in this study and appears capable of several functions, including acting as an RNA-binding protein within the testis. The IL-2 and IL-21 genes lie closest to the two strongly coeliac-associated SNPs, rs6840978 and rs6822844, with IL-21 being closest, flanked by these two SNPs.

This IL-2/21 region has recently been confirmed as associated with CD in an extensive second-generation GWA study (Dubois et al., 2010). In addition, the region has been associated with ulcerative colitis (Glas et al., 2009), and had a similar (though statistically non-significant) trend identified for its relationship with rheumatoid arthritis (Teixeira et al., 2009). In the light of there being no evidence for the association of the IL-2/21 region with schizophrenia, it may be worth considering the inverse relationship previously identified for rheumatoid arthritis and schizophrenia (Mors et al., 1999), which reinforces the hypothesis that while CD and schizophrenia may share common genetic factors, such factors might not lie along the established pathway currently defined for CD.

As a caveat, it should be noted that although the SNPs analyzed in this study showed the strongest association with CD in previous studies, there remains a possibility that the discounted SNPs (rs13151961 and rs13119723) were independently associated with CD, and that this association signal has been overlooked. Analysis of this region revealed that the four strongly-

associated SNPs were not in LD, so assumptions cannot be made regarding the relationship between these omitted loci and schizophrenia.

5.4.2 The MYO9B association is not replicated in a British schizophrenia sample group

While the MYO9B gene is an attractive candidate for CD, existing data have yet to satisfactorily confirm its involvement in the condition. The majority of studies assessing genetic association of MYO9B with CD have in fact failed to confirm the initial finding (Giordano et al., 2006, Hunt et al., 2006, Núñez et al., 2006),. One of the few positive associations comes from the work described by Wolters *et al.* (2007) who reported that the MYO9B association was detected in their study, but only in patients with refractory coeliac condition. This raises the possibility that the MYO9B association may be genuine, but specific to a subgroup of coeliac sufferers. A meta-analysis performed using all association studies up to year 2007 (Latiano et al., 2007) concluded that MYO9B did not play a significant role in CD, suggesting that the initial finding could represent a type 1 error. In this study, we have similarly failed to confirm the association of MYO9B with schizophrenia. Allele frequencies in our study matched closely with those published in the HapMap database, and again with those observed in the initial study by Jungerius *et al.*(2008), making population-specific differences seem an unlikely explanation. That considered, the fact that the original positive coeliac study and Jungerius' schizophrenia study were both performed in a Dutch population, and furthermore, shared 686 of their controls, could go some way to explaining why these results differ from those produced by subsequent studies. The fact that these two studies share a substantial component of their sample group ought to be considered carefully in the light of recent failures to replicate these initial findings.

Although the 3-SNP system did not show haplotypic association, we observed that, when rs8113494 was removed on the grounds of it being located in a separate haplotype block, the rs2305767(A)-rs1545620(A) haplotype did achieve significance prior to correction by permutation testing. This haplotype contains the A alleles found to be associated with increased risk of a several autoimmune conditions, including coeliac disease (Sánchez et al., 2007). The result suggests that there may be a functional variant at the MYO9B locus, but if so that its effect is small, and requires a larger sample size than our own to detect the association.

MYO9B has been found to be associated with a range of immune conditions besides CD, including rheumatoid arthritis [(Sánchez et al., 2007) and type 1 diabetes (Santiago et al., 2008). Interestingly, unlike CD, both rheumatoid arthritis and type 1 diabetes have an inverse relationship with schizophrenia (Juvonen et al., 2007, Mors et al., 1999), and are actually less common in this patient group. Regarding intestinal disorders, the potential association for MYO9B is not limited to coeliac disease; ulcerative colitis and Crohn's disease have also been reported to be associated with the MYO9B gene (Latiano et al., 2008, van Bodegraven et al., 2006), these conditions do not appear to share the much-documented link seen for CD and schizophrenia.

5.4.3 Coeliac-associated HLA haplotypes show equivalent frequency in schizophrenia patients and controls

No significant differences were observed in the frequencies of HLA-DQ2.2 or HLA-DQ2.5 variants, between the patient and the control group. Furthermore, those differences which were apparent, were fordecreased DQ2.2 and DQ2.5 in the patient group. This result strongly indicates that any shared genetic basis between CD and schizophrenia may be mediated by an alternative pathway, and that schizophrenia is unlikely to share the type of immune response against deamidated gluten-derived antigens that has been characterised for coeliac disease (Ráki et al., 2007, Jin et al., 2010, Dickerson et al., 2010, Samaroo et al., 2010). This also indicates that

any autoimmune mechanisms operating in schizophrenia are of a different nature, perhaps specific to this condition. It is worth noting that a GWA study has recently confirmed that the HLA-DQA*0501 and HLA-DQB*0201 alleles, which encode HLA-DQ2.5 molecules, are associated with a low risk of schizophrenia (Purcell et al., 2009), the DRB1*03-DQA1*05-DQB*02 haplotype was also less frequent in Chinese patients with schizophrenia (Li et al., 2001). This result in no way discredits the existing evidence for the involvement of HLA variants in schizophrenia, but rather highlights the need for a more thorough analysis of this region, in order to define the immune characteristics which are relevant to schizophrenia.

6 General Discussion

6.1 Introduction

Schizophrenia is a severe, usually lifelong, mental illness. With a prevalence of 0.5-1%, it is amongst the top ten causes of disability by cost (Murray and Lopez, 1997), yet it remains a poorly understood disorder for which treatments are available, but unsatisfactory (Kane and Correll, 2010). While it has been established that schizophrenia involves a substantial genetic component, relatively few genes have been confirmed to contribute to the disease; those genes that have been identified only play a minor part in disease development. Essentially a disorder of the brain, there is nonetheless interest in how other processes, especially the immune system, (Knight et al., 2007), might influence schizophrenia.

Amongst the hypotheses linking schizophrenia with other physiological conditions, is the long-standing correlation of the disease with gluten intolerance. Since the 1960s, there have been studies and anecdotal accounts detailing patients whose symptoms improved upon commencement of a gluten-free diet (De Santis et al., 1997). There are also population studies which report increased risk of auto-immune conditions – including coeliac disease – for individuals with schizophrenia. Although a number of researchers have attempted to investigate the gluten/auto-immune component proposed for schizophrenia, these studies have usually focused on symptoms or biomarkers, such as antibody levels. Given the high heritability of schizophrenia (estimated as 85%) - (Cardno et al., 1999)), this study sought to examine this hypothesis at a genetic level, looking for common genetic factors that might explain the link between gluten intolerance and schizophrenia.

The primary approach employed in this study was the testing of candidate genes in family sample groups, assessing transmission of specific alleles to determine the likelihood that a given

gene or region was associated with schizophrenia. Once genetic association had been established, follow-up studies were performed using both family and case/control sample sets, to examine possible functional evidence of association, including gene expression, influence of medication, and the analysis of previously reported antibody levels, in the context of the risk alleles identified by this work. Genes were selected either on the basis of prior association with coeliac disease, or on account of their having a confirmed or potential role in both coeliac and schizophrenia.

Four genomic regions were selected in total: the TGM2 gene, the MYO9B gene, the region between IL-2 and IL-21 genes, and the HLA-DQ locus corresponding to coeliac-associated HLA types. The TGM2 gene was selected because it encodes tissue transglutaminase (or transglutaminase 2), the enzyme responsible for converting gluten to a more immunogenic peptide in coeliac disease. The MYO9B gene was selected based on its initial association with coeliac disease (Monsuur et al., 2005), and a recent report of similar association in schizophrenia(Jungerius et al., 2008), thus representing the only such report (other than our own – (Bradford et al., 2009) of immune-relevant genetic factors associated with schizophrenia. The IL-2/-21 region had been found to be associated with coeliac disease (Adamovic et al., 2008) and was considered to be of additional interest, given the findings of studies concerning IL-2/sIL-2R dysregulation in schizophrenia (O'Donnell et al., 1996). Finally, typing of HLA-tagging SNPs known to capture the HLA-DQ2 variants was performed. These HLA types account for 95% of those seen in patients with coeliac disease and represent the best-characterised genetic factors for the disease (Petronzelli et al., 1997), making them exemplary candidates for the assessment of common genetic factors between coeliac disease and schizophrenia.

6.2 Summary of results

6.2.1 Chapter 3 summary

Chapter three documented the analysis, and resulting association of the TGM2 gene with schizophrenia. Eight SNPs across the gene were genotyped, and four of these achieved statistical significance (global p-value = 0.029 following 10,000 permutations). While a number of 2-SNP haplotypes were associated with schizophrenia, the 8-SNP haplotype analysis showed that only the A-T-A-A-T-G-A-G haplotype was excessively transmitted (corrected p-value = 0.0007) with an estimated relative risk of 2.13.

Hardy-Weinberg equilibrium (HWE) was maintained for all SNPs, except rs4811528. Although deviation from HWE can reflect genotyping errors, the low error rate determined through repeat-calls allows substantial confidence that this is not the case. When genuine, skewed allele frequencies such as this can be reflective of association in themselves(Nielsen et al., 1998); as such this result strengthens the argument for a functional variant in or near to TGM2.

Examination of the schizophrenia-associated 5' region of TGM2 yielded no obvious functional elements. A lack of evolutionary conservation, out with exons, suggested that any relevant mutation(s) might lie within the exons themselves, or result from alteration of regulatory elements in nearby introns. Regulatory elements within the 5' region of TGM2 have been described before, including a retinoid binding site which directed tissue-specific expression (Nagy et al., 1997), and hypermethylation within the region (and resulting decreased expression of TGM2) in glioma cancer (Dyer et al., 2011).

Two transcription factor binding sites were identified within 20kb of the associated SNPs; MRF-2 and MEF-2A. While the roles of these proteins; described as regulation of fat metabolism and muscle gene expression, respectively, are not obvious candidates for a role in schizophrenia, MEF-2A however may warrant further investigation. Two variants within MEF-2A (a Pro-Leu

substitution in exon 8 and a polyglutamine repeat in exon 12) have been associated with Alzheimer's disease, and the MEF-2 group of proteins are described as important regulators of apoptosis, acting as transcription factors for anti-apoptotic genes (González et al., 2007). While no reports have been published describing interaction between MEF-2A and TGM2, the TGM2 gene's own role in apoptosis, makes this binding site an interesting possibility for functional 5' variation.

The link between MEF-2A and Alzheimer's disease is especially interesting in light of the studies reporting TGM2 as a factor in neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis and Huntington's disease (Chun et al., 2001, Citron et al., 2001, Karpuj et al., 2002b, Lesort et al., 2000, Ruan and Johnson, 2007, Singer et al., 2002, Wilhelmus et al., 2009). Current thinking leans towards the ability of TGM2 to facilitate the formation of soluble protein complexes, as opposed to the insoluble aggregates previously thought to be causative in these conditions (Bailey and Johnson, 2005, Konno et al., 2005a, Konno et al., 2005b, Kuemmerle et al., 1999). Reports of increased TGM2 protein and mRNA expression in neurodegenerative conditions add further evidence that this gene may be relevant to such processes (Ruan and Johnson, 2007).

In contrast to the above associations with neurodegeneration, TGM2 is also important in the normal process of neurodevelopment. TGM2 is the key transglutaminase expressed in the CNS, where levels of both its mRNA and protein have been shown to vary in correlation with stages of development in mouse brain (Bailey and Johnson, 2004). The precise mechanism by which TGM2 influences development has not been determined, but it is thought to act through facilitating neurite outgrowth (Bailey and Johnson, 2004, Tucholski et al., 2001), a function also suggested to explain the impact of DISC1 in schizophrenia (Lee et al., 2011, Pletnikov et al., 2008) . These roles confirm the relevance of TGM2 to brain structure, and offer possibilities for its involvement in the dysregulation of such processes in schizophrenia.

This result provided substantial evidence that the TGM2 gene, the protein of which is so central to coeliac pathology, is associated with schizophrenia. Initially this appears to be a significant validation of the hypothesised link between coeliac disease and schizophrenia, and a highly promising candidate for explaining the relationship through common genetic factors for these two conditions. However, while the decision to test this gene was based upon this foundation, as yet no associations or mutations have been identified for TGM2 in coeliac disease itself. To confirm either the association for schizophrenia, or the relevance of TGM2 to its hypothesised relationship with coeliac disease, replication studies are needed for these SNPs in both conditions.

6.2.2 Chapter 4 summary

Chapter four described a series of investigations, designed to uncover evidence of a functional effect that would explain the association of TGM2 with schizophrenia, as described in chapter three.

Correlation of patient status with serum IL-2 levels was not observed in this study, in line with predictions based on a published meta-analysis of previous data (Potvin et al., 2008). When genotype at the associated TGM2 loci were taken into account, significance was achieved for the relationship between rs4811528 and IL-2 levels ($p = 0.022$), significance that was sustained following correction for age and sex ($p = 0.042$). The effect size seen was moderate; a 15% increase in mean serum IL-2 in the AA genotype group compared to the GG genotype group. It remains possible that this baseline difference is exacerbated under specific conditions.

Cytokine levels are notoriously susceptible to influence by environmental factors, such as infection, stress, diet and medication (Dod et al., 2010, Zhang et al., 2009, Xu et al., 1994). These factors are relevant, given the mental illness experienced by the patient group and the

medication prescribed to them. Reassuringly, previous comparisons of neuroleptic-treated and non-medicated patients within this sample group, showed no evidence that medication status influenced IL-2 measurements (Xu et al., 1994).

Interestingly, when the influence of clozapine on TGM2 expression was assessed in cell culture, there was evidence that it altered the gene's expression; with 1μg/ml treatment for 96 hours resulting in downregulation of TGM2 ($p = 0.013$ and $p= 0.003$ in original and repeat experiments respectively). In contrast, this result was not observed for the 2μg/ml treatment ($p = 0.880$). This lack of change at a higher concentration seems unlikely to result from a toxic effect, as the toxic level of clozapine is usually reported to be considerably higher (33-98 μg/ml). That considered, a study of supratherapeutic clozapine concentrations in U937 cells, found that metabolic activity was significantly decreased even at 12.5 μg/ml (Heiser et al., 2007). The repeat of the cDNA conversion and qRT-PCR stages for the 1 μg/ml experiment, confirm that any variation in data are unlikely to stem from errors introduced by these processes, however the inherent variability of cell culture (particularly with an immortalised line such as U937) and limited stability of mRNA can introduce errors of their own (Combes et al., 2009). Although this experiment was designed as a preliminary check, to detect any confounding effects when using samples from patients who had been prescribed this particular, the result is interesting in its own right.

The literature yields only one report regarding TGM2 in schizophrenia, and this is a patent rather than peer-reviewed publication. However, it does specifically mention altered expression of the gene within the anterior cingulate cortex (ACC) region of the brain of schizophrenia patients (Patent: WO/2004/053157). The ACC has been reported as having a number of abnormalities in schizophrenia; including a decrease in glial cells, decreased neuron density,

hypoactivity in response to task performance, and decreased dopamine D2 receptors (Benes et al., 2001, Suhara et al., 2002, Stark et al., 2004, Adams and David, 2007).

TGM2 carries out a variety of roles, but in the context of ACC disruption, it is its function as a regulator of apoptosis that may of greatest interest. Several lines of evidence suggest that apoptotic regulation may be dysregulated in the ACC in schizophrenia. In contrast to what might be expected given the reports of decreased grey matter in schizophrenia (Nenadic et al., 2010), the balance of pro- and anti-apoptotic genes expressed in the ACC of patients with schizophrenia, appears skewed towards a pro-survival pathway (Benes et al., 2006, Jarskog et al., 2005). At protein level, there is evidence of a vulnerability to apoptosis in the temporal cortex; the balance between the membrane-bound proteins Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic), being skewed so that Bax predominates. However, this is contrasted by unchanged (or even slightly decreased) levels of Caspase-3, an essential effector caspase which acts in the final stages of apoptosis regulation (Jarskog et al., 2004). DNA fragmentation (an initial step in the apoptotic pathway) is also decreased in schizophrenia, in contrast to findings for bipolar disorder (Benes et al., 2003, Buttner et al., 2007). What is less clear, is whether decreased apoptosis represents a primary dysfunction of the normal apoptotic response, or whether it a compensatory mechanism. Benes et al. (2003) suggested such a compensatory response could result from excess apoptosis in the early stages of the disease, a factor which could co-occur with the initial development of symptoms in early adulthood.

The description of upregulated TGM2, taken in the context on increased pro-survival gene expression, may indicate that the protein influences schizophrenia by acting in its capacity as an inhibitor of apoptosis. TGM2 is capable of facilitating apoptosis through the mitochondrial pathway (Milakovic et al., 2004), by interacting with the previously mentioned Bax protein (Ai et al., 2008), however this would be unlikely to have much impact if the downstream effects of Bax are blocked, as is suggested by the lack of increase in Caspase-3.

With regard to the effect of clozapine on TGM2 expression in U937 cells, the decrease in expression could reflect a process occurring in vivo, where neuroleptic drugs restore aberrant expression levels.

When TGM2 gene expression was compared in patients and controls, there was no significant variation observed ($p = 0.143$), in fact the measurements across both subjects and replicates were striking in their consistency. All patients were medicated with clozapine, which in light of the previous result, warranted consideration. Reassuringly, the non-significant trend was for slightly decreased TGM2 expression in patients compared to controls, so the influence of medication would be to enhance rather than mask any difference between the groups. While it cannot support the possibility of altered TGM2 expression in the brain, this result in PBMCs does not necessarily detract from it either.

The hypothesis that variation within the TGM2 gene might influence its expression was tested by dividing the control group according to genotype at the rs4811528 locus. The result confirmed no association between these factors ($p = 0.982$). This result is consistent with the overall stability observed for TGM2 expression, however it is valid only for PBMCs, and under these conditions. The possibility that TGM2 expression is altered under specific circumstances, perhaps by alteration of transcription factor binding sites, remains.

6.2.3 Chapter 5 summary

Chapter five describes the identification of coeliac-associated genes, and the replication of their analysis in our cohort of schizophrenia patients and controls. The first target was a region of chromosome four, located between the IL-2 and IL-21 genes. IL-2 has previously been a focus for researchers investigating immune system abnormalities in schizophrenia (Bessler et al., 1995, O'Donnell et al., 1996) and this genetic region had subsequently been found to by GWAS and

familial association studies to show significance for coeliac disease (Adamovic et al., 2008, van Heel et al., 2007).

Of the four SNPs selected (based on the previous study), two were discarded after a pilot period, due to low heterozygosity, failure for the sequence to map to the genome, and non-significance. Of the remaining two (originally the most statistically significant), neither showed association with schizophrenia ($p = 0.581$ and 0.52). The conclusion at the end of this experiment was that this gene region was unlikely to harbour any schizophrenia-relevant variants, and that the pathway through which these loci influence coeliac disease is not common to the two conditions.

Jungerius *et a.l* (2008) reported the association of the MYO9B gene with schizophrenia, a study initiated by prior association of this gene with coeliac disease (Monsuur et al., 2005). Although a mechanism for MYO9B was not confirmed for either disease, it was thought that MYO9B's role in actin remodelling of epithelial enterocytes might be relevant, with mutations in the gene leading to the compromise of intestinal permeability. We attempted to replicate the MYO9B finding in our own cohort, but found no evidence of allelic association as reported by Jungerius *et a.l* (2008). Only when the SNPs were divided according to haplotype block, was there any evidence of association; the A-A haplotype of rs2305767 and rs1545620 was significant ($p = 0.021$) but this significance did not withstand permutation testing (corrected $p = 0.084$). It is possible that these null findings represents a type 2 error, as our own sample group was smaller than that used in the original analysis; for the effect size observed by Jungerius *et al*, our sample group afforded 80% power to detect the association. It seems likely that if the original association is legitimate, that its actual effect size is smaller than anticipated.

Aside from the lack of association observed in this study, there may be other reasons to doubt the MYO9B association with schizophrenia, not least the fact that the original association with coeliac disease has itself been poorly replicated. Meta-analysis of studies published prior to

December 2006 found no evidence for association of any of the reported MYO9B loci with schizophrenia (Latiano et al., 2007), a result which calls into question the logic behind Jungerius *et al*'s own study. In addition, the original studies which found association of MYO9B with both schizophrenia and coeliac disease, shared a substantial proportion of their control groups, a fact which could explain why these data differ from the majority of subsequent reports.

The last analysis reported in this chapter, is of the HLA-DQ2.2 and DQ2.5 variants in schizophrenia. These variants are present in ~95% of patients with coeliac disease, and along with DQ8 (responsible for the remaining 5%), account for the largest identified genetic factor in the disorder to date. If schizophrenia was to be influenced by factors stemming from the classical coeliac pathway, it would be strongly expected that these haplotypes would be present at increased rates in the schizophrenia cohort. In our analysis of 650 patients and 686 controls however, this was not the case; HLA-DQ2.2 and –DQ2.5 variants were no more common in patients than controls ($p = 0.1$ and 0.329 respectively), in fact both variants were slightly less frequent in the patient group. This result offers convincing evidence that schizophrenia follows a distinct aetiological path, and though it may overlap with coeliac disease in some symptoms, it does not share the HLA-DQ2-mediated form of gluten response.

Consistent with this finding, is the GWA study performed by the International Schizophrenia Consortium (Purcell et al., 2009), which found that DQ2.5 alleles were actually associated with a decreased risk of schizophrenia. The significance of the HLA region, located on the short arm of chromosome 6, has been reported by other GWAS (Stefansson et al., 2009, Shi et al., 2009) and our negative finding re-affirms the importance of performing more detailed analysis of this region, in order to define the specific alleles or haplotypes which are relevant in schizophrenia.

6.2.4 Limitations and future research

As with any project, a number of factors limited the research that was performed and, by association, the strength of the conclusions to be drawn. These factors are discussed below.

The association of the TGM2 gene with schizophrenia constituted an exciting finding. Confirmation of the phenomenon, however, requires replication in additional sample groups. TGM2 has not been identified by GWAS in patients with schizophrenia, but such a result may be explained by the stringency with which GWAS data must be treated. While such stringency is undoubtedly necessary, the process can lead to the loss of significance for variants exerting a relatively small effect.

In the absence of an additional population in which to replicate the TGM2 finding, functional studies were designed to investigate the potential mechanisms behind the association, thereby clarifying both the validity and relevance of TGM2 in schizophrenia. These functional studies required mRNA and serum samples, in addition to DNA. These requirements meant that a new sample cohort needed to be recruited, but unfortunately, the collection of samples proved more challenging than expected. The combination of a relatively small geographical area, combined with seriously affected patients (many of whom were unsuitable for approach or were not interested in participating), meant that recruitment proceeded slowly, and could not be completed within the time period allowed for this project. As a result, only a small number of patient samples were available for analysis, limiting what could be assessed and indeed, what conclusions could be drawn from the findings. In particular, analysis of the relationship between TGM2 genotype, and antibody production, was not possible.

Sample size was, to a lesser extent, also an issue for some of the association studies performed, particularly the MYO9B replication. While the sample group available for this work represented many years of recruitment efforts and included a substantial number of family groups, genetic

analysis increasingly requires sample numbers in their thousands, not hundreds. This is especially true for multifactorial conditions such as schizophrenia, where individual variants are likely to exert only a small effect on disease risk, thereby requiring greater sample power for reliable detection. The approach used throughout this project, that of candidate gene analysis, minimized the impact of sample size, however the possibility of type 2 error cannot be entirely ruled out.

Gene expression work formed a logical next step following the association of TGM2 with schizophrenia, particularly given the 5' location of the associated SNPs. The consistency of replicates performed for subjects' samples was excellent, however the same consistency was not observed across the samples derived from cell culture. Repetition of the cDNA conversion and amplification stages yielded equivalent results, suggesting that the source of variability lay in the cell culture process rather than the expression analysis.

The impact of gene expression analyses was also limited by the samples used. The PBMCs obtained from participants provided mRNA, whilst requiring only a minimally invasive procedure. These samples also provided genotyping material, and the plasma needed for performing antibody analyses. Lastly, these cells were considered suitable for investigating the influence of TGM2 expression on gluten interaction in the peripheral system. Ideally, however, the analysis of gene expression would be performed in post-mortem tissue samples from the brain, although such sampling techniques carry their own frustrations; limited numbers and the immediacy with which mRNA can be extracted, in particular.

The analysis of HLA-DQ2 variants provided strong evidence that schizophrenia and coeliac disease do not stem from the same immune mechanism. The HLA region of chromosome 6 remains a prime target for research into schizophrenia, and ideally this analysis would be

extended to look at additional HLA variants, which might represent risk factors specific to schizophrenia.

6.3 Concluding remarks

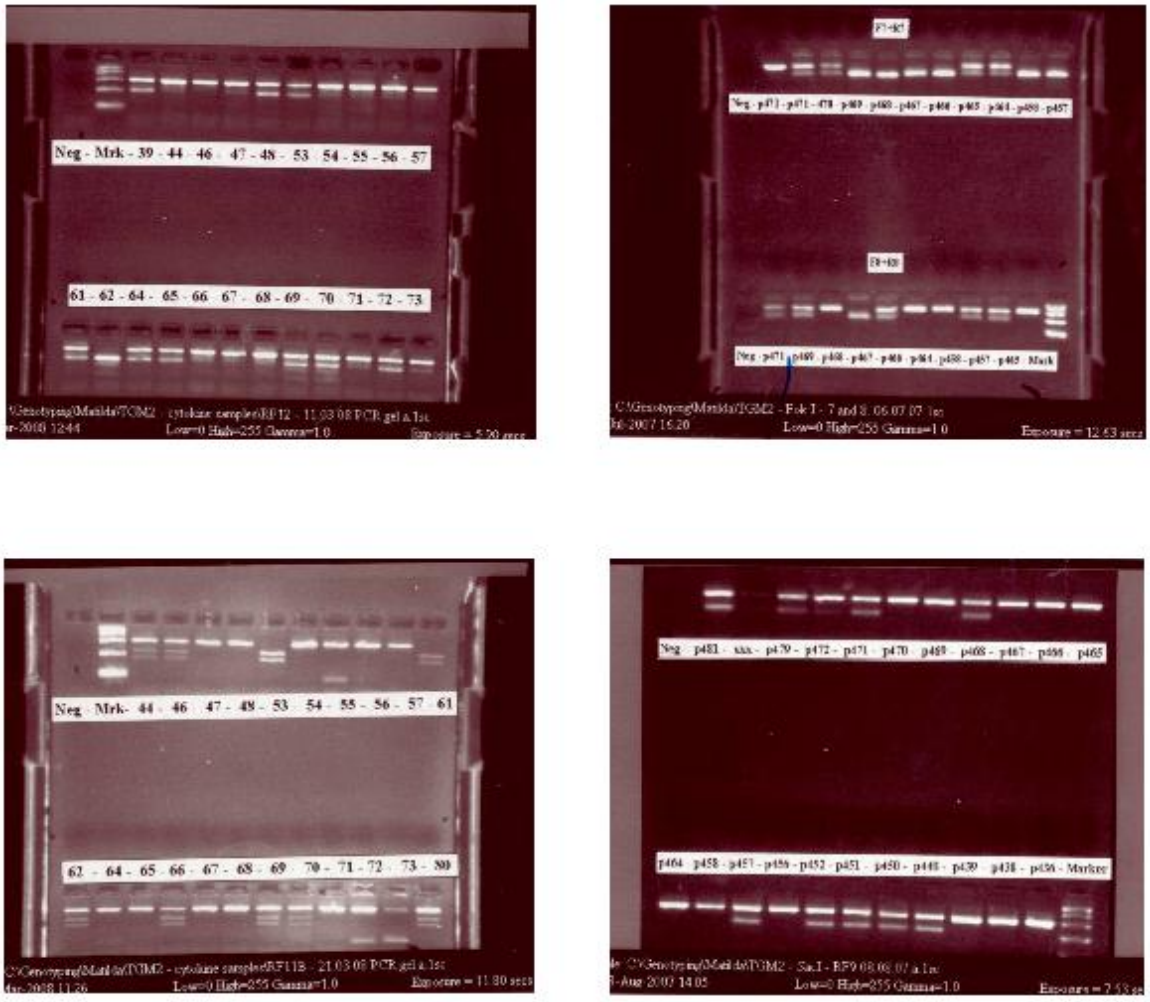
This project aimed to clarify the relationship between the autoimmune condition of coeliac disease, and the psychiatric illness, schizophrenia. The initial hypothesis was that shared genetic mutations might explain their co-occurrence, and that identifying these mutations would shed light on the pathology of schizophrenia. During the course of this research, the majority of data have confirmed the null hypothesis, with key genetic factors defined for coeliac disease being no more common in schizophrenia patients than in control subjects. The lack of association identified for MYO9B, the IL-2/-21 region, and most importantly, HLA-DQ2 haplotypes, provides strong evidence that the immuno-genetic pathways identified for coeliac disease are not shared with schizophrenia.

While the studies described here are not sufficient for any firm conclusions regarding the prevalence of anti-gliadin or anti-TGM2 antibodies in schizophrenia, the lower frequency of HLA-DQ2 variants in schizophrenia suggests that gluten reactivity and the resulting autoimmune response, as seen in coeliac disease, are highly unlikely to be relevant processes in the illness. If the prevalence of antibodies against gluten is increased in schizophrenia, as recently reported in other populations (Dickerson et al., 2010, Jin et al., 2010, Cascella et al., 2009), then the epitopes bound and the resulting pathways of immune response are likely to be distinct. The possibility of gluten reactivity as a risk factor has not been ruled out, but any model of this is likely specific to schizophrenia itself.

This study did detect association between TGM2 and schizophrenia, thereby defining a novel candidate gene for schizophrenia. However, this association has not been reported in studies of coeliac disease. While the antibodies directed against TGM2 are undoubtedly relevant to the process of coeliac disease, there is no evidence that a mutation within this gene influences their production. As such, TGM2 does not provide a clear link between the two conditions, but instead offers an intriguing option for research into other processes within schizophrenia. It seems likely that that the mechanism of TGM2's involvement with schizophrenia, lies in its neurological activity, not in its modification of gluten proteins within the gut. Neurologically, there is evidence that the dysregulation of apoptosis is involved in schizophrenia, particularly within the anterior cingulate. TGM2's role as a regulator of apoptosis, makes this a suitable line of enquiry for future research into its involvement of schizophrenia.

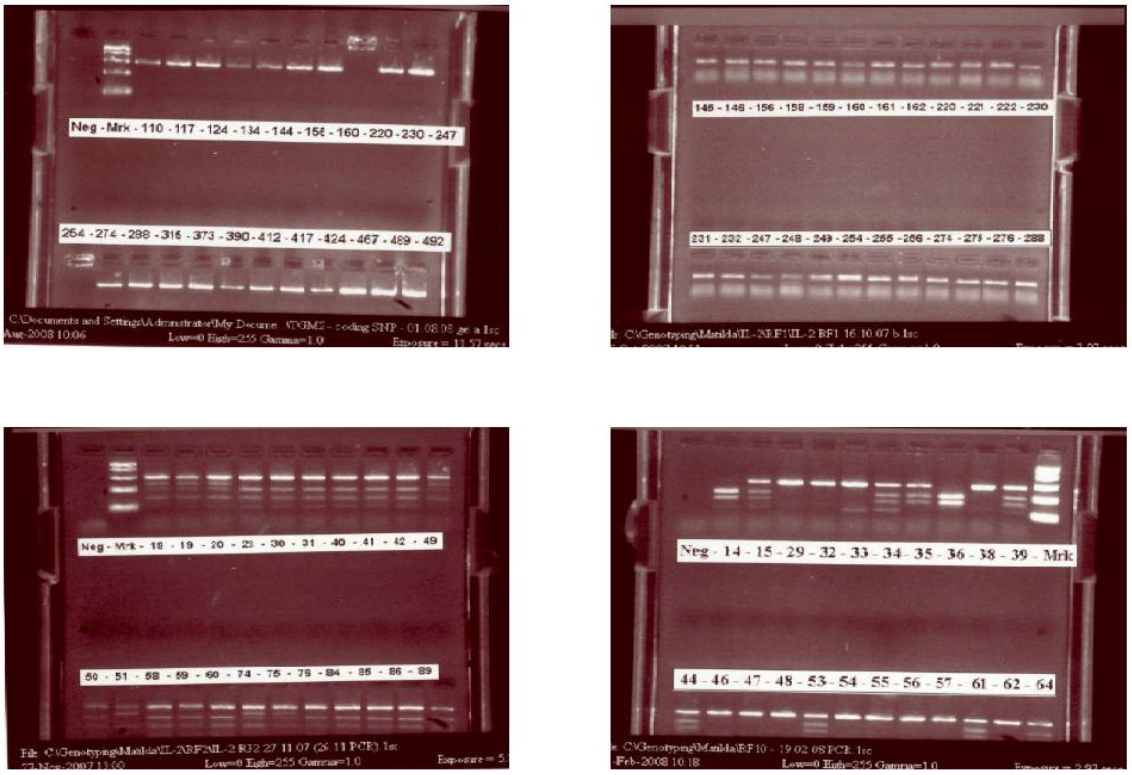
Appendices

Appendix 1 - Sample gels from TGM2 genotyping



Appendix 1 Examples of gel electrophoresis from genotyping of TGM2 SNPs; rs2720785, rs2284879, rs2268909 and rs17789158.

Appendix 2 – Sample gels from IL-2/21 region genotyping



Appendix 2 Examples of gel electrophoresis from genotyping of SNPs within the IL-2/21 region; rs1315961, rs13119723, rs6840978, rs6822844.

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2005. **SPSS for Windows, Rel. 14.0. 2005. Chicago: SPSS Inc.**

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