UNIVERSITY OF NEWCASTLE

The Complex Genetics of Multiple Sclerosis

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BSc (Biotech) (Hons)

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Declaration

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968.

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I hereby certify that this thesis is submitted in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author; and endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications

Mathew B. Cox Date

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Copyright statement

Additional publications which have relevance to the thesis

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Table of Contents

Abstract

Multiple sclerosis (MS) is an autoimmune disorder directed against the central nervous system (CNS). While it is known that lymphocytes can cross the blood brain barrier from the periphery, resulting in inflammatory lesions in the brain and spinal cord, the underlying aetiology of MS remains unknown. Current evidence suggests that the risk of developing MS is a result of both genetic and environmental factors. At least 57 genetic loci have been confirmed to be associated with MS, with DRB1*1501 showing the strongest effect. Alone these associated genes do not explain all of the predicted genetic contribution that underlies MS. Environmental factors, including low levels of vitamin D and smoking, have also been associated with an increased risk of developing MS, and together with genetic risk factors are thought to contribute significantly to the likelihood of developing disease.

The aim of this study was to investigate variation in the transcriptome and genome of MS patients, to identify genes, and pathways that interact with environmental factors that could potentially explain the risk of developing MS.

In this thesis, several levels of genetic investigation were undertaken which were then correlated with potential factors that interact with the environment. Initially a whole-genome gene expression study comparing MS cases against healthy controls was undertaken which revealed a predominance of genes involved in the immune system. Interestingly, four of the dysregulated genes form part of the plasminogen activation pathway, including *MMP9*, which is thought to be involved in the break-down of the blood brain barrier.

To investigate potential causes of this dysregulation in the plasminogen activation pathway, I performed a candidate gene SNP association study, investigating 17 common variants within these four genes, in over 4500 samples. There was no association of any variant investigated and MS risk, nor did genotype correlate with gene expression.

The role of miRNA and its association with gene expression in MS was undertaken where I identified an expression profile, unique to MS compared to healthy controls. Two of the miRNAs, miR-17 and miR-20a which were significantly under-expressed in MS were further investigated, and found to target genes involved in T cell activation. It appears from this study that the dysregulation of miRNAs observed in this study may result in increased T cell activation, as seen in MS.

Finally, a candidate gene SNP association study was performed to investigate two common variants in the vitamin D receptor gene (*VDR*). We identified some evidence of association of one variant in VDR with increased risk of MS. A second variant in VDR in combination with DRB1*1501, revealed a non-significant trend for an increased risk of MS.

The results presented in this thesis highlight the autoimmune nature of MS. Genes involved in the immune system are dysregulated in MS patients, and miRNAs that are able to regulate T cell activation genes are also dysregulated. Interestingly, we identified altered gene expression which may play a role in the breakdown of the blood brain barrier. We also identified a potential interaction between genetic factors associated with increased risk of MS.

Chapter One – Introduction: The Complex Genetics of Multiple Sclerosis

Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disorder that affects the central nervous system (CNS), resulting in inflammatory lesions within the brain and spinal cord. The aetiology underlying the development of MS remains unknown. However we do know that the disease acts through an aberrant immune response directed against the CNS. This results in the loss of nerve signal conduction, leading to a vast array of symptoms, ranging from fatigue, cognitive issues, spasticity, ataxia, sensory disturbances, neuropathic pain, bowel and bladder issues, visual disturbances, and depression. MS onset often occurs during the third or fourth decade of the patients' life, and is a lifelong disease. Females are affected more than males, with a ratio of 3.2:1, and it appears that this ratio is increasing (Greer and McCombe, 2011, Sadovnick, 2009).

Prevalence

The prevalence of the disease is approximately one in one thousand in populations of European descent, however this is lower in populations of Asian or African descent, suggesting a genetic component to the disease. MS is common in northern European countries, America and Australasia, but is less common in Africa, South America, India and the Orient (Compston, 1999). The prevalence rate in England has been estimated at 112 per 100,000 (Pudliatti et al., 2002), while in 1996 the prevalence in Newcastle, Australia was 59.1 per 100,000 (Barnett et al., 2003).

The prevalence of MS in Australia has also been shown to increase as distance from the equator increases, with the prevalence in 1981 ranging from 11.8 per 100,000 in Queensland, 36.6 per 100,000 in NSW and 75.6 per 100,000 in Tasmania (McLeod, 1997). This latitude gradient was confirmed in a recent study investigating first demyelination event in patients in Australia (Taylor et al., 2010). A recent meta-analysis confirmed the presence of a latitude gradient, with greater prevalence at higher latitudes in populations of European descent (Simpson et al., 2011). The latitude gradient has been confirmed through other locations world-wide such as England (Ramagopalan et al., 2011), South America (Risco et al., 2011), Scotland (Handel et al., 2011a), Sweden (Ahlgren et al., 2011) and The United States (Noonan et al., 2010). There have however been reports that the latitude gradient has diminished over time. An investigation of the incidence of MS across two time periods, in American NHS nurses born between 1920 to 1946, and 1947 to 1964, identified a latitude gradient only in the earlier cohort of nurses, which was not present in the later study (Hernan et al., 1999). A similar study

in American military personnel with MS identified a latitude gradient in MS incidence in US veterans of World War II (7/12/1944 to 31/12/1946) and the Korea conflict (27/6/1950 to 31/1/1955) (Wallin et al., 2004). However when investigated in a later cohort, of Vietnam (5/8/1964 to7/5/1975) and the Gulf War (2/8/1990 to 30/9/2003) veterans the strength of the association between latitude and incidence was decreased.

Subtypes

MS is subdivided into four subtypes, based on clinical features. Relapsing-remitting (RR) MS is the most common subtype, accounting for approximately 85% of cases. RRMS is characterised by relapses of neurological symptoms that may last for days to months, followed by a lessening of symptoms, and sometimes complete recovery. Relapse rate varies between patients, with some patients suffering multiple relapses per year, while other patients may remain relapse free for many years. After 25 years the majority of RRMS patients will have entered into a progressively worsening disease course, termed secondary progressive (SP) MS (Trapp and Nave, 2008). The SPMS phase is marked by progressive increase in neurological disability, unassociated with relapses.

Primary progressive (PP) MS is characterised by a gradual increase in the severity of the disease without periods of remission and accounts for only 15% of cases and the female:male ratio is 1:1 (Bove et al., 2012). Progressive relapsing (PR) MS is the least common form and is characterised by a progressive worsening of disease from onset with clear acute relapses, but without significant improvement. It remains unknown whether the subclasses represent subtle differences in the manifestation of a single disease, or if they are independent diseases with similar features.

Pathology

MS is characterised by inflammatory lesion formation within both the grey and white matter of the CNS. It has been proposed that the breakdown of the blood-brain barrier (BBB) allows auto-reactive lymphocyte infiltration into the CNS, resulting in inflammation, demyelination, axonal damage, oligodendrocyte loss and gliosis (Compston and Coles, 2008).

There are two main neurological features of multiple sclerosis: relapses and progressive neurological degeneration. Relapses represent the clinical manifestation of inflammatory events. It is thought that only a minority of inflammatory lesions result in clinical symptoms, as

only a minority of these lesions occur in a clinically eloquent pathway such as the optic nerve or the spinal cord (Trapp and Nave, 2008). The progressive phase of the disease may occur due to the accumulation of axonal loss and neuronal damage exceeding the brain's capacity to compensate for neuronal loss resulting in permanent neurological symptoms (Trapp and Nave, 2008, Dutta and Trapp, 2011). Evidence suggests though that axonal loss and neuronal damage may occur before clinical manifestations of the disease are present, with signs of degeneration, such as cognition and memory decline, occurring early in the disease course (Hoffmann et al., 2007, Wu and Alvarez, 2011).

Axons are surrounded by a multilayered lipid rich membrane, the myelin sheath, which allows rapid signal transduction (Kotter et al., 2011). Demyelination in MS occurs through both physical (phagocytosis) and chemical (nitric oxide and enzymatic) destruction, mediated by macrophages (Moore and Esiri, 2011). The demyelination results in reduction in the action potential efficiency for nerve signal transduction, and the subsequent clinical manifestations of the disease. Demyelination may result in axonal damage and transection, where there is complete loss of the axonal function. This inflammatory lesion formation in the white matter of the brain is the hallmark of MS pathology, and is a diagnostic feature on MRI. Cortical demyelination has recently been identified as playing an important role in the pathology of MS. Cortical lesions differ from white matter lesions due to the smaller number of inflammatory cell infiltrates (Bo et al., 2003a), lack of BBB breakdown and no oedema. Cortical demyelination often occurs in a much more structured form compared to white matter lesions; demyelination advances in a wave from the subpial region down to the 4^{th} layer where it usually stalls, and may extend in a ribbon structure along many gyri (Bo et al., 2003b). Cortical demyelination may also occur as a focal lesion without extending to the surface or white matter, or may extend across both the grey and white matter (Bo et al., 2003b). Due to the lack of inflammatory processes and different cellular densities, cortical demyelination is difficult to detect using current MRI methods (Kidd et al., 1999). In autopsy tissue, white matter demyelination is visible due to the destruction of the myelin, hence changing the colour of the tissue; cortical demyelination does not show such visible change and is only detectable using specific staining techniques. The extent and effect of cortical demyelination is still largely unknown. Up to 27% of the cortex has been found to be demyelinated in some studies.

Oligodendrocytes are responsible for the remyelination of axons, resulting in the return of the neuronal function, and provide essential trophic support for axons (Trapp and Nave, 2008). Oligodendrocyte loss is a common feature present in MS lesions although the cause of this remains unknown (Henderson et al., 2009). Oligodendrocyte apoptosis is thought to occur in

early lesions, rather than chronic active lesions (Breij et al., 2008). The capacity for remyelination decreases throughout the disease, until remyelination is no longer possible, due to oligodendrocyte damage or loss. Oligodendrocyte precursor cells are immature cells which can be recruited to the inflammatory lesion, to mature and replace damaged oligodendrocyte. The maturation has been found to be impaired in MS, resulting in the loss of the remyelination capacity and therefore leading to unrepaired neurodegeneration.

Another diagnostic tool is the lumbar puncture, confirming inflammation isolated in the CSF through positive oligoclonal bands (OCBs). OCB present in the cerebrospinal fluid (CSF) are a product of intrathecal B cell clonal expansion and IgG production. OCBs are present in ~90% of MS cases, but also demonstrate a latitudinal gradient, and are associated with a more severe disease outcome (Wu and Alvarez, 2011, Lechner-Scott et al., 2012). The presence of this immunoglobulin indicates a role for B-cells in the pathology of MS. Interestingly however, there is no consensus on the antigen for the IgG. Studies have failed to conclusively show that the IgG reacts to myelin components such as myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), or proteto-lipid protein (PLP) as would be expected if the disease was a result of acquired immunity (Wu and Alvarez, 2011).

Aetiology

The underlying aetiology of MS remains unknown, although both environmental and genetic factors are known to be involved in the disease. Studies using twins, siblings, half siblings, adoptees, as well as family history, have shown that the risk of developing MS decreases as the distance in genetic relationship increases(Sadovnick et al., 1996, Willer et al., 2003). The disease does not follow Mendelian genetics, indicating that it is not a monogenic disease, and it is likely that there are a number of interacting genes involved in the development of MS. The risk of developing MS within the general population is approximately 1:1000, while the risk increases to 1:40 for first degree relative and 1:4 for monozygotic twin. It has been found that most monozygotic twins are discordant, however there is a much greater risk compared to dizygotic twins or siblings. This indicates that although genetics plays a major role in MS, there are other factors involved with the disease (Dyment et al., 2004). A study using full- and half-siblings of MS patients showed that there was a higher incidence of MS in full-siblings compared to half-siblings indicating that the genetic relationship between the full-siblings is more important in the development of the disease compared to possible environmental factors (Sadovnick et al., 1996). The study also concluded that there were no specific

environmental exposures related to the familial aggregation of MS, as there was no significant difference between the risks of MS for half siblings raised apart compared to those raised together.

Nevertheless, differing rates of MS in different populations and locations, and apparent epidemics of MS indicated that there are environmental factors involved in the pathogenesis of the disease. Latitude, sunlight exposure and vitamin D have been studied, as has diet, cigarette smoking and viruses. However, vitamin D appears to have the strongest association with MS (Ascherio and Munger, 2007b, Ascherio and Munger, 2007a).

Environmental

Environmental factors are thought to be involved in the pathogenesis of the disease, and early studies identified a relationship between distance from the equator and MS incidence, with higher incidence at higher latitudes (Simpson et al., 2011). There is current debate though as to if this gradient has diminished in recent years. It has been proposed that this relationship may relate to sunlight exposure and subsequent production of vitamin D, which is involved in the regulation of the immune system. Infectious agents, including Chlamydia, Epstein-Barr virus (EBV) and human herpes virus 6, have been proposed as risk factors for the disease (Contini et al., 2010, Pawate and Sriram, 2010). EBV is the only infectious agent with consistent evidence for association, however the reason for the increased risk remains unknown. Cigarette smoking is associated with increased risk of developing MS (risk ratio = 1.5) and there is suggestive evidence of increased risk of conversion to SPMS and poorer prognosis (Handel et al., 2011b, Palacios et al., 2011). The mechanism by which smoking increases risk remains unknown, however it may be due to an immunomodulatory effect. No other endogenous agent has been associated with disease risk.

Vitamin D

A latitude gradient in the prevalence of MS, with higher prevalence at higher latitudes, has long been recognised, and recently confirmed by a large meta-analysis (Simpson et al., 2011). It has been proposed that this gradient is due to UV exposure, and subsequent vitamin D production. Vitamin D, as well as its role in calcium homeostasis, plays a role in immune regulation, and has been associated with a number of autoimmune disorders (Shoenfeld et al., 2009).

In humans, the majority of vitamin D3, cholecalciferol, is synthesised from 7-dehydrocholesterol, though a process catalysed by UV-B radiation, with only a small amount provided by diet. This 7-dehydrocholesterol is converted into 25-hydroxyvitamin D3, 25(OH)D3, before being further hydroxylated in the kidneys to 1,25-dihydroxyvitamin D3, 1,25(OH)2D3, the biologically active form of Vitamin D3 (Hanwell and Banwell, 2011, Muller et al., 2011). 1,25(OH)2D3 is able to bind to the vitamin D receptor (VDR), and mediate its effect through vitamin D responsive elements (VDREs) present within the promoter regions of genes.

High levels of circulating Vitamin D have been associated with decreased risk of developing MS (Munger et al., 2006, Kragt et al., 2009, Correale et al., 2009), and relapsing (Soilu-Hänninen et al., 2005, Soilu-Hänninen et al., 2008, Simpson et al., 2010, Mowry et al., 2010, Correale et al., 2009). One study found no difference between the vitamin D levels of healthy controls and PPMS patient (Correale et al., 2009). Other studies have failed to identify similar associations (Orton et al., 2008). Vitamin D is thought to act as an immunomodulator by increasing regulatory T cell counts (Prietl et al., 2010), inhibiting CD4+ T cell and MBP-peptide specific T cell proliferation (Correale et al., 2009). However, UV radiation alone stimulates immune-modulating and neuroendocrine pathways, and therefore may have an effect on MS separate from vitamin D production (Hanwell and Banwell, 2011). Large scale clinical trials investigating vitamin D as treatment or prophylaxis for MS have not yet been performed. Until these studies are completed, the question over the effect of vitamin D in MS will remain.

Epstein-Barr Virus

It has been proposed that MS is associated with viral or bacterial infection. Although a number of hypotheses and infectious agents have been proposed to explain the possible associations with MS, only Epstein-Barr Virus (EBV) stands up to rigorous testing. EBV infects B cells and usually presents as an asymptomatic latent infection that remains for life. Infection in the first decade of life is often asymptomatic; however infection in adolescents and adults may results in infectious mononucleosis (IM). In developed populations, the prevalence of infection is approximately 95%, which increases to almost 100% in MS cases (Wandinger et al., 2000). Past history of IM has been found to be significantly associated with increased risk of MS with odds ratio (OR) ranging from 2.0 to 2.3 (Thacker et al., 2006, Zaadstra et al., 2008, Ramagopalan et al., 2009b, Handel et al., 2010b).

A meta-analysis, with a total of 1779 cases and 2526 controls, showed that seronegativity for EBV antigens was protective against MS (OR 0.06 (0.03 – 0.13, $P < 10\text{-}9$) (Ascherio and Munger,

2007a). Santiago *et al.* (2010) expanded upon this to look at specific antibodies and found that MS was associated with exposure to EBV, measured by 3 specific EBV antibodies.

Studies have found that HLA-DRB1*15 is associated with greater levels of EBV antibodies levels (van der Mei et al., 2010). The risk attributed to the combination of the two factors is greater than the sum of the individual risks (Sundström et al., 2008, De Jager et al., 2008).

The mechanism by which EBV infection increases MS risk is unknown, however possible theories are: i) cross-reactivity where T-cells primed by EBV antigen exposure recognise and attack CNS antigens, ii) EBV infection of auto-reactive B cells results in both the production of pathogenic antibodies and co-stimulatory survival signalling for T cells, iii) the mistaken-self hypothesis, where there is no immune tolerance to a self-protein which is expressed after infection with EBV, resulting in auto-immunity, iv) homologous viral proteins altering signalling cascades, such as IL10, v) or that there is a common genetic determinate for both MS and EBV susceptibility (Lucas et al., 2011, Pender, 2009).

Genetics

The idea that MS has a genetic component to its aetiology initially developed through observations of multiplex families. Studies have shown that there is a familial component to MS, with the risk of developing disease increasing as relatedness to someone affected by MS increases. The concordance rate in monozygotic (MZ) twins is ~25%, which reduces to ~3% in dizygotic (DZ) twins and ~2.9% in siblings, which decreases to approximately 0.1% in the general population. The inheritance pattern in multiplex pedigrees indicates that the disease does not have a single rare variant that is causative in a Mendelian inheritance pattern, but rather that it is a complex polygenic disease.

HLA associations

The human leukocyte antigen (HLA) complex (otherwise known as the major histocompatibility complex or MHC), is a gene-dense region located at 6p21, which contains the HLA class I, II and III regions. The region is highly polymorphic and exhibits extensive linkage disequilibrium (Trowsdale, 2011). The HLA class I and II regions encode genes that are involved in self/non-self-antigen recognition: Class I, containing the HLA-A, -B and –C genes, presents exogenous antigens to $CD8^+$ cytotoxic T cells while class II, comprising the HLA-DP, -DQ and –DR genes, presents endogenous antigens to $CD4^+$ T Helper cells (Horton et al., 2004).

Linkage studies in the 1970s provided the first information on the underlying genetic aetiology of MS, identifying genes within the HLA complex associated with MS. The HLA-DR-2 haplotype was found to be significantly associated with an increased risk of developing MS. Numerous studies have examined this interaction and identified DRB1*1501 as the main susceptibility allele in populations of European descent. Fine mapping in genome wide association studies (GWAS) has confirmed this as well as confirming a secondary association in class II region, HLA-B (IMSGC, 2007, Fernandes et al., 2009, Orton et al., 2008, Ban et al., 2009). DRB1*17 was identified as a secondary association signal independent of DRB1*1501 (Masterman et al., 2000, Modin et al., 2004, Ramagopalan et al., 2007, Dyment et al., 2005) while DRB1*01, *10, *11 and *14 are protective (Ramagopalan et al., 2007, Dyment et al., 2005). DRB1*08 increases risk when in *trans* with 1501 (Ramagopalan et al., 2007, Dyment et al., 2005). In a recently published study, using the largest cohort to date, DRB1*1501 was identified as the strongest association with MS (OR 3.1, additive effect for each additional allele), but also confirmed the protective effect of a class I allele, HLA-A*0201 (OR 0.73) (IMSGC et al., 2011). Two further alleles within the MHC, DRB1*0301/DQB1*0204 and DRB1*1303 were identified as increasing disease risk (OR = 1.26 and OR =2.4, respectively).

The DRB1*1501 association is not however found across all populations. A study performed in a Sardinian population identified an association with rs2040406, which showed a strong correlation with the DRB1*0301-DQB1*0201 haplotype, while there was no significant association with rs3135388 (DRB1*1501 tagging SNP) (Sanna et al., 2010).

While it is undeniable that DRB1*1501 is associated with disease risk, it is still unclear as to whether this allele, and other HLA regions, are associated with disease phenotype. DRB1*1501 was found to influence disease severity, measured in four domains: decreased *N*-acetyl-aspartate concentrations in normal appearing white matter (NAWM), increased WM lesions, decreased brain volume and impairments in cognition (Okuda et al., 2009). Other studies have failed to replicate association with clinical course and HLA regions (Smestad et al., 2007, van Baarsen et al., 2006). DRB1*1501 has been associated with a younger age of onset (Masterman et al., 2000, Smestad et al., 2007, IMSGC et al., 2011), although this was not replicated by Barcellos et al., (2006). No difference in allele frequency of DRB1*1501 was found between benign MS (n = 112) and malignant MS (n = 51), while DRB1*01 was found to be significantly underrepresented in malignant MS (DeLuca et al., 2007).

The promoter region of the DRB1*15 gene contains a Vitamin D response element (VDRE), which is conserved in this haplotype, but contains significant variation in other DRB haplotypes

(Ramagopalan et al., 2009a). Expression of DRB1*15 was induced by the active form of vitamin D. This implies a direct link between known genetic and environmental factors, and may begin to explain the complex associations involved in the pathogenesis of MS.

Non-HLA associations

After the initial identification of the HLA association, it was expected that further linkage and family studies would rapidly provide the final clues as to the genetic architecture of the disease. However, this was complicated by the fact that large extended pedigrees with multiple affected members and generations are uncommon making linkage studies difficult (Dyment et al., 2002). A meta-analysis including 719 families with an average of 359 microsatellite markers, and the largest single study utilising 730 families with 4506 markers, failed to identify any regions of statistically significant linkage outside of the MHC (IMSGC et al., 2005, GAMES Consortium, 2003). This lead to a re-evaluation of the underlying genetic contribution to the disease, and the idea that the disease may be caused by a large number of genetic variations that each exert only a small effect on the risk of developing the disease. Candidate gene studies of common single nucleotide polymorphisms (SNPs) again were expected to provide definitive results. However these studies failed initially to consistently identify causative variants, likely due to the lack of statistical power of many studies. It was by now recognised that large-scale studies, with thousands of patients would be required to detect the small effects attributable to risk causing genes.

In 2007, the simultaneous publication of three reports confirmed the identification of three additional loci in two genes, associated with an increased risk of developing MS. A genome wide association study (GWAS) was performed by The International Multiple Sclerosis Genetics Consortium (2007) using a total of 12,360 subjects which identified one SNP in *IL7R* and two in *IL2RA*, as well as DRB1*1501, as significantly associated with MS. Gregory et al., (2007), examined SNPs in 3 genes identified in previous expression studies and identified *IL7R* as a significant risk factor for MS, using four large data sets (760 family trios, 438 MS patients and 479 controls from the US, 1338 MS patients plus parents from the UK and 1077 MS patients and 2752 controls from northern Europe). Lundmark et al., (2007) genotyped three SNPs, which were in linkage disequilibrium, within the *IL7R* gene in 1820 MS patients and 2634 controls of Nordic ancestry and 1210 MS patients and 1234 controls from Sweden, and found significant association between all SNPs and MS. The *IL7R* polymorphism causes a change in the protein structure and results in production of less membrane bound form of the protein

and higher levels of the soluble protein (Gregory et al., 2007). IL7R is important in the regulation of T-cell mediated immunity and in the generation of auto-reactive T-cells in MS (Peltonen, 2007). IL7R has previously been shown to play a role in autoimmunity and has been associated with Graves' disease and type 1 diabetes (Peltonen, 2007). However, the SNP in *ILR7* accounts for only a very small proportion of the risk of developing MS and has a very high frequency in the general population and therefore it is unlikely to be a major risk factor in the development of MS (Peltonen, 2007, Hafler et al., 2007). Although these SNPs are significantly associated with MS, (*IL7R*, $p=2.94*10^{-7}$, *IL2RA*, $p=2.96*10^{-8}$ and $p=2.16*10^{-7}$), they are thought to account for less than 0.2% of the variance present in the risk of developing MS (Hafler et al., 2007).

Following from this, a number of successive GWAS studies identified further susceptibility loci for MS. Although the number of confirmed genes associated with MS still remained relatively low, likely due to the lack of power of many of these studies. A novel study examining 14,500 non-synonymous SNPs, over four diseases (MS (n = 975), breast cancer, Ankylosing Spondylitis and Autoimmune Thyroid Disease, plus 1,466 controls), identified only two SNPs with suggestive evidence of association outside of the MHC region (Smolders et al., 2009). In a study of 978 cases with 883 controls, Baranzini et al., (2009) identified 13 SNPs with suggestive evidence of association ($P<10^{-5}$), as well as evidence of an association in HLA-class I region independent of the HLA-class II DRB1*1501. A meta-analysis combining two previous GWAS (IMSGC, 2007, Fernandes et al., 2009) and an additional 860 cases and 1,720 controls successfully confirmed the association of seven SNPs with MS, four of which were novel (Orton et al., 2008). A further seven SNPs also showed suggestive evidence of association. The majority of these genes are known to have immunologic functions. In our own GWAS (ANZgene, 2009), performed on a total of 3,847 cases and 5,723 controls, including replication analysis, we identified two regions with novel associations, 12q13-14 and 20q13 upstream of *CD40*, as well as replicating associations in five known loci. We conducted further studies to identify correlation between these variants and mRNA expression, confirming that the MS associated variant near *CD40* results in lower levels of mRNA expression and that the MS associated variant located at 12q13-14 is significantly associated with decreased expression levels of nearby *FAM119B* gene (Gandhi et al., 2010).

A meta-analysis combining the results of the GWAS by the IMSGC (2007), De Jager et al., (2009) and ANZgene (2009) with an additional 1,453 cases and 2,176 controls, for a total of 5,545 cases and 12,153 controls (Patsopoulos et al., 2011) identified three novel associations:

rs170934 (3p21.1), rs2150702 within *MLANA* (9p24.1) and rs6718520 (2p21) near *THADA*, and confirmed associations within *IL2RA*, *CD58* and *STAT3*.

Studies have been performed on genetically isolated populations, which may allow the identification of rare, high risk variations. Aulchenko et al., (2008) performed a GWAS on 45 cases and 195 controls from a genetically isolated Dutch population, but did not find any variants that reached genome wide significance. Following a suggestive association of a variant in *KIF1B,* with a replication cohort of 2,364 cases and 2,930 controls, genome wide significance was reached for the rs10492972 SNP, which was the first strong evidence for genetic association with a gene involved in neurodegeneration rather than inflammation. However, we were not able to replicate this association in a large study of 8,391 cases, 8,052 controls and 2,137 trio families (IMSGC et al., 2010), nor was the variant associated with any markers of neurodegeneration (Sombekke et al., 2011) or progressive forms of MS (Martinelli-Boneschi et al., 2010, Koutsis et al., 2011), which was confirmed by meta-analysis (Kudryavtseva et al., 2011).

In an isolate Finnish population with an increased MS prevalence, Jakkula et al., (2010) performed GWAS on 68 cases and 136 controls, replicating with a further 711 cases and 1029 controls from Finland, and validating in 3,859 cases and 9,110 controls. *STAT3* was associated with genome wide significance, which tags a protective haplotype that is also associated with increased risk of Crohn's Disease.

In a Sardinian population, a variant in *CBLB*, rs9657904, was the only SNP outside the MHC in the study to achieve genome wide significance (Sanna et al., 2010).

The small effect of each individual variant associated with MS, and the prediction that there may be approximately 50-100 genes or variants associated with disease susceptibility indicates that in order to achieve the statistical power required to detect further associations, the number of samples included in the studies must be increased. In the recently published report by the IMSGC (2011), we identified 29 novel susceptibility loci, 5 loci with suggestive evidence of association and replicated 23 known or suggestive loci, using 9,772 cases and 17,376 controls, for a total of 57 MS associated loci. Of the non-MHC loci, 30% are located within or close to genes with an immunological function, while only two loci are associated with genes involved in pathways of neurodegeneration, and approximately 1/3 of the loci overlap with known regions of association in other autoimmune disorders, indicating that the genetic predisposition is to an autoimmune disease rather than a neurodegenerative one.

No loci have been significantly associated with disease course or clinical features (IMSGC et al., 2011, Ban et al., 2009, Fernandes et al., 2009, Jensen et al., 2010).

The genetic studies indicate that MS is an autoimmune disorder, and highlight specific biological pathways, such as T cell activation and vitamin D metabolism, as involved in the pathogenesis of MS. However, there is still a large component of the underlying genetics of MS that remain unknown. Loci identified through GWAS are often not present within a gene, but are mapped to the closest gene, or the most likely biologically associated gene. Therefore the potential role of the variant in disease pathogenesis is often unknown. There have been few studies that examine the relationship between an associated loci and biological effect such as expressed quantitave traite loci (eQTL), Studies have started to investigate this, (Gandhi et al., 2010), but overall research is lacking. The IMSGC GWAS took over four years to complete and involved hundreds of researchers, and although this approach was immensely successful, a similar study approach is unlikely to be reproduced. Therefore, to decipher the remaining genetic tangles, different methodologies to supplement this approach are required. Single nucleotide variants may not be the only genetic factor influencing MS susceptibility, and copy number variations and epigenetics, such as methylation, must also be examined.

Gene Expression

Whole genome gene expression studies are able to measure the expression levels of the transcriptome. This has allowed the identification of gene expression profiles of diseases and therapies, and biomarkers of disease and progression for many diseases.

Previous microarray gene expression studies in MS have used differing methodological approaches (using either peripheral blood or brain tissue as a sample source), and examining the effect of treatment. Brain tissue is difficult to use due to the small number of samples available, differences in gene expression between various brain regions, and differences in the collection efficiency (i.e. post-mortem interval, tissue pH, storage of sample, age of subject, and cause of death), which may result in compromised RNA integrity (Chevyreva et al., 2008). To date all studies examining brain tissue have used very limited cohorts (Graumann et al., 2003, Tajouri et al., 2003, Lindberg et al., 2004, Baranzini et al., 2000, Lock et al., 2002, Whitney et al., 2001, Zeis et al., 2009, Zeis et al., 2008, Mycko et al., 2004). To compound these issues, the studies have also used different methodologies, comparing lesion tissue to non-affected white matter (NAWM) within the same individual, or using unaffected controls. Examining the pathological site of disease in the CNS will shed light on cortical events that

impact significantly on an affected individual. Given that GWAS data clearly implicates the immune system in MS peripheral blood represents an ideal tissue source.

To date, most studies examining the gene expression of MS patients compared to healthy controls using peripheral blood have suffered from relatively small sample sizes (Iglesias et al., 2004, Booth et al., 2005, Särkijärvi et al., 2006), combined MS subtypes (Bomprezzi et al., 2003, Achiron et al., 2004a, Iglesias et al., 2004, Booth et al., 2005, Särkijärvi et al., 2006, Satoh et al., 2005) or different disease modifying therapies (Achiron et al., 2004a, Iglesias et al., 2004, Booth et al., 2005, Särkijärvi et al., 2006) resulting in heterogeneous cohorts, or looked at small numbers of genes (Bomprezzi et al., 2003, Iglesias et al., 2004, Booth et al., 2005, Ramanathan et al., 2001, Särkijärvi et al., 2006, Satoh et al., 2005), thereby not providing a complete picture of the gene expression changes associated with MS.

The largely discordant results seen between these gene expression studies may be explained in part by these factors. Interestingly, no differential gene expression was observed in PBMCs from MS patients compared to controls, while 939 genes were differentially expressed in cells from CSF in MS patients compared to controls. Conversely, when comparing patients in relapse to patients in remission, no genes were differentially expressed in cells from CSF, while 266 genes were differentially expressed in PBMCs patients (Brynedal et al., 2010). It is thought that the underlying genetic component of MS is due to a large number of small variations each exerting a small effect, therefore larger cohort studies using whole genome gene expression analysis is required to identify what consequence genetic variation has on gene expression and hence disease aetiology.

There have been a number of studies focused on identifying the effect of treatment in MS patients, comparing MS patients receiving treatment to treatment naïve patients, examining patients pre- and post- treatment, or comparing treatment responders to non-responders. The majority of these studies have examined the response to interferon-beta therapy, and have identified different genes involved, not surprisingly a number of which are involved in interferon signalling. However, again differing methodologies have produced discordant results, possibly due to small sample sizes (Weinstock-Guttman et al., 2003, Fernald et al., 2007, Sturzebecher et al., 2003), combined MS subtypes (Gandhi et al., 2010), different disease modifying therapies (Achiron et al., 2004b), or small number of genes examined (Koike et al., 2003) (Weinstock-Guttman et al., 2003) (Weinstock-Guttman et al., 2008). However, these studies have shown that disease modifying therapies have an effect on the gene expression profile of MS, as would be expected in an effective treatment. This indicates a limitation in the

design of some studies, whereby the inclusion of MS cases with and without treatment may confound the MS gene expression signature (Achiron et al., 2004b).

Gene expression profiles in MS patients are altered during relapse compared to remission phase (Brynedal et al., 2010, Lindsey et al., 2011, Achiron et al., 2007). However it is possible that in the period prior to relapse, there could be a distinct change in the immune response leading to neuroinflammation, which may have subsided by the time the relapse is acute, with only the inflammatory response remaining. Apoptosis related signalling genes were found to be dysregulated during relapse in 22 MS patients compared to 20 control subjects (Achiron et al., 2007), which may signify impaired apoptosis in lymphocytes, allowing the inflammatory response to persist. A smaller study using 10 patients during relapse, 10 patients during remission and 25 controls, identified TGFalpha1, CD58 DBCI and ALOX5 as differentially regulated, however approximately 10% of the genes examined in the study were found to be differentially expressed (989 during relapse and 1317 during remission compared to controls) (Arthur et al., 2008).

MicroRNA

MicroRNAs are a species of non-coding RNA involved in post-transcriptional regulation. Mature miRNAs are produced through a multi-stage processing of a precursor transcribed by RNA polymerase II, the pri-miRNA (primary), with a characteristic hairpin structure (Guerau-de-Arellano et al., 2012). The pri-miRNA is cleaved by Drosher to produce pre-miRNA (precursor), which is further cleaved by Dicer/RISC complex, resulting in single strand miRNAs, one of which is able to bind the 3' UTR of target genes, while the complementary miRNA is often degraded. The short 18-22 nucleotide miRNAs bind to the 3' UTR of the target mRNA through sequence complementarity, resulting in mRNA degradation of the transcript or inhibition of translation.

miRNAs have been implicated in a number of diseases, through their effect on gene expression (Jansson and Lund, 2012, Tijsen et al., 2012, Schonrock and Gotz, 2012). miRNA expression arrays have allowed the large scale analysis of miRNA expression to be investigated in diseases and therapies, and correlated with gene expression data. The miRNA technologies have changed rapidly, with the increasing number of identified miRNAs.

miRNAs were first investigated in MS in 2009 (Otaegui et al., 2009) and since then, there has been a number of publications, investigating miRNAs in whole blood or cell subsets, and lesion tissue in MS patients compared to controls, and within relapsing compared to remitting

patients, and looking at combined or comparing subtypes. Unsurprisingly, the results of many of these studies have been discordant.

Otaegui et al., (2009) examined the miRNA expression profile in PBMCs in relapsing patients (n = 4), patients in remission (n = 9) and healthy controls (n = 8). miR-18b and miR-599 associated with relapse compared to controls while miR-96 to be an important candidate gene in remission. Pathway analysis of predicted targets of miR-96 was enriched for genes involved in the immune response (interleukin signalling pathway), and related to glutamate (metabotrophic glutamate receptor group I pathway, and Muscarinic acetylcholine receptor 1 and 3 signalling pathway) which has been associated with the pathomechanism of MS.

165 miRNAs were found to be differentially expressed in MS (n = 20) compared to healthy controls (n = 19), with a subset of 48 miRNAs to be accurate, sensitive and specific markers of disease (Keller et al., 2009). 43 of the dysregulated miRNAs had previously been associated with other diseases, while the remaining 122 miRNAs were novel disease associations. Although the effect of the dysregulation of miRNAs is unknown, it is proposed that dysregulation may serve as a biomarker of disease.

The first study to examine miRNAs in MS lesion tissue, using 16 active lesions, 5 inactive lesions and 9 normal white matter sections identified specific signatures of each lesion type (Junker et al., 2009). Interestingly, a number of these miRNAs target CD47, an inhibitor of macrophages, which may result in tissue destruction in MS.

Cell subset analysis of $CD4^+$, $CD8^+$ and B-lymphocytes identified 10, four and six miRNAs, respectively, differentially expressed in eight MS patients and 10 healthy controls (Lindberg et al., 2010). miR-17-5p which was up-regulated in $CD4^+$ cells, and has previously been associated with other autoimmune disorders. PI3K signalling, known to be important in T cell development, contains 15 genes which are predicted targets of mir-17-5p. Expression analysis of two of these genes, *PI3KR1* and *PTEN*, confirmed increased mRNA expression when miR17-5p expression was inhibited, while their expression was down-regulated in stimulated lymphocytes.

Epigenetics

There have been few studies examining epigenetics in MS, and similar to the early studies on linkage, SNPs and gene expression, these studies have been small, results have been

inconclusive and remain to be replicated. However, epigenetics may play an important role in the aetiology of MS with a separate role in the underlying genetics from common variations, and resulting in differential gene expression and therefore affecting disease expression. Epigenetics may partially explain the discordant disease seen in most MZ twins, who have identical DNA sequence, and largely, a similar exposure to environmental factors.

Hypomethylation of the *PAD2* promoter region was identified by Mastronardi et al., (2007), which was thought to explain their discovery of increased *PAD2* expression within NAWM, resulting in the loss of myelin stability. *MHC2TA*, a regulator of MHC class II expression, was found to contain no differences in methylation of the promoter region in PBMCs between discordant MZ twin pairs (Ramagopalan et al., 2008). Unique patterns of hypermethylation were found in MS cases, both in remission and relapse, compared to healthy controls, in an investigation of cell free plasma DNA, in an array of 56 gene promoter regions (Liggett et al., 2010). Handel et al., (2010a) examined benign and malignant MS cases (n = 48 and 20 respectively) for differences in methylation across DRB1*1501 and DRB5, however no differences were found. Three discordant monozygotic twin pairs were examined for gene expression analysis and genetic variations between twin pairs (Baranzini et al., 2010). No replicable differences in SNPs or mRNA expression, insertion/deletions or CNVs were found within co-twin pairs.

Differences in histone acetylation over disease duration was found in NAWM, with greater levels of acetylation in the late stages of the disease, which has also been shown to be involved in inhibitors of oligodendrocyte differentiation (Pedre et al., 2011).

Current disease modifying therapies and pharmacogenomics

The aim of current disease modifying therapies is to reduce the aberrant immune response that results in neurologic damage. Although the mode of action of some of the current therapies remains uncertain, research into these therapies add to the understanding of the disease.

Current first line treatments for MS are interferon beta-1a and -1b (IFN-beta), and glatiramer acetate (GA), which have shown similar efficacy in many head to head studies (Fontoura, 2010). Interferon beta was the first treatment available for MS, and was initially trialled as a therapeutic agent due to its antiviral properties. IFN-beta has been found to reduce both the relapse rate and formation of new lesions (Rudick and Goelz, 2011). While the mechanism of

action of interferon-beta in MS remains unknown, it is thought to activate signalling pathway involving Jak1 and Tyk2, resulting in transcriptional regulation of many genes (Rudick and Goelz, 2011).

GA was initially developed to induce the animal model of MS, EAE, and is composed of a mixture of four amino acids present in myelin basic protein (MBP). However it was found to suppress the development of EAE in the model animals. From this, human trials were undertaken, and GA was found to be effective in reducing relapse rate and disease progression compared to placebo. Although the mode of action of the drug is unknown, it is thought to alter the cytokine profile from pro-inflammatory to regulatory, modify the immune response through generation of suppressor T cells (Kala et al., 2011).

Natalizumab was registered for use in 2004 and Fingolimod in 2011, and both are regarded as second line therapies due to their greater efficacy and greater potential side-effects (Fontoura, 2010, Weinstock-Guttman et al., 2012). Both drugs act by reducing the lymphocyte infiltration into the CNS. Natalizumab is a monoclonal antibody to alpha4-integrin, which blocks the binding to VCAM1 and therefore prevents lymphocyte migration across the blood-brain barrier (Derwenskus, 2011). Fingolimod, after phosphorylation, binds to the sphingosine 1-phosphate receptor (S1PR) on lymphocyte cell surface, causing the internalisation of the receptor, and preventing the egress of the cells from the lymph nodes (Cohen and Chun, 2011). This results in a reduction in the circulating lymphocyte to approximately 20-30% which is thought to represent the effector memory T cell population, and reduction in recirculation of auto-aggressive cells into the CNS (Cohen et al., 2010, Kappos et al., 2010). Fingolimod may also have a neuroprotective role, with S1P receptors present on astrocytes, oligodendrocytes and neurons (Cohen and Chun, 2011).

Future

The understanding of the aetiology and pathogenesis of MS is increasing through research in many areas, and this knowledge provides further insights into the disease, such as the mechanism of action of DMT indicating disease process, and conversely, the identification of genes involved in MS and subsequent drug development. However our understanding of the genetics of MS is still incomplete and further studies with novel approaches are now needed. The completion of the largest GWAS in MS to date was a major achievement, but also highlights that these different approaches are required to fill the gaps in the knowledge, as it is unlikely another GWAS of such power will be conducted. Therefore, it is possible that

candidate gene studies, when conducted with sufficient power, and epigenetic studies will provide the remaining information of the underlying genetics of MS.

The research contained in this thesis has been conducted to pursue the following paths:

RNA expression studies may identify candidate genes (including miRNA) of interest in the pathogenesis of MS;

Candidate gene SNP association studies, when performed with appropriate power, may identify common variants associated with disease risk.

Chapter Two – RNA Expression Profiling in Multiple Sclerosis

Introduction

RNA expression profiling, including mRNA and miRNA expression, has the potential to identify the underlying pathomechanism of diseases and the biological effect of therapies, through the examination of the transcriptome. The results of differential expression analysis may not only identify specific dysregulated RNAs, but can also be used to identify molecular pathways that are enriched with dysregulated genes which may indicate a role in the disease. However, discordant results between studies may arise through heterogeneous cohorts, small sample sizes, or small number of genes examined.

Whole-genome gene expression studies in MS have investigated differential gene expression comparing MS and healthy controls, different MS subtypes, and treatment effects, examining whole blood, blood cell subsets, and brain tissue, and have therefore produced discordant results. However the 'big picture' of the dysregulated systems highlights the auto-immune nature of MS.

In studies of lesion tissue, differential expression was identified for genes associated with T cells (Baranzini et al., 2000, Lock et al., 2002, Tajouri et al., 2003, Lindberg et al., 2004), B cells (Baranzini et al., 2000, Lock et al., 2002), MHC class I (Tajouri et al., 2003) and class II genes (Baranzini et al., 2000, Lock et al., 2002), pro-inflammatory genes (Whitney et al., 2001, Lindberg et al., 2004, Mycko et al., 2003, Zeis et al., 2008), anti-inflammatory genes (Zeis et al., 2008), and cytokines (Lock et al., 2002). Other pathways likely to be associated with disease mechanisms, identified by altered gene expression, include metabolism (Graumann et al., 2003, Tajouri et al., 2003), oxidative stress (Graumann et al., 2003, Tajouri et al., 2003), and neuroprotection (Graumann et al., 2003).

Whole-genome gene expression studies in peripheral blood have identified common findings, again involving the dysregulation of genes involved in immune system and auto-immunity (Särkijärvi et al., 2006, Satoh et al., 2005, Mandel et al., 2004, Achiron et al., 2004a, Bomprezzi et al., 2003, Iglesias et al., 2004, Ramanathan et al., 2001), response to stimuli (Booth et al., 2005), oxidative stress (Satoh et al., 2005), amino acid phosphorylation (Booth et al., 2005), myelination or myelin degradation (Ramanathan et al., 2001, Särkijärvi et al., 2006), apoptosis (Satoh et al., 2005, Ramanathan et al., 2001, Mandel et al., 2004, Achiron et al., 2004a), DNA repair, replication and chromatin remodelling (Satoh et al., 2005), heat-shock proteins (Mandel et al., 2004), and signal transduction (Mandel et al., 2004).

As would be expected for an effective immunomodulatory therapy, treatment with interferon beta induced differential gene expression in MS patients. Pathways enriched for these genes included immune modulation (Reder et al., 2008, Sturzebecher et al., 2003, Weinstock-Guttman et al., 2003, Singh et al., 2007), IFN signalling (Reder et al., 2008, Weinstock-Guttman et al., 2003, van Baarsen et al., 2008, Serrano-Fernandez et al., 2010, Singh et al., 2007), and antiviral response (Reder et al., 2008, Weinstock-Guttman et al., 2003, Serrano-Fernandez et al., 2010).

These studies have provided further insight into the underlying disease pathomechanism, and identified specific areas that require further study. Unsurprisingly, the most common pathways enriched for differentially expressed genes are associated with the immune system. This confirms the autoimmune nature of the disease, however, we do not know whether the disease starts in the CNS or the periphery, and unfortunately, these studies are unable to shed further light on this issue.

Gene expression levels are in part controlled by miRNAs through translational repression, and therefore miRNAs may play an important role in disease pathogenesis. Studies first examined the association of miRNAs with MS in 2009, however initial studies investigated only a small number of miRNAs, and used small sample populations. Of four genome-wide miRNA studies completed prior to this thesis, two examined peripheral blood (Keller et al., 2009, Otaegui et al., 2009), one investigated blood cell subsets (Lindberg et al., 2010) and another investigated lesion tissue (Junker et al., 2009). This makes any comparison of the results difficult, however all studies identified dysregulated expression of miRNAs known, or predicted to target genes associated with the immune system (Keller et al., 2009, Lindberg et al., 2010, Otaegui et al., 2009, Junker et al., 2009).

Therefore it is clear that large, homogenous cohorts are required for whole-genome gene expression and miRNA expression studies to further investigate the complex genetics of MS. In this thesis, I examined multiple sclerosis whole-genome gene expression in 24 treatment naïve RRMS patients, 13 RRMS receiving interferon-beta therapy, and 39 healthy controls, and identified enrichment of dysregulated genes in the plasminogen activation cascade, including *MMP9*. I also examined miRNA expression in a heterogeneous population of 59 MS patients and 37 healthy controls identifying miR-17 and miR-20a dysregulation in MS, and further clarify the biological role of these two miRNAs in their regulation of T cell activation genes.

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Co-author Statement

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Altered expression of the plasminogen activation pathway in peripheral blood mononuclear cells in multiple sclerosis: possible pathomechanism of matrix metalloproteinase activation

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Abstract

Background: Multiple sclerosis (MS) is an autoimmune disorder where a breakdown in the integrity of the blood-brain barrier is thought to allow lymphocytes to enter the central nervous system.

Objectives: The purpose of this study was to examine gene expression profiles between MS patients and healthy controls to identify genes intimately involved in the pathobiology of MS.

Methods: Whole-genome gene expression analysis was performed using peripheral blood mononuclear cells from 39 healthy controls and 37 MS patients, 24 MS patients receiving no disease modifying therapy and 13 MS patients receiving interferon-beta (IFN-beta). Pathway analysis was performed to identify pathways dysregulated in MS.

Results: Gene expression profiling of MS identified a signature of predominately immune associated genes. The plasminogen activation pathway contained an over-representation of significantly differentially expressed genes, including matrix metallopeptidase 9 (*MMP9*). Treatment with IFN-beta ameliorated the over-expression of *MMP9*, however the expression of two genes, plasminogen activator urokinase (*PLAU*) and serpin peptidase inhibitor, clade B (ovalbumin), member 2 (*SERPINB2*), forming part of the plasminogen activation pathway were not affected by IFN-beta therapy.

Conclusions: High expression levels of MMP9 have been associated with MS and the breakdown of the blood-brain barrier, while IFN-beta therapy decreases MMP9 expression. We confirm altered *MMP9* expression in MS, and identify dysregulation within the plasminogen activation cascade, a pathway involved in the activation of MMP9.

Introduction

There is compelling evidence that multiple sclerosis (MS) is an autoimmune disorder in which lymphocyte infiltration into the central nervous system (CNS) results in inflammatory lesions within the brain and spinal cord, leading to demyelination and axonal damage.

The CNS is an immunoprivileged site, where the blood-brain barrier (BBB) separates the CNS from the periphery, controlling the passage of immune cells and small molecules. In MS lesions, the BBB is compromised, as can be imaged using gadolinium contrast in magnetic resonance imaging (MRI).[1] Evidence from clinical trials of natalizumab, a monoclonal antibody that prevents transmigration of T cells into the CNS,[2] suggests that the breakdown of the BBB

is integral to the formation of inflammatory lesions, rather than being a result of immunologic cell migration into the CNS.

Current first line disease modifying therapy (DMT), most commonly interferon-beta (IFN-beta; either beta-interferon 1a or 1b) and glatiramer acetate, are aimed at preventing and reducing the autoimmune inflammation that culminates in patient disability. These therapies have approximately 30% efficacy in reducing the relapse rate, although not all patients respond to treatment.[3, 4] The mechanism of action of these therapies remains to be fully elucidated, however it is thought that they modulate the immune system by altering the immune response from Th1 to Th2, thereby switching the immune system more towards an anti-inflammatory response.[3, 5]

Several studies have shown that there is a genetic component to MS, which comprises one major risk factor (HLA-DR2) with an odds ratio (OR) =2, and a large number of other genes, each with small effect sizes.[6, 7] The strongest genetic effect of DR2 has been fine-mapped to HLA-DRB1*15:01, which is present in approximately 50% of MS patients and confers a disease risk odds ratio of 3.1.[6] Genome wide association studies (GWAS) have identified and confirmed the association of an additional 57 variants, the effect sizes of which however are individually thought to be very small (OR <1.3).[6, 7]

Despite the success of GWAS in identifying the immunological nature of MS, there remains many questions about how the activated immune cells cross the blood-brain barrier. To gain a better understanding of the underlying aetiology of MS, different methodologies can be used in a convergent manner to fully explain how the genetic effects influence disease expression. Whole-genome gene expression analysis is able to identify differences in gene expression between disease and control subjects and as a consequence place any genetic findings into better context. Gene expression analysis has been effective in the identification of genes associated with MS, such as the identification of differential expression of IL7R in MS,[8] and the subsequent discovery of an associated single nucleotide polymorphism (SNP) in IL7R, that has been confirmed by several large genetic studies.[9-11]

This study provides a more extensive investigation into gene expression in MS than that previously reported.[12, 13] Whole-genome gene expression analysis (~24,000 mRNA transcripts) was used to ensure the entire transcriptome was assessed in the investigation, in a MS cohort selected for only relapsing–remitting multiple sclerosis (RRMS) disease, with all patients in remission phase at the time of collection. Patients free of any disease modifying therapy

(n=24) were selected for the main analysis, with an additional 13 patients receiving IFN-beta therapy included in the study, and 39 healthy controls matched for age and sex.

Methods

Peripheral blood was collected from 37 RRMS patients during remission phase of the disease, and 39 healthy control subjects matched for age and sex. All patients were diagnosed according to the McDonald criteria.[14] Twenty-four patients had not received any DMT for at least three months prior to blood collection ('treatment–naive'), and 13 patients were receiving IFN-beta 1a or 1b therapy at the time of blood collection.

Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) using TRIzol reagent (Gibco-BRL, USA) and the RNA purified using RNeasy MinElute clean-up kit (Qiagen, Australia). Total RNA was amplified and biotinylated using the Illumina TotalPrep RNA amplification kit (Ambion, USA), and hybridized to Illumina HumanRef8-V2 microarrays (Illumina, USA). The transcript expression results were cubic spline normalized using BeadStudio 2.0 software (Illumina, USA), and the remaining analysis was performed using GeneSpring GX 7.0. To account for bias or skewing of expression results all the gene expression profiles and each individual gene were normalized to the median resulting in two-way normalization. For visualization of the results the data was log transformed.

Differential gene expression analysis was performed between treatment-naïve RRMS cases and healthy controls, treatment-naïve RRMS cases and RRMS patients receiving IFN-beta therapy, and RRMS patients receiving IFN-beta therapy and healthy controls, with genes showing a statistically significant difference in expression (Welch's approximate t-test), ≥2 fold change selected for further analysis. Four genes differentially expressed between RRMS patients receiving no therapy and healthy controls, interleukin 8 (*IL8*), matrix metallopeptidase 9 (*MMP9*), plasminogen activator urokinase (*PLAU*), Fc receptor-like A (*FCRLA*) were selected for quantitative real-time polymerase chain reaction (RT-PCR) confirmation, as genes of interest (due to the previous association of *IL8* with MS, *MMP9* and *PLAU* associated with the same pathway and *FCRLA* as the only gene down-regulated). Briefly, 500 ng of total RNA was reverse-transcribed using the High Capacity Reverse Transcription kit (Applied Biosystems) and a 1:20 dilution of the resultant cDNA was used in triplicate for each sample. Expression was measured in triplicate and normalized to Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (ΔCt) using TaqMan gene expression assays (Applied Biosystems) and a 7500 RT-PCR system (Applied Biosystems). Relative expression was calculated using the $2^{-\Delta Ct}$ method. The

Kruskal-Wallis test (GraphPad Prism, California, USA) was used to test for differences in relative expression.

Pathway analysis was performed on differentially expressed genes using the PANTHER 7.0 classification system[15, 16] with Bonferroni multiple testing correction, to identify pathways associated with the differentially expressed genes, and to classify the genes according to biological process.

This study conformed to the Declaration of Helsinki and was approved by the Hunter Area Health Human Research Ethics Committee. Written informed consent was obtained from all participants. Participants were recruited through the Multiple Sclerosis Clinic, John Hunter Hospital, Newcastle, Australia.

Results

Patient characteristics are shown in Table 1. There was no significant difference in the gender ratio (chi square), age, Expanded Disability Status Scale (EDSS) or disease duration by t-test between any group (Bonferroni multiple testing correction, data not shown). The average duration of treatment was 2.7 years (standard deviation 2.7) in the treated group. Treatment-naïve patients had not received any disease modifying therapy for at least three months, with 16 patients having no history of any DMT. Control subjects were matched for age and weighted with more females than males.

Gene expression profiling of 24 treatment-naïve RRMS patients compared to healthy controls revealed 50 genes with increased expression in the RRMS patients, and one gene with decreased expression (≥2 fold-change, $p<0.05$) (Supplementary Table 1). The most significant gene expression changes were found in HLA-DRB5 (11.58 times higher in MS), *DEFA1B* (4.4 times higher in MS) and HLA-DRB1 (3.90 times higher in MS), while *FCRLA* was the only under-expressed gene (2.02 times lower in MS patients). RT-PCR confirmed statistical differential gene expression of *MMP9*, *PLAU* and *IL8*; fold changes were similar for all genes respective to their expression in the whole-genome analysis (Table 2).

Gene ontology classification by biological process [15]revealed a significant number of genes involved in immune response or signalling (Table 3 and Supplementary Table 2). Pathway analysis revealed four pathways with a significantly higher number of genes showing altered expression than expected, these included plasminogen activating cascade, blood coagulation, inflammation mediated chemokine and cytokine signalling pathway, and Alzheimer disease-

presenilin pathway (Supplementary Table 3). The plasminogen activation cascade and blood coagulation had three genes in common; *PLAU* (or *uPA*), PLAU receptor (*PLAUR* or *uPAR*) and serpin peptidase inhibitor, clade B (ovalbumin), member 2 (*SERPINB2* or *PIA-2*). *MMP9* appears to be involved in both the plasminogen activation cascade and Alzheimer disease-presenilin pathway.

Three related genes, HLA-DRB1, HLA-DRB5 and HLA-C, were found to have higher expression in the RRMS cases compared to the controls. These genes, and HLA-DRB4, were also over-expressed in RRMS patients receiving INF-β therapy.

The effects of treatment were examined by comparing those patients receiving IFN-beta treatment against untreated patients and normal healthy control subjects. There were 106 genes differentially expressed (20 down-regulated and 86 up-regulated) between RRMS patients receiving IFN-beta therapy and healthy controls. A significant number of these differentially expressed genes are involved in immunity and defence, IFN mediated immunity, and T-cell mediated immunity (Table 3 and Supplementary Table 2). Only 15 genes identified in the treatment-naïve RRMS group compared to the healthy controls analysis were in common with this analysis (Figure 1), indicating that while the treatment has 'normalized' the majority of the genes differentially expressed between the treatment-naïve cases and the healthy controls, it has altered the expression of a number of other genes.

A total of 63 genes were differentially expressed between RRMS patients receiving IFN-beta therapy and RRMS patients receiving no therapy (41 genes up-regulated and 22 genes down-regulated in patients receiving treatment). Similarly, a large proportion of these genes are involved in immune system/ response or signalling. Interestingly, *PLAU* and *SERPINB2*, both involved in the plasminogen activation cascade show higher expression in patients receiving no treatment compared to those receiving IFN-beta, while *MMP9* and *PLAUR* have similar levels of expression between the two groups.

A number of genes known to be induced by IFNs, such as 2'-5'-oligoadenylate synthetase 1 (*OASI*), and the interferon inducible genes (IFIs) showed increased expression in patients treated with IFN-beta, as expected.

Discussion

Gene expression profiling in MS using PBMCs has the potential to identify genes intimately involved in the pathobiology of this disease. The evidence to date is overwhelmingly in favour

of MS being an auto-immune disorder that culminates in demyelination and axonal loss. Exactly how immune-reactive cells are capable of crossing the blood-brain barrier in MS remains to be elucidated, although it is thought that a breakdown in the blood-brain barrier allows peripheral immune cells to enter the CNS. By examining the gene expression profiles of peripheral blood lymphocytes derived from MS patients that are under treatment and comparing them to treatment-naïve patients, insights into how the disease is inhibited will shed light on the molecular events that underlie this disease.

Previous microarray gene expression studies in MS have used differing methodological approaches (using either peripheral blood or brain tissue as a sample source), and examining the effect of treatment. Brain tissue is difficult to use due to the small number of samples available, differences in gene expression between various brain regions, and in the collection efficiency (i.e. post-mortem interval, tissue pH, storage of sample, age of subject, and cause of death) all of which have the potential to compromise RNA integrity and consequently data interpretation.[17] To date, all studies examining brain tissue have used very limited cohorts.[18] To compound these issues, these studies have used different methodologies, comparing lesion tissue to non-affected white matter (NAWM) within the same individual, or using unaffected controls. Given that GWAS data clearly implicates the immune system in MS, peripheral blood remains the tissue of choice for investigating the pathological events underlying MS.

To date, most studies examining the gene expression of MS patients compared to healthy controls using peripheral blood have suffered from relatively small sample sizes, combined MS subtypes or different disease modifying therapies resulting in heterogeneous cohorts, or looked at small numbers of genes thereby not providing a full picture of the gene expression changes associated with MS.[12, 13] The largely discordant results seen between gene expression studies in MS may be explained in part by these factors. The current concepts about the pathobiology of MS is that the underlying genetic component of disease is due to a large number of small variations each exerting a small effect, therefore larger cohort studies using whole-genome gene expression analysis are required to identify what consequence genetic variation has on gene expression and hence disease progression.

We identified a gene expression signature in treatment-naïve RRMS patients and compared it to one from healthy controls. There were 51 genes identified as being differentially expressed in treatment-naïve patients of which only one was down regulated. A large proportion of the differentially expressed genes were involved in immunity or cell signalling.

Three of these genes differentially expressed in RRMS treatment-naïve patients and those receiving IFN-beta treatment compared to controls were within the HLA region. HLA-DRB1, known to be associated with MS for over 30 years, and the gene conferring the strongest susceptibility to MS was over-expressed in all RRMS cases irrespective of treatment status. HLA-DRB5 and HLA-C, both of which have previously been associated with MS,[9, 19, 20] were also over-expressed in the RRMS cases, regardless of treatment status.

Of particular interest revealed in the current study was the finding that two matrix metallopeptidase genes, *MMP9* and *MMP25* were found to have increased expression in the RRMS patients receiving no treatment compared to healthy controls. MMP9 is associated with MS through its role in altering the integrity of the blood-brain barrier, therefore allowing lymphocytes to pass from the periphery into the cerebral space, as well as being capable of cleaving myelin basic protein (MBP).[21, 22] MMP9 is produced as an inactive precursor, which must be activated via cleavage of the precursor protein, which can be performed by plasmin. Interestingly, four genes involved in the plasminogen activation cascade, *PLAU*, *PLAUR*, *SERPINB2* and *MMP9* were up-regulated in RRMS patients. Plasmin is activated through the cleavage of plasminogen by the PLAU/PLAUR complex, which in turn can be activated by plasmin. SERPINB2, is a negative regulator of the pathway, and inhibits plasmin activity (Figure 2). It is of interest that *PLAU*, *PLAUR*, *SERPINB2* and *MMP9* are all up-regulated, as there is a negative feedback regulatory system involved in the pathway. Intriguingly the levels of PLAU and SERPINB2 in MS patients receiving IFN-beta therapy are less than that observed in patients receiving no treatment, suggesting that one effect of therapy is to reduce the expression of these genes. *PLAUR* and *SERPINB2* have previously been found to be altered in gene expression analysis of not only MS but also lupus patients compared to healthy controls.[23] Mandel postulates increased MMP activity may be involved in autoimmunity, due to dysregulation of genes involved in the regulation of MMPs, as found in their study. We can confirm this hypothesis documenting increased expression in *MMP25* and *MMP9*, plus altered expression of genes involved in MMP9 activation. Studies in lesion tissue have identified increased levels of PLAU, PLAUR and MMP in active lesions, with the proteins localized to mononuclear cells and monocytes. [24]

IFN-beta can inhibit production and expression of *MMP9* at the mRNA level [25] in T cells, [26] specifically $CD4^+$ T cells,[27] and myeloid dendritic cells.[28] IFN-beta suppresses *MMP9* transcription through the recruitment of histone deacetylase 1 to the *MMP9* promoter.[29] *MMP9*, while up-regulated in RRMS patients receiving no treatment, is normalized in RRMS patients receiving IFN-beta, consistent with a therapeutic effect of IFN-beta. Although it is of

interest that there was no differential expression of *MMP9* between patients treated with interferon-beta and treatment naïve patients, and patients treated with interferon-beta and healthy controls. This may be due to only small differences in the expression of the gene, in the three different patient populations. The suppression of *MMP9* by IFN-beta may also be transient.[30]

MMP9 activity is inhibited by TIMP-1, and it has been shown that while MMP9 levels are increased in MS, there is no increase in tissue inhibitors of metalloproteinases (TIMP-1), [31] indicating that the regulatory mechanism is not operating correctly. Serum levels of TIMP-1 are not affected by IFN-beta therapy.[32] Currently the mechanism behind the increased expression of MMP9 in MS remains undetermined. The altered expression of genes in the plasminogen activation cascade in untreated MS patients provides a new insight into the possible mechanisms of this dysregulation.

The results presented from this study are consistent with the autoimmune nature of the disease, with the majority of differentially expressed genes involved in immune cell signalling or trafficking. Further evidence for the role of MMPs and the pathway by which they are activated, the plasminogen activation cascade, in the pathogenesis of MS is provided by the identification of increased gene expression in MS patients, which is counter-regulated by IFN-beta therapy. Further functional studies are required to demonstrate that the plasminogen activation pathway is important for the integrity of the blood-brain barrier.

In summary, from our analysis, patients who are not treated present with a gene expression profile that appears to suggest a key problem in this disorder is disruption of the blood/brain barrier, allowing immune cells to access the CNS and then presumably to orchestrate the autoimmune inflammatory response culminating in the degradation of the myelin sheath.

Article Notes

Conflicts of interest: J Lechner-Scott's institution has received non-directed funding from Bayer Australia, Biogen Idec Australia, CSL Biotherapies, Merck Serono Australia and Novartis Pharmaceuticals Australia, and honoraria from Bayer Australia, Biogen Idec Australia, CSL Biotherapies, Merck Serono and Novartis Pharmaceuticals Australia.

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Table 1. Patient characteristics.

	Number (F:M)	Average Age (SD)	EDSS (SD)	Disease Duration (SD)	Treatment duration (SD)
RRMS (no Treatment)	24 (17:7)	48.8 (±12.8)	3.8 (±1.8)	14.4 (±9.7)	-
RRMS (Interferon-β)	13 (11:2)	41.6 (±10.8)	2.7 (±1.1)	10.8 (±7.2)	2.7 (±2.8)
Healthy control	39 (24:15)	48.1 (±11.5)	-	-	-

Table 2. Quantitative real-time PCR results for selected differentially expressed genes.

Gene symbol	Gene name	RRMS No treatment vs. Healthy control	
		p-value	Fold change
MMP9	matrix metallopeptidase 9	0.001	3.80
PLAU	Plasminogen activator, urokinase	0.023	4.36
FCRLA	Fc receptor-like A	0.205	-1.33
IL8	Interleukin 8	0.002	4.42

Table 3. Biological process containing a significant number of differentially expressed genes, common between all analyses (full details in supplementary Table 2).

Biological Process	NoTx vs. HC			IFN-B vs. HC			IFN-B vs. NoTx		
	# of genes	over/ under	P-value	# of genes	over/ under	P-value	# of genes	over/ under	P-value
Immune system process	22	+	1.18E-11	25	+	2.64E-07	19	+	3.49E-07
Response to stimulus	16	+	5.28E-09	19	+	1.41E-06	14	+	5.47E-06
Response to interferon-gamma	7	+	1.57E-04	11	+	1.94E-05	8	+	1.00E-04
Immune response	4	+	4.07E-03	6	+	1.82E-03	4	+	8.85E-03

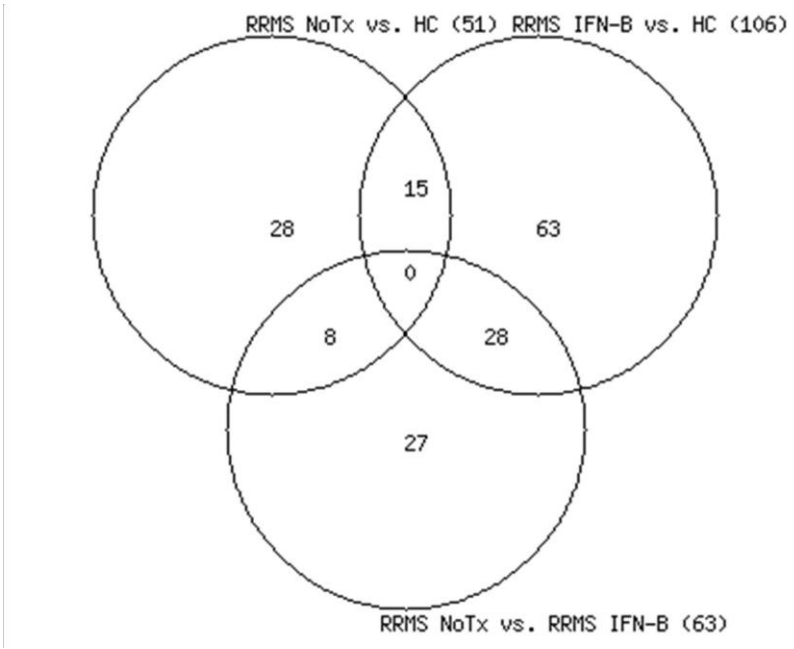

Figure 1. Venn diagram of differentially expressed genes.

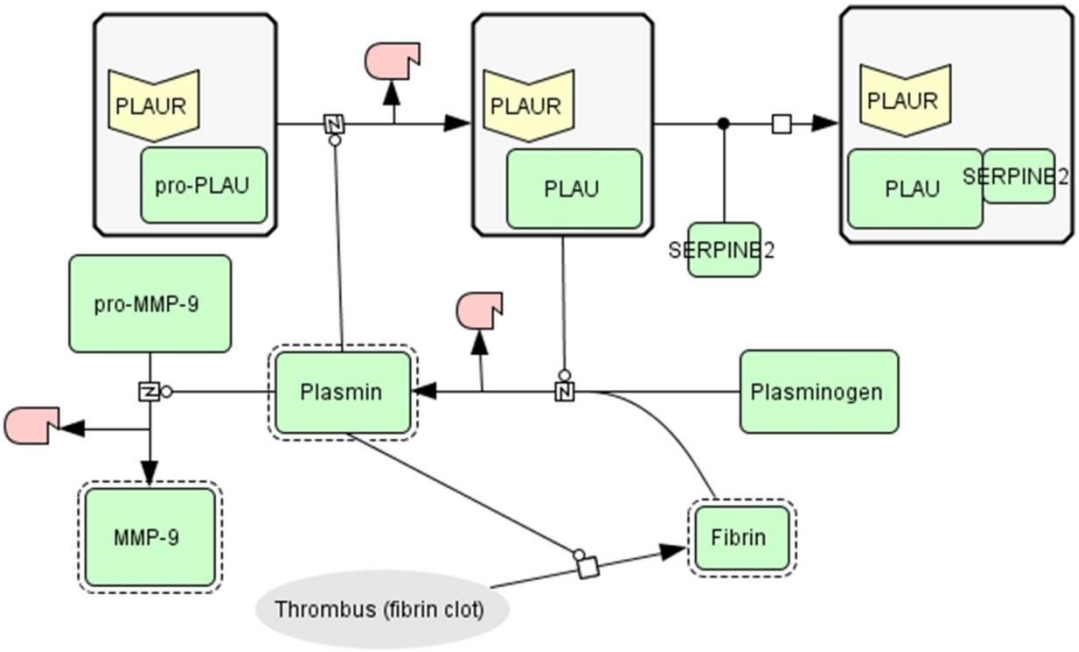

Figure 2. Plasminogen activation pathway, showing activation of MMP9 by plasmin thorough interactions of PLAU, PLAUR and SERPINB2. Open circles represent catalytic reaction, open square represents heterodimer association, square with zigzag represents truncation reaction.

Publication Two

Co-author statement

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I attest that Mathew B. Cox was involved in the conception and design of the study, the collection of patient samples, performed the microRNA expression experiment, analysed the Q-RTPCR data, performed the whole genome gene expression experiment and performed the analysis, and was involved in the writing of the manuscript.

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MicroRNAs miR-17 and mir-20a inhibit T cell activation genes and are under-expressed in MS whole blood

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Abstract

It is well established that Multiple Sclerosis (MS) is an immune mediated disease. Little is known about what drives the differential control of the immune system in MS patients compared to unaffected individuals. MicroRNAs (miRNAs) are small non-coding nucleic acids that are involved in the control of gene expression. Their potential role in T cell activation and neurodegenerative disease has recently been recognised and they are therefore excellent candidates for further studies in MS.

We investigated the transcriptome of currently known miRNAs using miRNA microarray analysis in peripheral blood samples of 59 treatment naïve MS patients and 37 controls. Of these 59, 18 had a primary progressive, 17 a secondary progressive and 24 a relapsing remitting disease course. In all MS subtypes miR-17 and miR-20a were significantly under-expressed in MS, confirmed by RT-PCR. We demonstrate that these miRNAs modulate T cell activation genes in a knock-in and knock-down T cell model. The same T cell activation genes are also up-regulated in MS whole blood mRNA, suggesting these miRNAs or their analogues may provide useful targets for new therapeutic approaches.

Introduction

Multiple Sclerosis (MS) is a disease which affects mainly young people that often leaves them disabled in their most productive years. It is a relatively common disease with an incidence somewhere between 1-2 per 1000 and the rate appears to be increasing [1]. It is assumed that a T cell dysregulation results in demyelination and axonal loss throughout the central nervous system [2]. Most patients have a relapsing remitting course (RRMS), which is unpredictable and is observed as episodes of acute inflammation that results in neurological dysfunction, which in the majority of cases responds to immunomodulatory steroid treatment. Relapsing remitting disease is characterized by some level of myelin repair, whereas in the progressive form myelin repair seems to be insufficient or ineffectual resulting in progressive disability without any observable signs of recovery.

In recent years genome wide association studies have identified that not only is there an association with haplotype in the Human Leukocyte Antigen (HLA) region, but also in both the *IL-2* and the *IL-7* receptor genes, *CD56*, *CD226* and *CLEC16A*, which together are considered to contribute to a predisposition to develop the disease [3,4,5].

In the largest single genome wide association study searching for genetic risk factors for MS, polymorphisms associated with the disease confirmed the importance of immune dysfunction [5]. These identified polymorphisms alone or in combination do not explain the significant differences in immune function associated with this disease.

MicroRNAs (miRNAs) have recently been discovered to be regulatory modulators of gene expression [6]. A striking feature of these mRNA regulators is their evolutionary conservation, indicating their level of importance in the control of gene expression [7]. MiRNAs bind to the 3' UTR of target mRNA through base pairing, resulting in target mRNA cleavage or translation inhibition [8]. On average each miRNA regulates about 200 genes, and the outcome of regulation is cell state and type specific [9]. Their dysregulation has been associated with many diseases, and the potential for modulating their action by therapeutic intervention has excited much interest [10].

This is particularly so in immune-related diseases. The miRNA transcriptome of immune cell subsets are distinct, suggesting that naïve, effector and central memory T cell [11] and regulatory T cell [12] function is dependent on the miRNA regulation. Gross changes to mouse miRNA regulation by deletion of the genes which mediate it, Dicer and Drosher, results in T cell abnormalities and autoimmune disease [13]. Autoimmune diseases such as MS are ameliorated by drugs modulating T cell function like interferon beta and glatiramer acetate, and with monoclonal antibodies specific to T cell surface markers [14]. The genes which are associated with this, and other autoimmune diseases, are predominately expressed in antigen presenting cells and T cells, and there is a marked T cell activation gene expression pattern in MS whole blood [15,16,17]. One of the difficulties of studying multiple sclerosis is the acquisition of samples unaffected by the influence of immunomodulatory treatment. In this report we have investigated miRNA expression profiles of a series of effectively treatment naïve MS patients (i.e. all patients were free of disease modifying therapy for at least 3 months) compared to a healthy age-matched control group. The findings of this study demonstrate the differences between the immune function in MS without the influence of any treatment regiment.

Results

We measured the known miRNA transcriptome in whole blood from 59 MS patients and 37 healthy controls using the Illumina sentrix array matrix. Of these 59 patients, 18 had a primary progressive (PPMS), 17 a secondary progressive (SPMS) and 24 a relapsing remitting course.

The patient demographics are shown in table S1. To ensure we were not measuring effects due to diurnal variation in immune function, all samples were collected between the hours of 9am -1pm for both the MS and healthy control populations. All of our patients were of Caucasian origin, and had not received any immunomodulatory therapy within the previous 3 months. The female to male ratio was 2:1 similar to the disease incidence in the general MS population. The age range for the entire group was 32 to 81 years with a mean age of 54 years, mean time since diagnosis was 20 years, ranging from 0 to 58 years, mean expanded disability status scale (EDSS) was 4.5 ranging from 0 to 6. There was no significant difference in the demographics between the group assessed by microarray and the one assessed by RT-PCR. As expected the disease duration and the EDSS was higher in the SP and PP group compared to the RR group.

MiRNA expression analysis revealed that out of 733 miRNAs assessed, 26 were down-regulated and 1 up-regulated in MS whole blood, based on a false discovery rate of less than 1% (Table 1). The fold change ranged between 1.36 and -1.59. The down-regulation of miRNAs was across all the subtypes of MS. Out of the 27 we focused on miR-17 and miR-20a as the differential expression analysis revealed that these miRNAs were the most significantly different in the MS group compared to the controls. Both miRNAs are known to be involved in immune function and their importance was confirmed by RT-PCR performed between 3 and 6 times. Both miRNAs were differently expressed across all MS subtypes (Figure 1). Most significantly different was miR-17 with a p-value of 7.61 e-05 (see Table 2).

To better define the effects of miR-17 and miR-20a on gene expression we conducted knock-in (with synthetic miRNA) and knock-down (with LNA modified anitmiRs) experiments using Jurkat cells (performed in triplicate). Genes down-regulated by the miR-17 transformants, and up-regulated by the transformants of small interfering RNAs (siRNAs) corresponding to it were identified using Illumina HT12 microarrays and differential expression analysis (table S2). Similarly, genes potentially regulated by miR-20a were identified (table S3). Putative targets for miRNAs can be identified from their sequences, and we identified these using target prediction metadata collated in the miRecords database (http://mirecords.umn.edu/miRecords).

Genes that were differentially expressed as a result of knock-in or knock-down were compared against the predicted targets of the respective miRNA using *in silico* analysis. The resulting lists of genes were considered more likely to be directly affected by the changes in miRNA.

Gene expression data was used to identify gene pathways over-represented in the set of dysregulated genes in the transformed Jurkat cells using GeneGo maps module of Metacore

(Genego, MI) (tables S4, S5). Translation ($P<10^{-4}$ for miR-20a), cholesterol biosynthesis ($P<10^{-5}$ for miR-20a, 10^{-4} for miR-17) and immune response pathways ($P<10^{-4}$ for miR-17) were over-represented in the list of genes dysregulated by changes in miRNA expression.

We then tested if the genes regulated by the miRNAs miR-17 and 20a in Jurkat cells were also over-represented in MS whole blood. We previously reported, from a comparison of whole blood transcriptomes of 150 MS patients and controls, that T cell activation genes were markedly up-regulated in MS ($P<10^{-14}$) [15]. We compared the gene expression profiles from transformed Jurkat cells against non-transformed cells. Putative miR-17 or miR-20a target genes were then compared with gene expression profiles from the MS whole blood samples to identify genes likely to be affected by these miRNAs.

From just the mRNA and transformed Jurkat cell list, the pathways represented were very similar to the pathways over-represented in mRNA alone (Figure 2). Genes which were on all three lists were the most likely to be regulated by the miRNA in MS. From all 3 lists, transcription ($P<10^{-6}$) and immune response pathways ($P<10^{-5}$) were over-represented for miR-20a; and the Th17 immune response pathway ($P<10^{-3}$) for miR-17.

Discussion

Dysregulation of the immune system in MS is considered to be a fundamental aspect of disease initiation and development in this neurodegenerative disease. In this report we have investigated the miRNA transcriptome in a series of MS patients who were not on current immunomodulatory treatment. 59 clinically definite MS patients (mean age of 54.2 years), enriched for PP subtype (n =18), with 24 RR and 17 SP were included in the analysis compared to 37 controls (mean age 48.0 years). The mean disease duration even for the relapsing remitting group was over 15 years. The selection of treatment naïve patients suggests that they were not a group with high disease activity. It could be argued that the significance of the findings of miR-17 and miR-20a being down regulated is underestimated compared to a more active northern European MS population. From the miRNA transcriptome comprising 733 miRNA species only one was up-regulated whereas 26 miRNAs were down-regulated with a false discovery rate less than one. From this data we focused on two miRNAs, miR-17 and miR-20a, as these were all under-expressed in all subtypes of MS and are known to be involved in the control of immune function [18].

Although miR-17 and miR-20a are encoded in the same cistron (miR-17-92), other miRNAs from this cistron were not under-expressed, and these two miRNAs were not identically expressed. There appears to be post-transcriptional regulation of the cistron miRNAs, allowing them to regulate different processes [19]. The translation pathway of lymphocytes is orchestrated by miR-17 and miR-20a [20]. Elevated expression of miR-17 and related miRNAs has been found to be associated with lymphoma and other cancers, and is probably important in maintaining an undifferentiated phenotype and resisting apoptosis [21,22]. A reduction of miR-17-92 miRNAs observed in the present study, has also been associated with differentiation [23] and could potentially be involved in MS-associated T cell activation. We have previously demonstrated that mRNAs from these pathways were up-regulated in all clinical subtypes of MS [15]. In order to test whether these down-regulated miRNAs, miR-17 and 20a inhibit the expression of these T cell mRNAs we used knock in (with the miRNAs) and knock-down (with the antisense or siRNAs) assays of Jurkat cells, a model for T cells.

Jurkat cells are derived from a T cell lymphoma patient, and have traits of a partially differentiated T cell line [24,25]. The transformed Jurkat cells were not stimulated, whereas the T cells in whole blood represent a myriad of subsets in various states of stimulation. However, as these miRNAs under-expressed in MS were known to increase transcription of genes involved with translation and immune response pathways, we expected they may also contribute to the pattern of gene dysregulation seen in MS whole blood mRNA. The set of genes down-regulated by miR-17 and miR-20a in transformed Jurkat cells overlap with the genes and the pathways they are predicted to control, suggesting these miRNAs contribute to the gene dysregulation observed in whole blood.

To our knowledge there have been only five other publications investigating the role of miRNA in MS [26,27,28,29,30], four of which focus on the immune system in MS and the other on active and inactive central nervous system lesions. Otagui *et al.* [29] focused more on up-regulation during relapse. As they only examined small number of patients, their qPCR did not reach statistical significance. Keller *et al.* [28] examined 20 RRMS patients on treatment and identified a number of miRNAs that were up or down regulated. The report by Du *et al*. [26] identified miR-326 as a major determinant of disease in Chinese MS but not neuromyelitis optica (an inflammatory demyelinating disorder thought to be mediated by antibodies targeting aquaporin 4 [31]). In our study we did not identify any statistically significant change in miR-326 between MS patients and controls. Furthermore, subgroup analysis confirmed this finding. Since we do not know why this miRNA was chosen for analysis by Du *et al*. [26] we are

unable to put forward any functional explanation to describe our discrepant results apart from differences observed in MS patients from Asian or Caucasian origin.

The results of this investigation indicate that miR-17 and miR-20a are implicated in the development of MS. Of special interest is miR-17 which we found to be down regulated in peripheral blood. Interestingly, Lindberg *et al.* [30] also identified miR-17 as being associated with MS in $CD4^+$ cells, but the relationship was in the opposite direction to what we observed. This is likely to be a result of a type 1 error given that only 8 patients and 10 controls were examined by Lindberg *et al.* in their initial miRNA expression analysis and only 15 patients and 10 controls in their confirmatory study. This in combination with our study design that included the three major subgroups of MS whereas the patients participating in the Lindberg report were diagnosed with relapsing remitting disease only could potentially explain the differences observed in miR-17 expression. In our analysis we have examined 59 cases against 37 controls thereby providing a more robust statistical analysis. Another possibility for the discrepancy is that we examined expression in whole blood collected in PAXgene RNA tubes, which stabilizes RNA on collection, and includes a high proportion of neutrophil RNA. One possibility is that miR-17 is very labile, and if it degraded in the 90 minutes or so longer it took Lindberg *et al* [30] to purify their miRNA - it could have degraded preferentially in the healthy control cells because of the different regulatory environment. Also, it may be that miR-17 is down-regulated in MS neutrophils cf controls, so that the net miR-17 we measured was less, even if it was more abundant in MS CD3 cells.

Even if the miRNAs under-expressed in MS were not directly contributing to the immune cell signature observed in MS whole blood, the excessive T cell activation signature seen in MS [15,16,17]; and other autoimmune diseases [32] suggest agents which can reduce this activity may be therapeutically beneficial. This study suggests, that the miRNAs identified here as under-expressed in MS, especially miR-17 and miR-20a, are regulators of genes involved in T cell activation.

Methods

Ethics Statement

Human Research Ethics Committees from the Sydney West Area Health Service, The Hunter New England Area Health Service and the Gold Coast Hospital approved this study. Written informed consent was given by all participants.

Patients and sample collection

Peripheral blood samples were collected from MS patients and healthy controls in PAXgene™ Blood RNA tubes (Qiagen, Germany). Patients were recruited from Westmead Millennium Institute, Sydney (NSW), John Hunter Hospital, Newcastle (NSW) and Griffith University Gold Coast (QLD). Healthy controls, with no history of any autoimmune disease or any immunomodulatory therapy, were recruited from Westmead Millenium Institute, Sydney (NSW) and the John Hunter Hospital, Newcastle, (NSW). Patient and healthy controls samples were collected between 0900h and 1300h over the period of one year and collected in PAXgene tubes (PAXgene™ Blood RNA kit Qiagen, Hilden, Germany). Patients had received no immunomodulatory therapy within the last 3 months. MS diagnosis was according to Poser and McDonald criteria. The mean age of the MS patients was 54.2 years, and the controls 40.8 years. The mean age of the PPMS patients was 57.1 years; the mean age of the SPMS patients was 57.2 years and the mean age of the RRMS patients was 49.9 years (see suppl. Table 1).

MiRNA expression analysis

RNA extraction

Patient and healthy control samples were processed concurrently to prevent batch effects. Frozen PAXgene blood RNA samples were thawed overnight at room temperature, centrifuged (4 minutes, 3,000g), and the supernatant discarded. The pellet was resuspended in 1ml TRIzol reagent (Invitrogen), briefly vortexed and homogenised. TRIzol RNA extraction was continued according to manufacturer's instructions. Briefly, 200µl chloroform was added, mixed thoroughly, incubated for 2 minutes at room temperature and centrifuged (15 minutes, 12,000g, 4°C). The aqueous phase containing the RNA was removed and added to 500µl isopropanol, incubated for 10 minutes at room temperature and centrifuged (10 minutes, 12,000g, 4°C). The supernatant was removed and the RNA pellet washed in 1ml 75% ethanol and centrifuged (5 minutes, 7,500g, 4°C) (repeated once). The supernatant was removed and the RNA resuspended in 20µl water.

The total RNA concentration was determined using RiboGreen (Ambion, TX) quantitation according to manufacturer's protocol.

Illumina miRNA SAM methods

MiRNA microarray assay using Illumina sentrix array matrix (SAM) was performed according to manufacturer's instructions (Illumina, CA) using 59 MS patients (24 RRMS, 17 SPMS and 18 PPMS) and 37 healthy controls. Briefly, 200ng total RNA was polyadenylated, reverse-transcribed and biotinylated. The cDNA was used in a second strand synthesis reaction, and universal amplification reaction involving the incorporation of a fluorescent marker. The labelled product was hybridised to the SAM and imaged using the Illumina BeadArray reader.

The microarray data was submitted to the gene expression omnibus (www.ncbi.nlm.nih.gov/geo/) under access number GSE21079.

Differential Expression Analysis

Data was quantile normalised by Illumina's BeadStudio V3. The differentially expressed miRNAs between MS and HC were identified using Significance Analysis of Microarray at a false discovery rate of less than 1%.

Real time PCR confirmation

Two miRNAs, miR-17 and miR-20a, were selected for quantitative PCR confirmation, using endogenous control U49. The sample cohort differs slightly from the cohort used in the microarray experiment (see supplementary table 1) due to small amount of RNA isolated from some samples, and the inclusion of extra samples which were not used on the 96 sample microarray. Final samples numbers were 57 MS (25 RRMS, 14 SPMS and 18 PPMS), and 34 healthy controls.

Reverse transcription of miRNA was performed on 11.35ng total RNA using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems), and pooled miRNA-specific primers. qPCR was performed using TaqMan miRNA assays (Applied Biosystems, CA) specific for each miRNA in triplicate for each sample. The sample was repeated if the standard deviation between triplicates was greater than 0.33.

The relative expression level was calculated using the comparative Ct method, and an unpaired, one-tailed t-test performed to test for significant difference between the entire MS cohort, as well as subtypes compared to the healthy controls.

miRNA-perturbed gene expression analysis

Cell transfection

Jurkat cell cultures were maintained in suspension at 37°C with 5% CO_2 and 90% humidity in RPMI 1640 supplemented with 10% (vol/vol) foetal calf serum and 2 mM L-glutamine. Synthetic miRNA (Sigma) or LNA modified antimiRs (Integrated DNA Technologies, CA) were delivered by electroporation (1µg oligonucleotide per $1x10^6$ cells) using a Nucleofector device and reagent V according to the manufacturer's instructions (Amaxa, Quantum Scientific, QLD), performed in triplicate. After 24 hours, cells were harvested by centrifugation and washed in PBS. Total RNA was then extracted from the cell pellets in TRIzol according to the manufacturer's instructions (Invitrogen, CA).

Whole genome gene expression analysis

500ng total RNA from each sample was biotinylated and amplified using Illumina TotalPrep RNA Amplification Kit (Ambion, TX) according to manufacturer's instructions. The cRNA yield was measured at using RiboGreen RNA quantitation kit (Invitrogen, CA) and 750ng of cRNA sample was hybridized on a human HT-12 expression beadchip (Illumina, CA) profiling 48,804 transcripts per sample. The chips were stained with streptavidin and scanned using an Illumina BeadArray Reader. Three biological replicates were performed for each transfection experiment.

Differential gene expression analysis

BeadStudio V3 was used to cubic spline normalise the data, with Quantile normalisation and baseline transformation to median of all samples performed in GeneSpring GX10. Gene expression profiles from miRNA and antimiRs electroporated Jurkat cells were generated and compared with profiles derived from untreated cells, and those electroporated with non-targeting control oligonucleotides using GeneSpring (unpaired t-test, $p<0.05$). Genes down regulated in response to the introduction of synthetic miRNA or up regulated in response to the introduction of the miRNA targeting anitmiR were cross-matched with the respective miRNA target genes predicted by a range of target algorithms supported by the miRecords target gene meta site (http://mirecords.umn.edu/miRecords/prediction_query.php). In order to capture a broad range of potential target sites, the conditions were set such that each target only need to satisfy the criteria for two out of a possible 11 different search algorithms. The predicted target genes found to be modulated in response to specific changes miRNA

expression in electroporated cells were then examined to determine their potential for biological implications in the context of MS.

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Table 1. miRNAs dysregulated in MS whole blood

Gene Name	Fold-Change	D Value
hsa-miR-768-3p:11.0	1.36	3.81
HS_265.1	-1.13	-3.06
hsa-let-7d	-1.14	-2.85
hsa-let-7f	-1.34	-3.81
hsa-let-7g	-1.19	-3.66
hsa-let-7i	-1.11	-3.27
hsa-miR-106a:9.1	-1.3	-4.09
hsa-miR-126	-1.22	-3.68
hsa-miR-126*	-1.51	-3.66
hsa-miR-140-5p	-1.32	-2.87
hsa-miR-15a	-1.12	-2.89
hsa-miR-15b	-1.07	-3.14
hsa-miR-16	-1.08	-3.18
hsa-miR-17	-1.59	-4.72
hsa-miR-20a	-1.18	-4.44
hsa-miR-20b	-1.25	-3.31
hsa-miR-211	-1.09	-2.92
hsa-miR-27a	-1.32	-3.27
hsa-miR-27b	-1.26	-3.47
hsa-miR-374a	-1.26	-4.27
hsa-miR-454	-1.54	-3.94
hsa-miR-510	-1.13	-2.98
hsa-miR-579	-1.18	-2.77
hsa-miR-623	-1.17	-2.95
hsa-miR-624*	-1.25	-2.83
hsa-miR-93	-1.08	-3.72
hsa-miR-98	-1.49	-4.18

Table 2. P values of relative expression of target miRNAs

	miR-17	**miR-20a**
$MS_{(total)}$	7.61E-05	9.43E-04
RR	4.21E-03	1.95E-02
SP	1.39E-02	5.00E-02
PP	3.20E-02	4.68E-02

P-values of the relative expression as determined by Q-RTPCR of miR-17 and miR-20a in MS patients as a whole and their respective subgroups compared against a healthy aged-matched population.

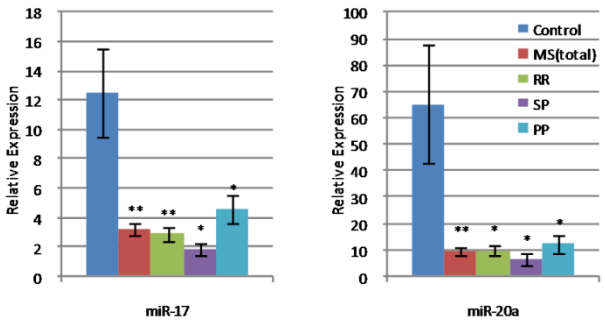

Figure 1. Relative expression of target miRNA.

Relative expression of target miRNA compared to endogenous control miRNA in MS subtypes and healthy controls, validation by Q-RTPCR. The two miRNAs were significantly under-expressed in MS patients compared to healthy controls (*P<0.05, **P<0.01), error bars ± SEM

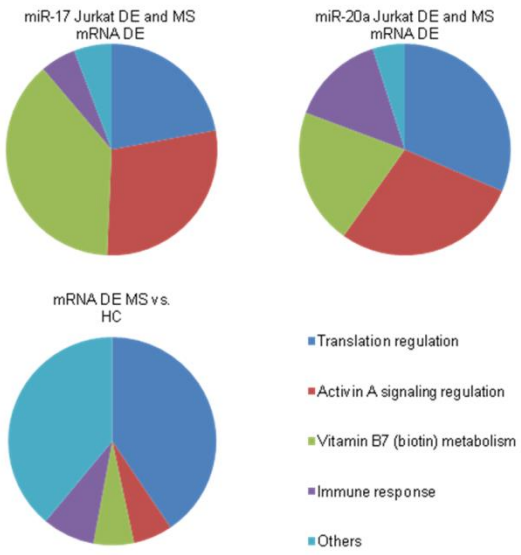

Figure 2. Gene networks dysregulated in MS are regulated by miR-17 and miR-20a miRNAs.

The pathways enriched with genes both dysregulated in MS-whole blood and modulated in miR-17/20a transformed Jurkat cells were identified using Metacore (Tables S4 and S5). Most significant pathways are represented in the form of pie-charts where each slice represents $-\log_{10}$ of the p-value of that pathway as a proportion of the sum of the –log10 of the p-values of the over-represented pathways ($P<0.05$ for miR-17, $p<0.001$ for miR-20a and mRNA (MS vs. HC)). The p-values were determined by Metacore Pathway analysis based on the chi squared value for the expected compared to observed number of genes identified from that pathway in the list of dysregulated genes. (DE differential expression).

Chapter Three - Candidate Gene SNP Association Studies in Multiple Sclerosis

Introduction

Single nucleotide polymorphisms (SNPs) are among the most common genetic variations present within the genome (Sherry et al., 2001). A minority of these variants are non-synonymous and may cause changes in the translated protein through mis-sense or non-sense changes, resulting in a non-functional protein, alternate splice variants or altered gene expression. These non-synonymous variants are often associated with phenotypic differences. The remaining majority of single base changes are synonymous, having no effect on the translated protein code. Synonymous SNPS may also play a role in disease, affect gene expression, or may tag other variants that are associated with disease.

Investigations of SNPs in MS initially yielded discordant results and were conducted using only small sample sizes. It is now known that the effect size of any variant associated with MS is quite small, therefore studies require large samples sizes to reliably identify results, and early studies were thus under-powered. Genome-wide association studies allow the investigation of hundreds of thousands of variants, but also require thousands of samples. These studies have proved successful in identifying a number of variants associated with disease (Patsopoulos et al., 2011), however these variants only account for a small proportion of the underlying genetic risk of the disease (Watson et al., 2012). Candidate gene studies, when performed with appropriate statistical power, have been successful in identifying variants associated with a number of diseases, and require fewer samples than GWAS.

In this thesis I have performed two candidate SNP association studies in MS, examining candidate variants identified through two different approaches:

i. Variants in genes differentially expressed in a whole-genome gene expression study
ii. Variants in a gene with a plausible biological role in the pathogenesis of the disease.

A large sample population, from the Cambridge Neuroscience Group, University of Cambridge and the John Hunter Hospital, Newcastle, Australia, consisting of over 4,500 samples was used.

I have previously identified altered expression of four genes involved in the plasminogen activation cascade, including *MMP9*. Candidate SNPs from within these four genes were genotyped to test for disease association and association with the expression levels of their respective gene. Identifying a relationship between a genetic variant and gene expression within a disease would provide important functional and biological information regarding the pathomechanism of the disease.

Low levels of vitamin D are associated with increased disease risk and risk of relapse (Correale et al., 2009). Vitamin D is able to bind to the vitamin D receptor (VDR) and regulate gene expression through interaction with a vitamin D response element (VDRE) present within the promoter of target genes. The *VDR* has previously been investigated for common variants associated with MS. However these studies have utilised small sample populations and therefore produced inconclusive results. I have investigated two SNPs present within the *VDR* for association with MS. The first variant, rs2228570 (FokI), is a non-synonymous variant that results in a longer, less active protein. rs731236 (TaqI) is a synonymous variant that tags a large LD block.

The importance, and effect, of single base variants is exemplified in diseases such as sickle cell anaemia (caused by the Hb S variant in the haemoglobin gene) (Bunn, 2013) or Cystic fibrosis (caused by variants in the *CFTR* gene) (Lubamba et al., 2012). However many diseases such as MS are thought to have a complex genetic background, involving many genes each imparting only a small risk. Further to this, it is expected that these genes may interact to affect both disease risk and disease expression. To investigate this, we examined epistatic interactions between the *VDR* variations and DRB1*1501, following from work showing that vitamin D is able to influence expression of DRB1*15, through a VDRE present in the promoter (Ramagopalan et al., 2009a).

Publication Three

Co-author statement

Common genetic variants in the plasminogen activation pathway are not associated with multiple sclerosis 2013 *Multiple Sclerosis Journal* Submitted 11 April 2013

Cox, M.B., Bowden, N.A., Scott, R.J., and Lechner-Scott, J.

I attest that Mathew B. Cox was involved in the conception and design of the study, the collection of patient sample, performed all laboratory work, analyses and wrote the manuscript.

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Common genetic variants in the plasminogen activation pathway are not associated with multiple sclerosis

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Keywords: Multiple sclerosis, plasminogen activation pathway, MMP9, matrix metalloproteinase 9

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Abstract

Background: MMP9 is involved in multiple sclerosis aetiology. Previously we identified differential gene expression of plasminogen activation cascade genes in MS patients.

Objectives: Based on our gene expression results we wanted to identify if polymorphisms in the genes associated with this pathway could predict MS risk.

Methods: 1153 trio families, 727 MS cases and 604 healthy controls were genotyped for 17 polymorphisms in MMP9, PLAU, PLAUR and SERPINB2.

Results: No associations were found between the 17 polymorphisms and MS. Also, gene expression levels were analysed according to genotype. No associations were observed.

Conclusions: Despite the consistent evidence for the role of MMP9 and the plasminogen activation cascade in MS, no association between genotype or gene expression was found. This suggests there are other potentially modifiable factors influencing gene expression in MS.

Introduction

Multiple sclerosis (MS) is the most common neurological disorder in young adults, characterised by inflammatory demyelination within the central nervous system (CNS). An aberrant immune response results in the formation of inflammatory lesions within the CNS, causing neuronal damage and axonal loss.

We have previously identified a differential gene expression profile of MS compared to healthy controls (1). This profile contained four genes from the plasminogen activation cascade that showed increased expression in MS compared to healthy controls: matrix metalloproteinase 9 (*MMP9*), plasminogen activator urokinase (*PLAU*), plasminogen activator urokinase receptor (*PLAUR*) and serpin peptidase inhibitor, clade B (ovalbumin), member 2 (*SERPINB2*). The plasminogen activation cascade is involved in the activation of MMP9, and MMP9 is known to be expressed at high levels in MS lesions, in serum, and cerebrospinal fluid (CSF) of relapsing-remitting MS patients (2, 3). MMP9 is involved in the breakdown of the blood-brain barrier, thereby allowing migration of T cells into the CNS. It has been shown that MMP9 is required for the migration of T cells into the CNS parenchyma in experimental autoimmune encephalomyelitis (EAE), a mouse model of CNS inflammation (4). MMP9 can also degrade myelin basic protein (MBP), a key protein in the myelin sheath surrounding axons.

Single nucleotide polymorphisms (SNPs) may affect the expression level of mRNA and proteins, or cause conformation changes of the translated protein, and therefore may have a direct effect on function, resulting in disease. To determine if there was any association with MS disease risk, we examined 17 single nucleotide polymorphisms (SNPs) present in four genes involved in the plasminogen activation cascade pathway; seven in *MMP9*, five in *PLAU*, four in *PLAUR* and one in *SERPINB2*.

Methods

1153 trio families, 727 MS cases and 604 healthy controls from the UK and Australia were used for this study. All MS patients fulfilled the diagnostic McDonald criteria for affection status and informed written consent was obtained from all individuals. Controls recruited in Australia self-reported no MS or family history of MS. The study was approved by the ethics committees of the University of Cambridge and Hunter New England Health Service.

Common variants present within *MMP9*, *PLAU*, *PLAUR* and *SERPINB2* were selected for analysis (table 1) based on a minor allele frequency >0.05, likelihood of tagging large regions of linkage disequilibrium (LD) present within the genes, based on the HAPMAP project data, and the possibility to impact the gene due to location within the gene.

Genotyping was performed using an Applied Biosystems (Foster City, California) platform utilising TaqMan SNP genotyping assays, ABI 7900 HT real-time detection system and SDS 2.1 software as per the manufacturer's instructions. Genotyping was repeated on 277 and 94 samples from the UK and Australian cohorts, respectively, as a quality control measure to ensure genotype accuracy.

Individuals were removed from the analysis if they failed genotyping for greater than 50% of SNPs. In the trio family dataset, all three individuals were removed from the analysis if any individual within the trio was not successfully genotyped for greater than 50% of SNPs. Trio family data was tested for Mendelian errors using PedCheck. Statistical analysis was performed using Unphased version 3.1.4, testing for allelic association, with 1,000 permutations to control for multiple testing correction.

The level of gene expression, as measured previously by whole-genome gene expression analysis (1), was tested for correlation with genotype in a subset of samples included in both gene expression and genotyping studies. This numbered 17 MS patients without disease modifying therapy, 11 MS patient on interferon-beta therapy, and 37 healthy controls.

Statistics were performed in GraphPad Prism, using Mann Whitney or Kruskal Wallis test for association, depending on number of groups (genotypes).

Results and discussion

We examined >4,500 samples to test for MS disease association of 17 common variations across four genes, *MMP9*, *PLAU*, *PLAUR* and *SERPINB2*. 1124 trio families and 556 MS cases/473 healthy controls (UK) and 147 MS cases/122 healthy controls (Australia) passed quality control measures and were included in the analysis.

We found no association between allele frequency and disease risk for any SNP investigated (table 1). Furthermore, none of the genes demonstrated a correlation between expression level and their respective SNP genotypes (data not shown). High levels of MMP9 in serum, CSF and lesions have previously been associated with MS, and we have previously identified increased *MMP9* mRNA expression in MS. However, we have demonstrated that this increased expression of *MMP9* and plasminogen activation pathway genes is not a consequence of the variants tested in this study.

None of the variants investigated in this study have been reported to be associated with MS in GWAS performed to date (5). Of the 17 polymorphisms, only rs2227564 in PLAU has previously been reported to be associated with inflammatory bowel disease in any GWAS (5, 6).

Previous studies have produced discordant results regarding association of two variants in the promoter region of MMP9, a microsatellite CA repeat and the SNP rs3918242. There have been reports of association between rs3918242 with plasma levels of the MMP9 protein (7), as well as increased disease disability and susceptibility to the disease (8, 9) while other studies have found no association (10, 11). There have been no reports of MS disease association of rs3918242 in GWAS reported to date (5). However, neither of these variants have been investigated in the HAPMAP project, and were not investigated in this study.

In conclusion the SNPs investigated in this study show no association with MS disease risk, and do not alter the expression levels of their respective genes. Further studies are required to investigate if for example epigenetic changes like methylation, miRNA or histone modification are responsible for the altered expression.

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Table 1. Unphased output for SNP association testing, with Empirical 5% quantile p-value threshold = 0.004777.

Gene	SNP	Minor Allele	Trio family		Case/Control		p value	Odds ratio (95% CI)
			Transmitted Frequency	Untransmitted Frequency	Case Frequency	Control Frequency		
MMP-9	rs13969	A	0.383	0.390	0.361	0.386	0.124	0.935 (0.857 - 1.019)
MMP-9	rs2274756	A	0.147	0.149	0.141	0.149	0.553	0.946 (0.855 - 1.087)
MMP-9	rs13925	A	0.148	0.151	0.139	0.146	0.398	0.950 (0.842 - 1.071)
MMP-9	rs17576	G	0.346	0.351	0.325	0.358	0.129	0.934 (0.855 - 1.020)
MMP-9	rs3787268	A	0.199	0.200	0.190	0.212	0.456	0.961 (0.864 - 1.068)
MMP-9	rs3918253	C	0.419	0.420	0.388	0.420	0.116	0.935 (0.859 - 0.017)
MMP-9	rs3918241	A	0.145	0.150	0.141	0.146	0.429	0.953 (0.845 - 1.074)
PLAU	rs2227564	T	0.261	0.249	0.256	0.261	0.501	1.033 (0.940 - 1.136)
PLAU	rs2227562	A	0.147	0.138	0.147	0.142	0.363	1.057 (0.939 - 1.189)
PLAU	rs4065	C	0.428	0.412	0.425	0.428	0.378	1.039 (0.954 - 1.131)
PLAU	rs2227551	G	0.285	0.275	0.284	0.291	0.552	1.028 (0.937 - 1.128)
PLAU	rs2227566	C	0.436	0.421	0.438	0.433	0.274	1.048 (0.963 - 1.139)
PLAUR	rs4760	G	0.141	0.162	0.165	0.170	0.434	0.955 (0.851 - 1.072)
PLAUR	rs344787	A	0.459	0.489	0.489	0.492	0.381	0.963 (0.887 - 1.047)
PLAUR	rs2302524	C	0.171	0.172	0.163	0.157	0.679	0.977 (0.873 - 1.092)
PLAUR	rs4251854	C	0.128	0.126	0.118	0.129	0.625	0.970 (0.856 - 1.098)
SERPINB2	rs6104	G	0.222	0.215	0.197	0.207	0.500	0.965 (0.870 - 1.071)

Publication Four

Co-author statement

Potential association of vitamin D receptor polymorphism Taq1 with multiple sclerosis 2012 *Multiple Sclerosis Journal,* 18(1):16-22

Cox, M.B., Ban, M., Bowden, N.A., Baker, A., Scott, R.J., and Lechner-Scott, J.

I attest that Mathew B. Cox was involved in the conception and design of the study, the collection of patient sample, and conduct of the laboratory work, performed all analyses and wrote the manuscript.

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Potential association of vitamin D receptor polymorphism Taq1 with multiple sclerosis.

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Keywords

Epistasis, genetic, genetics, HLA DRB1*1501, human, multiple sclerosis, single nucleotide polymorphism vitamin D receptor

Abstract

Background: The environmental influence of sun exposure and vitamin D in particular and its implication with multiple sclerosis (MS) has recently received considerable attention. Current evidence based on genetic and epidemiological studies indicate that vitamin D is implicated in the aetiology of this disease.

Methods: We examined two common variants in the vitamin D receptor (*VDR*) gene in 1153 trio families and 726 cases and 604 controls. We also examined epistatic interactions between the *VDR* SNPs rs731236 and rs2228570 with the tagging single nucleotide polymorphism (SNP) rs3135388 for the HLA-DRB*1501 locus containing a highly conserved vitamin D responsive element within its promoter region.

Results: We found weak evidence for an association between the rs731236C allele and MS, while there was no direct association with rs2228570. When examining the interaction between the VDR gene variations and the DRB1*1501 tagging SNP a more complex relationship was observed. Although the interaction was not statistically significant, there appeared to be a trend of increasing risk of MS in participants who were homozygous for the HLA-DRB1*1501 allele in association with the more active form of the VDR (Fok1).

Conclusion: We have identified weak evidence of an association between a common variation within the *VDR* gene and MS, in the largest study reported to date of this candidate gene. There appears to be a relationship between polymorphisms in the VDR and the risk of MS, which is potentially modified by HLA-DRB1*1501.

Introduction

Multiple sclerosis (MS) is an auto-immune disease of the central nervous system, affecting approximately 1 in 1000 people in the northern European population, representing the most common disabling neurological condition in young adults. Onset of disease is often between the ages of 25 and 35 years and three times as many women are affected compared to men.[1]

While the aetiology of the MS remains elusive, both genetic and environmental factors are thought to be involved in the risk of developing disease. Genome-wide association studies (GWAS) have identified several genes implicated in the aetiology of MS; however, these genes are thought to only exert a small effect on disease risk with individual odds ratios (ORs) typically in the range of 1.1 to 1.3.[2] Approximately 30 years ago the human leucocyte antigen (HLA) region was found to be associated with MS.[3] Despite extensive investigations, the HLA-

DRB1*1501 allele has remained the strongest genetic risk factor for developing MS.[3] A recent meta-analysis of GWAS data identified rs3135388, the tagging SNP of DRB1*1501, as the most significantly associated SNP in MS ($p = 3.8 \times 10{-}225$, OR 2.75).[4]

Environmental factors are also implicated in disease risk and there is evidence supporting an association of infectious agents with MS, in particular Epstein–Barr virus (EBV). A history of EBV infection is more common among patients with MS and such a history is associated with a 15 times greater risk compared to those never infected.[5] Cigarette smoking has been shown to be associated with increased susceptibility to MS, the risk increasing with number of cigarettes smoked. A recent meta-analysis confirmed the increased risk (relative risk 1.48, 95% CI 1.35–1.63) of MS associated with smoking, which supports a relationship with increased susceptibility in younger patients.[6]

The prevalence of MS has been found to vary according to a latitude gradient,[7] with greater prevalence in more polar latitudes compared to more equatorial ones.[8–10] The latitude gradient has been shown to be strongest in populations of European descent, but absent in populations of non-European descent, which suggests gene–environment interactions.[7] It is possible that the observed latitude gradient in MS prevalence may be associated with UV-light exposure and subsequent 1,25-hydroxy vitamin D [$1,25(OH)_2D_3$] production, suggesting an intimate relationship between vitamin D synthesis and function with disease risk. In humans, the majority of the active form of vitamin D, $1,25(OH)_2D_3$, is synthesised through a reaction pathway requiring exposure to ultraviolet-B (UV-B) light. Lower $25(OH)D_3$ and $1,25(OH)_2D_3$ serum levels have not only been found to increase the risk of MS, but also to be associated with relapse.[11–13] $1,25(OH)_2D_3$ inhibits proliferation of both $CD4^+$ T cells and myelin basic protein (MBP)-specific T cells of MS patients. It enhances anti-proliferative cytokines and up-regulates T reg cells.[11]

$1,25(OH)_2D_3$ regulates gene expression through its binding to the vitamin D receptor (VDR), which in turn interacts with the vitamin D responsive element (VDRE) present within the promoter region of a number of target genes. Recently, a VDRE within the promoter region of the HLA class II histocompatibility complex antigen β subunit (HLA-DRB1) has been described, and shown to be a binding target for VDR.[14] This VDRE is conserved in DRB1*15 haplotypes whereas non-DRB1*15 haplotypes have significant variation in the VDRE sequence. The active form of vitamin D, $1,25\text{-}(OH)_2D_3$, has also been found to stimulate expression of the DRB1*1501 promoter. This potentially provides a direct link between HLA-DRB1*1501 and exposure to UV light, and subsequent vitamin D production with MS susceptibility.

Due to its potential role in MS pathogenesis, *VDR* has been considered an excellent candidate gene in many single nucleotide polymorphism (SNP) association studies. Many of the reports of the association studies between *VDR* and MS have often been limited due to small patient groups resulting in a series of contradictory associations.[15–25] We have examined rs2228570 (Fok1), a non-synonymous SNP located in the translation start site and rs731236 (Taq1), a synonymous variation in the ligand binding domain of the *VDR* gene in approximately 4500 samples, composed of a case–control population and a large family trio dataset. This is the largest candidate gene study to date to investigate the association of these two common variants in the *VDR* gene with MS. In addition we investigated if an increased risk of developing MS was due to epistatic interactions between variations in the *VDR* gene in combination with the known MS risk factor, HLA-DRB1*1501.

Patients and methods

Patients

We investigated 1153 trio families (affected individual and both parents), 560 MS cases and 480 healthy controls from the UK collected through the University of Cambridge, UK, as described previously;26 and 167 MS cases and 124 healthy controls from Australia, recruited from the John Hunter Hospital Multiple Sclerosis Clinic, Newcastle, Australia (Table 1). All MS patients fulfilled the diagnostic McDonald criteria for affection status27 and informed written consent was obtained from all individuals. Controls recruited in Australia self-reported no MS or family history of MS. The study was approved by the ethics committees of the University of Cambridge and Hunter New England Health Service

Methods

Statistical power was determined using Quanto version 1.2.4,[28] based on the HAPMAP-CEU population (Utah residents with Northern and Western European Ancestry) Minor allele frequency (MAF) of 0.44 for both *VDR* SNPs and an odds ratio of 1.2. For the trio cohort, there was greater than 85% power to detect an association, while in the case–control cohort, we had greater than 64% power to detect a difference.

Two SNPs in the *VDR* gene, rs2228570 (Fok1) and rs731236 (Taq1) residing at positions 48272895 and 48238757 on chromosome 12 (NCBI, build 37.1), were assessed to determine if their frequency was different in the MS population compared to the control population.

Genotyping was performed using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, California, USA) and analysed using an ABI 7900 HT real-time detection system and SDS 2.1 software as per the manufacturer's instructions. Genotyping was repeated on 277 and 94 samples from the UK and Australian cohorts, respectively, as a quality control measure to ensure genotype accuracy.

Individuals were removed from the analysis if they failed genotyping for both SNPs. In the trio family dataset all three individuals were removed from the analysis if any individual within the trio was not successfully genotyped for both SNPs. Trio family data was tested for Mendelian errors using PedCheck.[29] Statistical analysis was performed using Unphased 3.1.4, with 1000 permutations to calculate empirical 5% significance thresholds within each dataset.[30] Linkage disequilibrium (LD) was calculated using HaploView 4.1.31

Odds ratios and p-values for previous studies were calculated using InStat software (Graphpad, San Diego, CA, USA).

The DRB1*1501 tagging SNP, rs3135388 was typed in the Australian cohort using a TaqMan SNP genotyping assay and the UK samples were genotyped as described previously.[32] Gene–gene interaction analysis between rs3135388 and both *VDR* SNPs, rs2228570 and rs731236, was performed using Unphased 3.1.4. Unphased is capable of handling both nuclear family data and unrelated subjects data simultaneously, and implements a maximum likelihood inference on haplotype and genotype effects.[30] The gene–gene interaction test compares the odds ratio of the full haplotype to that expected if the conditioning and test haplotypes had independent risks.[33] Power was calculated for the gene–gene interaction using Quanto, based on the *VDR* SNPs as described previously, and the rs3135388 HAPMAP CEU MAF of 0.19, and OR of 2.75. In the case–control cohort we had >80% power to detect an interaction with an OR = 1.5, while in the Trio data set >93% power to detect an interaction with an OR of 1.3.

Results

In total, 1153 trio families, 727 MS cases and 604 healthy controls were genotyped for two common SNPs in the *VDR* gene. Genotypes for both SNPs were successfully obtained for greater than 97% of samples in both the trio family cohort and the case–control cohort. There was no significant deviation from Hardy–Weinberg equilibrium, and no Mendelian errors present in the trio family cohort. There was no LD between the two SNPs in the *VDR* gene (case–control $R^2 = 0.0$, $D' = 0.004$, trio cohort $R^2 = 0.0$, $D' = 0.005$) (Table 1).

The minor allele (C) of rs731236 was found to be over-represented in MS in the combined case–control and trio family dataset (p = 0.036, OR = 1.096 (1.006–1.193)) which remained significant after permutative correction (empirical threshold = 0.038) (Table 2). In the separate cohorts, there was a higher frequency of the minor allele in cases compared to healthy controls and a trend of over-transmission in the trio cohort; however, this did not reach statistical significance for either cohort (p = 0.100 and p = 0.115, respectively).

There was no evidence of disease association with the *VDR* SNP rs2228570 in the combined cohort (p = 0.804, OR = 0.989 (0.908–1.078)). Similarly, there was no association in the case–control analysis, or over-transmission of either allele in the trio family dataset (Table 2).

Analysis of the combined case–control dataset and trio family dataset identified a trend in gene–gene interaction between rs2228570 and rs3135388 with MS risk (p = 0.011). However, this remained slightly above the adjusted empirical 5% quantile significance threshold (p = 0.010). The presence of the DRB1*1501 tagging SNP, rs3135388A, increased disease risk, whether heterozygous or homozygous (Table 3). DRB1*1501 was associated with increased MS risk regardless of FokI genotype (OR 2.783, CI 1.824–4.246). When considering the two genotypes together, a seven-fold increased odds ratio (7.198 CI, 2.62–19.78) of MS was observed for those who were DR15A/A and FokIC/C compared to the most common genotype (DR15TT, FokIC/T). There was no evidence of gene–gene interaction between rs3135388 with rs2228570 in the separate case–control dataset (p = 0.06, adjusted threshold = 0.03) or in the trio family dataset (p = 0.01, adjusted threshold = 0.005). There was also no evidence of interaction between rs731236 and rs3135388 (p = 0.043).

Discussion

While it is known that there is a significant genetic component involved in the risk of developing MS, most genes which have conclusively been shown to be associated with disease have only small effect sizes. The main environmental factor that has been consistently demonstrated to be associated with disease risk is the latitude gradient8 which correlates with sunlight exposure and, by association, vitamin D production. Since vitamin D activity requires a receptor mediated response any differences in the vitamin D receptor may play a role in the aetiology of the disease.

Previous studies have focused on four main variations within the *VDR* gene, rs2228570 (Fok1), rs731236 (Taq1), rs1544410 (Bsm1) and rs7975232 (Apa1), and increased disease risk has been

associated with the three SNPs, rs731236[20], rs1544410[15] and rs7975232.[15,16,20] These studies, however, have only used small sample cohorts and, as such, the positive associations may represent type 1 error, or conversely, the negative reports may be due to the lack of power these studies had to detect weak effects (Table 4[).17–25] We therefore have examined two of these SNPs, rs2228570, which encodes a functional variant, and rs731236, which is in LD with rs1544410 and rs7975232, in the largest candidate gene study of these variations to date.

In the current study, rs731236C within the *VDR* gene was found to have weak evidence of association with an increased risk of developing MS, although with only a small OR of 1.1 (95% CI 1.006–1.193). rs731236 is a synonymous variation present in exon 9 of the *VDR* gene, and has been reported to be in strong LD with rs1544410 and rs7975232 and part of a LD block of ~13 kb.[34] These SNPs have previously been examined in GWAS studies, but associations have not been reported. This region is part of the ligand-binding domain of the VDR and therefore any structural variation in the protein may result in differential binding specificity of vitamin D. Although rs731236 is synonymous, and rs1544410 and rs79752322 are intronic and therefore unlikely to affect the protein structure, they may be in LD with another variant which may result in structural change.

rs2228570 is a non-synonymous SNP located at the translation start site. The T>C change ameliorates the start codon, resulting in a three amino acid shorter protein translated using a start codon at position +10 to +12.[35] The shorter version of the protein has been shown to have greater transcriptional activity, due to increased binding affinity with TFIIb.[36] $1,25(OH)_2D_3$ was also shown to induce higher levels of transcription in the presence of the short version of the protein compared to the wild-type longer version. rs2228570 has previously been associated with an increased risk of Graves' disease[37] and cancer,[38] as well as affecting immune cell behaviour.[39] rs2228570 is not in LD with any other SNP,[18,20–24] and is not present on the Illumina 370K or 610K SNP array, or the Affymetrix 500k array, and therefore has not previously been directly examined in any MS GWAS to date.[40–43] Previous candidate gene studies have not found any association with disease risk;[18,20–25] however, the longer protein variant has been associated with increased disease severity.[44]

In this large case–control and trio family study, we have identified an association between a LD block tagging SNP for Taq1 with increased risk of developing MS. There was a trend for an epistatic interaction between Taq1 (rs731236) and the HLA-DRB1*1501 tagging SNP; however, this did not reach significance thresholds.

rs2228570 was not found to be associated with an increased risk of developing MS. However, based on an OR of 1.2, the power of our study to identify an effect (at p = 0.05) in the case–control group is 64%, due to the high minor allele frequency (MAF) and in the trio cohort is >85%, therefore we cannot rule out that variations in this gene may have a smaller effect on susceptibility that could not be detected with the sample size investigated.

The recent finding of a VDRE in the HLA-DRB1 promoter, and the ability of vitamin D to stimulate expression of the *VDR* promoter, may indicate a link between the known DRB1*1501 association, and the vitamin D link with MS. The association between genetic variation in the VDR gene and DRB1*1501 has previously been examined in 71 patients with MS and 67 controls. DRB1*1501 was more prevalent in rs7975232G MS cases compared to the rs7975232G controls and the rs7975232T MS cases; however, this was not significant after multiple testing correction.[16] Similar results were found for the rs7975232G/rs1544410A haplotype. Gene–gene interaction between the DRB1 locus with three *VDR* SNPs, rs2228570, rs731236 and rs11568820, and MS risk was also examined in a larger cohort;[22] however, no association was found.

In the current study, gene–gene interactions between rs2228570 and rs3135388 in the combined data set approached significance for association with MS; however, this was not supported in either the separate case–control or trio family datasets. Interestingly, risk does not appear to differ according to rs2228570 genotype when the major (T) allele of rs3135388 is present. However, in patients homozygous for rs3135388A (i.e. DRB1*1501 positive), risk appears to increase as the number of rs2228570C alleles increase (i.e. the shorter version of the protein with greater transcriptional activity). This appears to support the notion that the vitamin D response element on HLADR1*1501 is influenced by the more active form of the Fok1 allele.

In light of these results, and taking previous studies into account, the *VDR* gene may be involved in disease risk. It is also possible that the variations in the gene may play a role in disease risk under certain circumstances, such as vitamin D insufficiency or lack of sunlight.[21] Investigations of these effects as well as a possible role in disease progression would be of interest in further studies in much larger sample sizes.

Article notes

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Table 1: Patient demographics for the Australian and UK cohorts.

	Australia	UK
Average age of onset	34.16	29.17
Status (%)		
RR	66.1	59.6
SP	18.2	29.2
PP	7.3	11.3
CIS	8.5	0
EDSS (%)		
<3	33.9	30.4
3-6	38.2	24.8
≥6	26.7	44.9

Table 2: Statistical results for VDR SNPs rs2228570 and rs731236 in the combined case/control and trio-family cohort, and the cohorts separately. Empirical 5% quantile threshold calculated by permutative testing, for both SNPs in each cohort. MAF = minor allele frequency.

SNP	Dataset	MAF Case	MAF Control	χ2	P value	Empirical 5% quantile threshold	OR (CI 0.95)
rs2228570 (FokI)	Combined	-	-	0.061	0.804	0.038	0.989 (0.908–1.078
	Case/ Control	0.39	0.38	0.001	0.970	0.025	1.003 (0.855-1.177)
	Trio families	-	-	0.527	0.468	0.288	0.956 (0.846-1.08)
rs731236 (TaqI)	Combined	-	-	4.385	**0.036**	0.038	1.096 (1.006–1.193)
	Case/ Control	0.42	0.38	2.662	0.100	0.025	1.141 (0.9736-1.338)
	Trio families	-	-	2.483	0.115	0.288	1.103 (0.976-1.247)

Table 3: Gene-gene interaction between the HLA-DRB1*1501 tagging SNP (rs3135388), and the *VDR* gene polymorphisms, for the combined case/control and trio data sets. Empirical 5% quantile significance threshold = 0.010. The most common genotype in the control population (rs3135388 T/T, rs2228570 C/T and rs731236) were used as the reference genotype for calculation of the odds ratio.

rs3135388	**rs2228570**	**OR (95% CI)**	**P**
T/T	T/T	1.042 (0.8617 - 1.259)	0.011
T/T	C/T	1	
T/T	C/C	1.136 (0.8827 - 1.463)	
A/T	T/T	3.064 (2.518 - 3.728)	
A/T	C/T	3.206 (2.665 - 3.857)	
A/T	C/C	2.284 (1.75 - 2.982)	
A/A	T/T	2.783 (1.824 - 4.246)	
A/A	C/T	5.499 (3.855 - 7.843)	
A/A	C/C	7.198 (2.62 - 19.78)	
rs3135388	**rs731236**	**OR (95% CI)**	**P**
T/T	T/T	1.114 (0.8798 - 1.411)	0.036
T/T	C/T	1	
T/T	C/C	0.7441 (0.6103 - 0.9073)	
A/T	T/T	2.744 (2.118 - 3.554)	
A/T	C/T	2.553 (2.124 - 3.069)	
A/T	C/C	2.804 (2.299 - 3.419)	
A/A	T/T	4.468 (2.383 - 8.377)	
A/A	C/T	4.867 (3.234 - 7.324)	
A/A	C/C	3.014 (2.019 - 4.498)	

Table 4: Previous association studies of common SNPs in the VDR gene with MS, with study cohort size and association P-values.

Cohort Characteristics	**VDR gene polymorphisms**				**Reference**
	rs2228570 (FokI)	**rs731236 (TaqI)**	**rs1544410 (BsmI)**	**rs7975232 (ApaI)**	
77 MS 95 HC (Japanese)	-	-	OR 2.53 (p = 0.07)	OR 1.61 (p = 0.14)	15, 16
419 MS, 422 HC (northern European Caucasian)	OR 1.14 (p = 0.28)	OR 1.00 (p = 0.97)	-	-	18
104 MS , 104 HC (northern European Caucasian, Australian)	OR 1.3 (p = 0.26)	OR 0.59 (p = 0.02)	-	OR 1.5 (p = 0.05)	20
150 twin sets (Canada)	OR 0.68 (p = 0.15)	-	-	-	21
212 MS, 289 HC (Netherlands)	OR 0.97 (p = 0.87)	OR 0.96 (p = 0.84)	-	OR 0.89 (p = 0.39)	23, 24
136 MS, 235 HC (Tasmania, Australia)	OR 1.24 (p = 0.20)	OR 1.16 (p = 0.36)	-	-	22
214 MS, 428 HC (>90% self-reported white ancestry, USA)	OR 0.83 (p = 0.41)	OR 1.24 (p = 0.35)	OR 1.2 (p = 0.4)	OR 0.91 (p = 0.70)	25

Chapter Four - Discussion

Discussion

Multiple sclerosis is an autoimmune disorder directed against the central nervous system (CNS). While there is a large amount of knowledge regarding the mechanism of disease activity, the aetiology of the disease remains elusive. It is known that both genetic and environmental factors are involved in the disease. While there has been great progress in the identification of these factors, it is estimated that the genetic loci currently associated with MS, account for only ~30% of the genetic risk (Watson et al., 2012). The main risk factors for developing MS are vitamin D deficiency and presence of the DRB1*1501 allele. There are at least 56 other genetic loci associated with MS, but there remains an unknown genetic contribution to the disease (IMSGC et al., 2011).

In this thesis, I have investigated the genetics of MS through two methods, RNA expression analysis and candidate SNP association studies. The aim of this has been:

> To identify genes and miRNAs, and their associated biological pathways, that are dysregulated in MS patients. This has been achieved through the use of whole-genome gene expression arrays and miRNA expression arrays in well-characterised MS case and control populations.
>
> To identify common variants in candidate genes that are associated with disease risk. This was performed using case-control and trio family approaches.

Gene expression

Whole-genome gene expression studies have been performed with the aim of identifying genes and pathways associated with the aetiology of MS, and the effect of treatment. Few of the studies published to date are directly comparable due to the different methodologies employed and thus far no cohesive gene expression pattern has been identified. The main reported outcome of these studies, however, has been differential expression of genes that are implicated in the immune system. In this thesis I have similarly reported on the identification of an enrichment of dysregulated genes from whole blood involved in immune system processes in MS cases compared to healthy controls. We also identified dysregulation of gene expression in the plasminogen activation cascade and *MMP9*, which was partially ameliorated by treatment with interferon-beta.

The identification of dysregulation of the plasminogen activation cascade in MS provides information on a potential disease associated pathway in MS. MMP9 is known to be expressed at higher levels in MS compared to healthy controls, and is thought to be involved in the breakdown of the blood brain barrier, which is confirmed by higher expression after stroke (Agrawal et al., 2008), allowing lymphocytes egress into the CNS. The cause of the increased MMP9 gene and protein expression is unknown. Our data identified dysregulated expression in the plasminogen activation cascade, one of the pathways involved in MMP9 activation. This may provide insight in to the cause of the dysregulation of MMP9, and therefore the pathomechanism of MS.

Recent whole-genome gene expression studies in MS have started investigating novel approaches to examine the gene expression profile of MS under different conditions. However these studies may still have similar issues to earlier studies utilising small or heterogeneous sample populations.

In one study, whole-genome gene expression was investigated in 31 benign multiple sclerosis patients compared to 36 RRMS patients (Achiron et al., 2012). The RNA-polymerase-I (POL-1) pathway was enriched for genes under-expressed in benign MS compared to RRMS. This decreased activity of the POL-I pathway may result in lower disease activity through increased apoptosis, specifically of autoimmune cells. Although this study investigated a relatively large sample population, the patients in both groups were heterogeneous in terms of therapies, which therefore does not allow for direct comparisons.

A more recent study, stratifying MS patients by DRB1*1501 status compared to healthy controls identified large numbers of genes differentially expressed in MS patients, with only a few differentially expressed genes in both the DRB1*1501 positive and negative individuals (Apperson et al., 2013). This suggests a difference in the underlying disease mechanism of MS based on DRB1*1501 status, and the response to environmental factors. However, the study was based on only 20 cases and 20 controls, such that when more detailed sub-categorization is undertaken the number of samples in each group is reduced (for example by stratifying by DRB1*1501 status) and robust statistical significance disappears.

Whole-genome gene expression studies in MS clearly show the complex nature of MS, through the complex, discordant results produced. These studies do, however, confirm the autoimmune nature of the disease, with altered expression of many immune-related genes. There is also the potential to identify areas for further investigation, such as the plasminogen

activation pathway, and its role in the disease. Through well designed studies, investigating specific questions and novel findings may shed new light on the pathomechanism of disease.

miRNA expression

miRNAs are a small species of RNA that are involved in the post-transcriptional regulation of mRNA expression. miRNAs are transcribed similarly to mRNA, but undergo post-transcription modification into mature miRNA. miRNAs are involved in gene expression control by inhibiting translation of their target mRNA through translation inhibition or mRNA degradation. There is therefore a potential for miRNAs to be associated with altered gene expression and disease pathogenesis.

During the course of my studies into the role of miRNAs in MS I have identified the differential expression of 27 miRNAs in whole blood of MS patients compared to healthy controls. The effect of two miRNAs that were under-expressed in MS, miR-17 and miR20a, was investigated through consilience of an MS whole-genome gene expression profile dataset and a cell transfection model, to confirm the target genes of these two miRNAs and define their role in MS. These two miRNAs are encoded in the same cistron, miR-17-92, and others have confirmed an association of the miRNAs in this cistron with MS. However the miRNAs have been reported to be over expressed in T cells (De Santis et al., 2010, Lindberg et al., 2010), and under-expressed in B cells (Sievers et al., 2012) and whole blood (Cox et al., 2010). These findings support the likely involvement of these miRNAs in the regulation of T cell activation, with decreased expression resulting in excessive T cell activation.

Similar to gene expressions studies, numerous studies have identified miRNAs differentially expressed in MS and there have been some functional studies to confirm predicted roles in translational regulation or disease pathogenesis. Many miRNA expression studies to date have produced discordant results, with little overlap; different miRNAs are differentially expressed and in some cases expressed in the opposing direction (Huynh and Casaccia, 2013). Nevertheless, several studies have identified altered expression of miRNA that are likely to result in an increased pro-inflammatory T cell state or are associated with T cell activation or regulation (Huynh and Casaccia, 2013, Guerau-de-Arellano et al., 2012). Since the publication of my study other investigators have continued to identify and define the role of miRNAs in MS.

Under-expression of miR-15b, miR-23a, and miR-223 in serum of MS patients compared to healthy controls was not replicated in PBMCs (Ridolfi et al., 2013). The latter comparison showed miR-23a and miR-223 were over-expressed in MS compared to controls, and miR-15b showed no differential expression (Ridolfi et al., 2013). This may be due to the small sample size of their study, or as the authors suggest, a result of sequestration of these miRNAs in cells culminating in decreased serum miRNA (Ridolfi et al., 2013).

Circulating miRNAs from plasma were investigated in four MS patients and matched controls (Siege et al., 2012), identifying seven miRNAs differentially expressed, two of which, miR-22 and miR-422a, have previously been reported. Interestingly, while five of the dysregulated miRNAs were predicted to target genes associated with immunity, miR-648 was predicted to target myelin-associated oligodendrocyte basic protein (*MOBP*), and miR-572 predicted to target neural cell adhesion molecule 1 (*NCAM1*).

A larger study investigating circulating miRNAs in 10 RRMS, 9 SPMS and 9 healthy controls replicated findings of blood cell and brain tissue studies (Gandhi et al., 2013). miRNAs in the let-7 family, the miR-17-92 family and miR-454 were differentially expressed in MS, and miR-92a-1* and miR-454 were also associated with EDSS and disease duration (Gandhi et al., 2013).

More studies are investigating the relationship between mRNA and miRNA expression simultaneously, using both biological and bioinformatics approached. 104 miRNAs were differentially expressed in whole blood in MS compared to health controls, with Let-7g and miR-150 showing the strongest association (Martinelli-Boneschi et al., 2012). The functional role of these two miRNAs was examined through *in silico* analysis of predicted targets and a direct approach correlating miRNA expression with whole-genome gene expression from the same patients. However there was no apparent overlap between the predicted target genes identified by the two approaches.

Although there are differences in the results of these studies, it is clear that miRNA dysregulation in MS may lead to altered expression of genes involved in the immune system, specifically genes associated with T cell activation. It is also apparent that different cell types have different miRNA expression profiles. Although this results in a greater complexity, it indicates that there is very specific and sensitive gene expression regulation occurring. This may make potential therapeutic targeting of miRNA difficult, however the identification of the expression profiles from all cell subsets may provide evidence as to the underlying biological disruption occurring in MS.

Vitamin D Receptor

Low levels of vitamin D are associated with an increased risk of developing MS. Vitamin D exerts its biological influence by binding to the vitamin D receptor (VDR), forming a complex that then binds to the vitamin D response element (VDRE) present within the promoter region of its target genes. Due to the known vitamin D association with MS risk, the VDR has been considered a potential candidate gene in the pathogenesis of MS.

During the course of my work I have identified some evidence of an association between a polymorphism (rs731236 otherwise known as TaqI) in the *VDR* and MS (p = 0.036, OR 1.096 (1.006 – 1.193)) in a combined case/control and trio family patient population. This association was not identified in the separate case/control and family trio populations. There was no evidence of an association between a second VDR polymorphism (rs2228570) and MS.

Since the publication of my study, our data contributed to a meta-analysis of *VDR* variants in MS. This study failed to identify any association with MS, when examining case control studies (Huang and Xie, 2012). A meta-analysis of six studies that included a total of 1775 cases and 1830 controls, no disease association with rs2228570 (OR = 0.99 (0.87 – 1.14), p = 0.93) was observed. In a second meta-analysis, (that included eight studies, comprising 2472 cases and 2446 controls), no association with rs731236 (OR = 1.12 (1.00–1.26) p = 0.06 was found. Although this study appears to contradict our findings of a disease association with rs731236, only our case-control data was included in the meta-analysis. In our own study, the association was identified in the combined case/control and trio sample population consisting of over 4,500 individuals, which was larger than that investigated in the meta-analysis. A family based study, involving large, extended families, composed of 1,360 MS patients and 1,677 unaffected family members examining 41 *VDR* SNPs, including rs731236 and rs2228570, revealed no association with MS (Orton et al., 2011). There are a number of confounding factors associated with these types of study, the main one being the paucity of environmental data and how this is likely to influence any genetic association. Extension of this study would require the accurate assessment of vitamin D levels for each and every patient such that association data could be adjusted for variance in this important environmental factor.

I also identified a trend of an epistatic interaction between rs3135388A (i.e. DRB1*1501 positive patients) and rs2228570C where, in DRB1*1501 positive individuals, the presence of rs2228570C is associated with increased risk in a dose dependent manner. The rs2228570 variation causes a functional change, with the C allele producing a slightly smaller protein that has greater transcriptional activity (Jurutka et al., 2000). This finding of increased risk of MS for

carriers of both rs3135388A (i.e. DRB1*1501 positive patients) and rs2228570C alleles, provide evidence of an interaction between the DRB1*1501 allele and rs2228570 allele. Interestingly, in another study there was over-transmission of the rs2228570T in DRB1*1501 negative patients, while there was no difference in DRB1*1501 positive patients (Orton et al., 2011). Although not directly comparable to our results, this provides further evidence of interactions between the *VDR* variant and DRB1*1501 in MS.

In a co-segregation study of three *VDR* variants, rs2228570, rs7321236, and rs1989969 with DRB1*1501, there was no association found for rs2228570 and rs1989969 (Agliardi et al., 2011). However, in a study population of 641 MS and 558 healthy controls, DRB1*15 positive MS patients had a lower frequency of the rs731236T allele compared to DRB1*15 positive healthy controls, with a protective effect against MS ($p = 0.039$, OR 0.73 (0.54 – 0.98)). There was no significant difference in rs731236 allele frequency between unstratified MS cases and healthy controls, or DRB1*15 negative MS cases and healthy controls. Higher mRNA expression levels of *VDR* were found in TT and CT genotypes compared to CC genotypes in MS patients.

Although these three studies have identified different genetic interactions between the variants in *VDR* and DRB1*15, they provide further evidence of an interaction between these two genes. The interaction of the largest genetic risk factor, DRB1*1501, with the receptor of a known environmental factor, vitamin D, could provide important information as to the underlying pathobiology of MS, and provide a link between the environmental and genetic risks. Investigation of this with vitamin D levels data from patients could further allow the development of interventions to reduce the risk of MS in genetically susceptible people.

Plasminogen Activation Pathway

The dysregulated gene expression of *MMP9*, *PLAU*, *PLAUR* and *SERPINB2* in MS patients compared to healthy controls identifies the plasminogen activation cascade as a potential candidate pathway in the pathogenesis of MS. With the known association of MMP9 with MS, it is important to identify the cause of the increased expression in MS patients. For this reason, the dysregulation of genes in one of the pathways involved in the activation of MMP9, is an important discovery.

To investigate potential causes of altered gene expression, we performed a SNP association study investigating common variants present within these four genes. We found no evidence of an association of any of these variants with MS. We also investigated the relationship

between gene expression and genotype; No association was observed between the SNP genotype and level of gene expression.

Despite consistent evidence of higher levels of MMP9 in MS, we conclude that the variants investigated are not associated with disease, or gene expression levels. It is well known that gene expression is controlled and affected in many ways, including miRNAs, methylation and other epigenetic factors. It is possible that these other factors regulating gene expression , which need to be further investigated.

Conclusion

Multiple sclerosis is an autoimmune disorder with an underlying genetic basis. Although 57 genetic loci have been associated with disease risk, there remains further genetic contribution still to be identified (IMSGC et al., 2011).

In this thesis, I have reported on the altered expression of miRNAs and genes associated with MS. The miRNAs identified in this thesis are likely to play a role in the pathogenesis of MS through the dysregulation of genes involved in the immune system, and specifically increased T cell activation. Similarly, the altered gene expression profile identified is enriched for genes involved in the immune system. The identification of altered expression in the plasminogen activation pathway provides interesting evidence for the cause of the known increased expression of MMP9, and its role in the pathogenesis of MS. No evidence was found of an association between variants in the plasminogen activation pathway genes and MS, or gene expression levels.

The role of variants of the *VDR* in the aetiology of MS remains unknown. Large candidate gene studies have failed to conclusively show evidence either way, although no evidence of association has been identified through GWAS. It is possible that the *VDR* may only play a role in the disease in a subgroup of patients, either with vitamin D deficiency, or with other genetic factors, such as DRB1*1501.

Future Directions

The aetiology of MS is a complex interplay of genetic and environmental factors. To further understand the cause of this disease, studies must use the information already available to guide further research. For instance, while RNA expression profiling studies have produced

complex results, they have clearly identified differences in the immune system in MS patients. However, it is the other findings, such as differential expression of plasminogen activation pathway genes or interactions of *VDR* which may prove to be of more value.

We have identified altered gene expression in the plasminogen activation pathway, which may lead to the known up-regulation of MMP9 in MS. Although the cause of the altered expression remains unknown, this is a pathway that requires further study in MS. Identifying the cause of MMP9 over-expression and subsequent BBB breakdown may lead to the production of targeted therapies to reduce immune cell transit across this barrier.

While miRNAs are still a relatively new field of genetic research, and we have not yet been able to harness their multiple uses, we have a significant knowledge of their dysregulation in MS, and the effects this may cause. Further clarification of the miRNAs dysregulation in MS, and their biological role will shed light on the pathomechanism of MS. These studies though must be directed towards answering specific questions, rather than the broad 'fishing expedition' approach which has been valuable in identifying new avenues of investigation but has not provided any specificity with respect to understanding the pathomechanisms associated with disease.

A relationship between *VDR* variants and DRB1*1501 has been suggested in this thesis, and by others (Agliardi et al., 2011, Orton et al., 2011, Ramagopalan et al., 2009a), and it is clear from these studies that larger study populations are required to have enough statistical power to identify such an interaction. The information currently available from multiple GWAS should be mined to investigate this relationship.

The combined effect of environmental and genetic factors must be further investigated. This is most important since at the moment the vitamin D association is being actively studied. Future studies need to investigate the relationship between vitamin D deficiency, and specific the genetic factors, DRB1*1501 and *VDR* variants. This may identify a risk factor in a specific patient group, which can be easily, and cheaply, modified by a dietary supplement which is widely available.

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Appendices

Appendix One: Supplementary material for Publication One

Altered expression of the plasminogen activation pathway in peripheral blood mononuclear cells in multiple sclerosis: possible pathomechanism of matrix metalloproteinase activation

Supplementary Table 1: Differentially expressed genes in each comparison

Gene Symbol	RRMS NoTx vs. HC	RRMS IFN-B vs. HC	RRMS NoTx vs. RRMS IFN-B
ACOT2		2.137	
ACTG1		3.195	-2.75482094
ADM	2.391		
AHCY		2.160	
AKIRIN1		2.364	
ALPL	3.139		
ANKDD1A			2.04
AP1G1			2.057
AQP9	2.279		
BST2		2.463	-2.74725275
C10ORF32		2.110	
C15ORF48	2.166		
C19ORF10		2.500	-2.43309002
C1QA			-2.04081633
C22ORF34		-2.007	
C5AR1	2.317		
C7ORF41		-2.041	
CA4	3.882	2.427	
CAMP	2.368	2.717	
CARHSP1		2.132	
CCL2	2.013		
CD302		2.101	
CD38			-2.35294118
CD63		2.119	
CLC		3.571	
CMTM2	2.337		
CRIPAK		-2.050	
CSF3R	2.548		
CTSB		2.000	
CYP4F3	3.068		2.152
DDAH2		2.179	
DEFA1B	4.403	4.032	
DEFA4	2.183		
DOT1L			2.119
DUSP6	2.087	2.445	
ECGF1		3.135	-2.57731959
EDN1			2.114
EPSTI1		2.252	-2.42130751
ERAF	2.443		
FAM200A		2.155	
FAM63A		2.123	
FBXO6			-2.05338809
FCGR3B	2.963	3.279	
FCRLA	-2.024		
FFAR2	2.387	2.128	
FLJ11783		-2.352	
G0S2	2.911	2.421	
GPBAR1			-2.09205021
GPR84	2.101		
GPR97	2.225		
GRN		2.198	

Gene Symbol	RRMS NoTx vs. HC	RRMS IFN-B vs. HC	RRMS NoTx vs. RRMS IFN-B
GSDMDC1			-2.07900208
GZMB			-2.25225225
GZMM		2.183	-2.48756219
HBA2	2.270	2.188	
HBB	2.017		
HBG1			2.91
HBG2			2.837
HES4		2.353	
HIST1H1E		2.577	-2.45700246
HLA-C	2.081	2.841	
HLA-DRB1	3.902	4.032	
HLA-DRB4		2.294	
HLA-DRB5	11.580	18.215	
HMGN2		2.033	
ICAM1	2.049		
IER3	2.454		
IFI27		7.353	-6.66666667
IFI35			-2.1141649
IFI44L		4.255	-3.89105058
IFI6		2.342	-2.3255814
IFIT1		2.667	-2.04498978
IFIT2		2.004	
IFIT3		3.413	-2.17391304
IFITM3		3.704	-2.95857988
IGFBP7		2.208	-2.04081633
IL1R2	2.318		2.028
IL1RN	2.380		
IL8	2.654	2.110	
IRF7			-2.0746888
ISG15		2.604	-3.26797386
ISG20L2		2.128	
ITGA2B		-2.054	
KCTD20		2.092	
KIAA1632		-2.375	2.228
KRT72		-2.204	
KRT73		-2.036	
LGALS3BP		2.404	-2.3364486
LILRA3		2.066	
LILRA5		3.040	
LMOD3		2.151	
LOC201175		2.227	
LOC653994		2.994	
LOC727877		2.151	-2.15053763
LONRF1			2.112
LRG1	2.424		
LRRC25		2.632	
LY6E		2.020	-2.00400802
LYRM2		2.169	
LYZ	2.059	2.849	
MAT2A		-2.440	
MCOLN2		-2.052	
MGEA5		-2.085	
MIR600HG		-2.046	
MMP25	2.661		
MMP9	3.356		
MNF1			-2.01207243
MRPS18B		2.123	-2.19298246
MXD1	2.009		
NAMPT	2.960	2.110	
NDUFA10		2.110	
NFKB2			2.098
NRGN		-2.173	
OAS1		2.793	-3.14465409
OASL		2.137	
ORM1	2.094		2.151
OSM	2.179		

Gene Symbol	RRMS NoTx vs. HC	RRMS IFN-B vs. HC	RRMS NoTx vs. RRMS IFN-B
OTOF		2.451	-2.2675737
PGAM1		2.632	-2.03252033
PGK1		2.053	
PGLYRP1	2.424		
PHACTR1	2.088		
PHLDA1	2.305	2.016	
PI3	2.666		2.336
PILRA		2.096	
PKD1		-2.032	
PLAC8			-2.22222222
PLAU	3.207		3.138
PLAUR	2.113		
PLEKHB2		2.463	
PMVK			-2.10084034
POLS		-2.096	
PPM1K		2.203	
PSENEN		2.075	
PTAFR		2.114	
PTGES			2.106
PTPRCAP			-2.17864924
QPCT	2.111		
RAB7L1			-2.08333333
RICTOR			2.033
RN28S1		2.358	
RNASE2		2.639	-2.04918033
RNF125		2.075	
RP2		2.410	
RPS4Y1		-3.111	
RRAS			-2.15982721
S100A11		2.404	
S100P	2.505		
SERPINB2	2.312		2.417
SERPING1		2.370	
SH2B2		2.028	
SH3BGRL2		-2.213	
SLC11A2		-2.173	2.044
SLC3A2		2.075	
SND1-IT1		-2.403	
SPATS2L		2.262	-2.14592275
TCN2		2.273	
TGM3	2.101		2.084
THBS1	2.004		2.166
TLE3	2.300		
TMEM41B			2.112
TNFSF13B		2.193	
TPM3		2.128	
UPB1			2.699
XAF1			-2.61096606
ZBP1		2.439	-2.3364486
ZNF337		-2.057	
ZNF394		2.165	
ZNF428		2.037	-2.04498978

Supplementary Table 2: Biological processes with significant number of genes differentially expressed between the test groups, with number of genes differentially expressed for each biological process.

	RRMS NoTx vs. HC			RRMS IFN-B vs. HC			RRMS IFN-B vs. RRMS NoTx		
Biological Process	# of genes	over/ under	P-value	# of genes	over/ under	P-value	# of genes	over/ under	P-value
immune system process	22	+	1.18E-11	25	+	2.64E-07	19	+	3.49E-07
response to stimulus	16	+	5.28E-09	19	+	1.41E-06	14	+	5.47E-06
response to interferon-gamma	7	+	1.57E-04	11	+	1.94E-05	8	+	1.00E-04
immune response	4	+	4.07E-03	6	+	1.82E-03	4	+	8.85E-03
cytokine-mediated signalling pathway	3	+	7.69E-04	3	+	5.26E-03	0	-	NS
cellular defence response	4	+	7.93E-04	3	+	4.81E-02	1	+	NS
natural killer cell activation	2	+	3.44E-03	2	+	1.26E-02	0	-	NS
antigen processing and presentation	2	+	2.68E-02	4	+	1.63E-03	1	+	NS
antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	2	+	2.35E-02	1	+	NS	2	+	3.53E-02
macrophage activation	6	+	4.38E-05	2	+	NS	1	+	NS
glycolysis	4	+	2.63E-04	2	+	NS	0	-	NS
purine base metabolic process	18	+	9.40E-04	16	-	NS	9	-	NS
monosaccharide metabolic process	11	+	1.71E-03	7	-	NS	3	-	NS
DNA replication	18	+	1.72E-03	16	-	NS	9	-	NS
RNA catabolic process	8	-	2.40E-03	34	-	NS	23	+	NS
blood circulation	3	+	7.01E-03	1	+	NS	2	+	NS
transcription	3	+	7.01E-03	1	+	NS	2	+	NS
transcription from RNA polymerase II promoter	2	-	8.01E-03	18	+	NS	8	-	NS
complement activation	6	+	1.87E-02	6	+	NS	2	-	NS
developmental process	2	+	2.04E-02	1	+	NS	1	+	NS
protein transport	2	+	2.44E-02	2	+	NS	0	-	NS
intracellular protein transport	5	+	2.55E-02	3	-	NS	2	-	NS
JNK cascade	2	+	2.58E-02	0	-	NS	0	-	NS
B cell mediated immunity	0	-	4.13E-02	2	-	NS	3	-	NS
cell-cell signalling	0	-	4.13E-02	2	-	NS	3	-	NS
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	19	+	4.45E-02	26	+	NS	12	-	NS
JAK-STAT cascade	1	+	NS	6	+	5.94E-06	5	+	9.59E-06
cell communication	1	+	NS	3	+	5.26E-03	1	+	NS

cell surface receptor linked signal transduction	1	+	NS	3	+	3.70E-02	1	+	NS
signal transduction	0	-	NS	3	+	3.95E-02	0	-	NS
cellular process	0	-	NS	3	+	1.28E-02	0	-	NS
Unclassified	0	-	NS	2	+	4.31E-02	1	+	NS
blood coagulation	2	-	NS	5	-	NS	2	-	4.89E-02
response to external stimulus	2	-	NS	5	-	NS	2	-	4.96E-02
G-protein coupled receptor protein signalling pathway	5	-	NS	10	-	NS	2	-	3.82E-02
hemopoiesis	0	-	NS	1	+	NS	2	+	2.13E-02

Supplementary table 3: Pathways containing statistically significant numbers of differentially expressed genes

Pathway	RRMS NoTx vs. HC		RRMS IFN-B vs. HC		RRMS IFN-B vs. RRMS NoTx	
	# of genes	P value	# of genes	P value	# of genes	P value
Plasminogen activating cascade	4	1.37E-07	0	NS	2	1.44E-03
Blood coagulation	3	2.38E-04	1	NS	2	9.68E-03
Inflammation mediated by chemokine and cytokine signalling pathway	3	2.95E-02	3	NS	3	NS
Alzheimer disease-presenilin pathway	2	3.65E-02	2	NS	1	NS
Salvage pyrimidine deoxyribonucleotides	0	NS	1	1.91E-02	1	1.22E-02
Glycolysis	0	NS	2	6.15E-03	1	NS
S adenosyl methionine biosynthesis	0	NS	1	1.44E-02	0	NS
Pyrimidine Metabolism	0	NS	1	NS	2	1.01E-03
Toll receptor signalling pathway	0	NS	0	NS	2	1.30E-02
Cholesterol biosynthesis	0	NS	0	NS	1	3.91E-02

NS = non-significant

Appendix Two: Supplementary material for Publication Two

MicroRNAs miR-17 miR-20a Inhibit T Cell activation Genes and Are Under-Expressed in MS Whole Blood

Table S1. Demographics of multiple sclerosis and control individuals

		miRNA microarray							q RT PCR						
		number of samples	average age (years)	age range	average EDSS	EDSS range	average disease duration (years)	disease duration range (years)	number of samples	average age (years)	age range (years)	average EDSS	EDSS range	average disease duration (years)	disease duration range (years)
MS	Total	59	54.2	32 - 81	4.5	0 - 5.8	20.3	1 - 58	57	53.8	32 - 81	4.4	0 - 8.5	23.1	1 - 66
	Male	19	53.1	32 – 66	5.4	1.5 - 8.5	21.7	1 - 58	18	52.7	32 - 66	5.3	1.5 - 8.5	31.1	1 - 66
	Female	40	54.7	34 – 81	4.1	0 - 8.5	19.1	1 - 53	39	54.3	34 - 81	4.0	0 - 8.5	19.8	1 - 62
RRMS	Total	24	49.9	33 – 64	2.4	0 - 6.5	16.8	1 - 36	25	48.8	33 - 64	2.4	0 - 6.5	15.9	1 - 36
	Male	3	42.3	33 – 52	3.5	1.5 - 6	13.0	1 - 25	3	42.3	33 - 52	3.5	1.5 - 6	13.0	1 - 25
	Female	21	51.0	35 – 64	2.2	0 - 6.5	17.3	1 - 36	22	49.7	35 - 64	2.3	0 - 6.5	16.3	1 - 36
SPMS	Total	17	57.2	34 – 73	6.4	4 - 8.5	23.2	2 - 53	14	56.6	34 - 73	6.5	4 - 8.5	20.6	2 - 37
	Male	4	54.8	44 – 63	6.5	4 - 8.5	22.8	21 - 26	4	54.8	44 - 63	6.5	4 - 8.5	22.8	21 - 26
	Female	13	58.0	34 – 73	6.4	4 - 8.5	23.3	2 - 53	10	57.3	34 - 73	6.5	4 - 8.5	19.7	2 - 37
PPMS	Total	18	57.1	32 – 81	5.4	2 - 8	22.9	4 - 58	18	58.9	32 - 81	5.6	3.5 - 8	36.6	4 - 66
	Male	12	55.3	32 – 66	5.5	3.5 - 8	28.8	5 - 58	11	55.0	32 - 66	5.4	3.5 - 8	40.8	5 - 66
	Female	6	60.7	36 – 81	5.4	2 - 7.5	14.0	4 - 34	7	64.4	56 - 81	5.9	5 - 7	31.3	4 - 62
Control	Total	37	48.0	23 – 77	-	-	-	-	34	48.3	23 - 77	-	-	-	-
	Male	16	54.2	26 – 69	-	-	-	-	15	56.1	26 - 69	-	-	-	-
	Female	21	43.2	23 – 77	-	-	-	-	19	42.1	23 - 77	-	-	-	-

MS Multiple Sclerosis; RRMS relapsing remitting MS; SPMS secondary progressive MS; PPMS primary progressive MS; EDSS Expanded disability status scale.

Table S2. Genes dysregulated in miR-17 knock-in and knock-down Jurkat transformants

miR-17 (Jurkat DE + predicted target genes of	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
MARCH5	C17orf39	C17orf39
SEPT7	CAP1	CAP1
AADAC	CASC3	CASC3
ABAT	CEBPB	CEBPB
ABCD3	DLST	FRAT1
ABHD13	DPF2	HLA-E
ABL1	FRAT1	IDS
ABTB1	FTL	ITGB5
ACAT2	HIST1H2BD	MXD1
ACSS2	HIST1H2BK	MYADM
ACTR10	HLA-E	NINJ1
ACVR2B	IDS	NUMB
ADAR	ITGB5	PRCP
ADM	MXD1	SLBP
ADM2	NUMB	TSC22D1
ADSSL1	PILRA	TXNIP
AGFG2	PRCP	UBAP1
AKAP13	PRSS7	UPF3B
ALDH3A2	SEC14L1	ZNF213
ALKBH1	SNX30	APOOL
AMDHD1	STAU1	ARMET
AMPD2	STK40	C6orf48
ANKS4B	STX11	CDKN2C
ANXA6	TSC22D1	COMMD3
AP4E1	TST	COQ5
APOBEC3F	TXNIP	DARS
APOOL	UBAP1	DUSP8
APPL2	UPF3B	IL23A
ARF6	ZNF213	MRPS22
ARFGEF2	ASF1B	WDR33
ARHGEF10	CDKN1B	ZNF740
ARID4B	CORO1A	BPGM
ARL4A	MYADM	PTPRCAP
ARL5B	NINJ1	RPL32
ARMET	SLBP	
ARNT2	APEX1	
ASL	APOOL	
ATCAY	ARMET	
ATF3	C19orf48	
ATF5	C6orf48	
ATP5SL	CCDC53	
ATP6V1H	CCDC84	
ATPAF1	CCNB1IP1	
ATRX	CDKN2C	
ATXN7	CHPF	
B3GAT1	CIP29	
B3GNT1	COMMD3	
BBS7	COQ5	
BCL2A1	DARS	
BCL2L11	DRG1	
BMPR1A	DUSP8	
BMPR2	EIF2B4	
BPGM	EIF3D	
BPTF	HEMGN	
BRUNOL5	HYLS1	
BTN1A1	IL23A	
BZW1	LSM5	

miR-17 (Jurkat DE + predicted target genes of	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
C10orf58	MED10	
C11orf63	MRFAP1L1	
C12orf44	MRPS21	
C12orf48	MRPS22	
C12orf49	NOSIP	
C14orf138	NUP37	
C16orf58	RIOK2	
C16orf59	RPL17	
C17orf39	RPLP0	
C17orf48	STK16	
C19orf59	STOML2	
C1orf106	WDR33	
C1orf107	WDR61	
C1orf124	ZNF740	
C1orf135	ZNHIT3	
C1orf21	ATP5J	
C1orf220	BPGM	
C1orf9	CDC42	
C1orf91	PRDX1	
C1orf95	PTPRCAP	
C20orf111	RPL13A	
C2orf30	RPL32	
C4orf3	TAF15	
C4orf34	TCEAL4	
C4orf49	UQCRFS1	
C5orf41		
C5orf45		
C5orf51		
C6orf48		
C6orf62		
C7orf26		
C7orf43		
C7orf53		
C9orf102		
C9orf72		
CACNB2		
CACNB2		
CACNB4		
CADM2		
CAMK2D		
CAP1		
CAPN3		
CAPN3		
CAPRIN2		
CAPZA1		
CASC1		
CASC3		
CAV3		
CCDC109A		
CCDC127		
CCDC150		
CCDC3		
CCDC62		
CCDC86		
CCDC90B		
CCL5		
CCL8		
CCND2		
CCRK		
CCS		

miR-17 (Jurkat DE + predicted target genes of	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
CD14		
CD163		
CD38		
CD5		
CD82		
CD83		
CDC2L5		
CDH26		
CDK8		
CDKN2C		
CDYL2		
CEBPB		
CER1		
CES3		
CH25H		
CHCHD4		
CHIC1		
CHIC2		
CHRNB1		
CLEC4A		
CLSTN1		
CLUAP1		
COG3		
COG5		
COL2A1		
COMMD3		
COPS8		
COQ10B		
COQ5		
COX11		
CPEB4		
CPZ		
CRCP		
CREB3L2		
CRK		
CRMP1		
CRTAP		
CRTC1		
CRYZL1		
CSNK2A2		
CTAGE5		
CTPS2		
CUX2		
CXCL12		
CXorf1		
CYTIP		
DAG1		
DARS		
DCDC2		
DCLRE1C		
DDHD1		
DDX17		
DEDD		
DENND5A		
DGUOK		
DGUOK		
DHRS2		
DHX57		
DIABLO		
DKK2		

miR-17 (Jurkat DE + predicted target genes of DLEC1	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
DLG5		
DMD		
DNAJB5		
DNAJB6		
DNAJB9		
DNAJC27		
DNALI1		
DNASE2		
DNMT3A		
DNMT3A		
DOCK4		
DPY19L4		
DRD2		
DUOX1		
DUSP8		
DUT		
DUXA		
DYNC1I1		
DYRK3		
DZIP3		
EBF1		
EFHA2		
EFTUD1		
EHMT1		
EIF1AX		
EIF2B2		
ELMO1		
ELMOD3		
EML1		
EMP1		
ENO3		
ERO1L		
ERO1LB		
ESRRG		
ETV3L		
ETV5		
EVI1		
EXOC7		
FADS1		
FAM123A		
FAM12B		
FAM165B		
FAM19A4		
FAM24B		
FAM38B2		
FAM63A		
FAM71E1		
FAM82A2		
FBLIM1		
FBXO22		
FBXO41		
FBXW8		
FCF1		
FEN1		
FLJ41603		
FMNL3		
FMO5		
FNBP4		
FOXD4L2		

miR-17 (Jurkat DE + predicted target genes of FOXO1	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
FOXP4		
FRAT1		
FUNDC1		
GABARAPL1		
GABRA1		
GABRB2		
GADD45B		
GALK2		
GAS8		
GBF1		
GBP6		
GDAP1		
GDF6		
GGPS1		
GIMAP2		
GIMAP4		
GLT25D2		
GM2A		
GNAO1		
GNG7		
GOT1		
GPATCH8		
GPR83		
GPR89A		
GRB10		
GRB2		
GRLF1		
H1F0		
HAUS1		
HAUS2		
HBEGF		
HBP1		
HDDC3		
HES7		
HIF3A		
HINT3		
HIP1		
HLA-E		
HMGA1		
HMGCS1		
HOMEZ		
HOOK3		
HOXC6		
HPS4		
HRH1		
HSH2D		
HTN1		
HTR7		
HVCN1		
HYOU1		
IDS		
IFI44L		
IFIT3		
IFNA17		
IFT140		
IGF1		
IGF2BP1		
IGFBP5		
IGSF3		

miR-17 (Jurkat DE + predicted target genes of IL23A	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
INA		
ING1		
ING4		
INO80D		
INSIG1		
INSM2		
IPO13		
ISX		
ITGB5		
ITM2C		
ITPR1		
JAG2		
JOSD1		
KCNAB1		
KCNH5		
KCNH7		
KCNJ10		
KCNK3		
KCNMA1		
KCNMB2		
KDM3B		
KHDC1		
KHK		
KIAA0774		
KIAA1370		
KIF3B		
KLC4		
KLF10		
KLHL2		
KLHL25		
KLHL28		
KLK5		
KLK7		
KLRD1		
KRBA2		
L3MBTL4		
LAMA3		
LARP2		
LATS2		
LDLR		
LEPREL1		
LEPROTL1		
LIAS		
LIF		
LILRB3		
LIMA1		
LIPH		
LMBRD1		
LRRC1		
LSS		
LYPD5		
LYPD6		
LYZ		
MAGEA5		
MAP2K3		
MARVELD3		
MATN3		
MCFD2		
MFAP3L		

miR-17 (Jurkat DE + predicted target genes of	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
MFAP5		
MLC1		
MLF2		
MMP2		
MPHOSPH8		
MPP5		
MRAP2		
MRGPRX3		
MRPL4		
MRPS22		
MRRF		
MST150		
MTMR14		
MTPAP		
MTUS1		
MUC17		
MUDENG		
MUT		
MVD		
MXD1		
MYADM		
MYO1E		
MYOM2		
NAIF1		
NAP1L2		
NAP5		
NCAPG2		
NCBP2		
NCOA5		
NDUFV3		
NFATC2IP		
NGFRAP1		
NINJ1		
NLRP3		
NME6		
NMNAT2		
NMUR1		
NOVA2		
NRXN1		
NSFL1C		
NSL1		
NUDT16		
NUFIP1		
NUMB		
NUP54		
OGT		
OPTN		
OR7D2		
ORAOV1		
ORC5L		
OSM		
OXCT1		
PASD1		
PASK		
PAX8		
PBOV1		
PCDH10		
PCDHGC3		
PCDHGC3		
PCGF6		

miR-17 (Jurkat DE + predicted target genes of	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
PDCD6		
PDE1A		
PDGFRA		
PEX13		
PEX16		
PEX19		
PGBD5		
PGM1		
PHC3		
PHF17		
PHF21A		
PHYHIP		
PIWIL2		
PKNOX1		
PLCXD2		
PLEKHO2		
PLGLB1		
PLS1		
PMEPA1		
PMM1		
PNPLA3		
POLDIP2		
POLDIP3		
POLE		
PPARA		
PPP1R1C		
PPP1R3F		
PPP2R2A		
PPP3CA		
PPP4R1		
PPP4R2		
PRCP		
PREPL		
PRKACB		
PRKCE		
PRNP		
PRR16		
PRRC1		
PRSS16		
PSD		
PTBP2		
PTEN		
PTGER3		
PTK2		
PTP4A1		
PTPRCAP		
PUM1		
PUS7L		
RAB11FIP4		
RAB14		
RAB30		
RAB3GAP1		
RAB43		
RALB		
RANBP17		
RASA1		
RBAK		
RBM16		
RCN2		
RCOR2		

miR-17 (Jurkat DE + predicted target genes of REL	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
REPS2		
REXO1L1		
RFC3		
RFFL		
RFFL		
RG9MTD3		
RGS3		
RHBDD2		
RIT1		
RNASE6		
RNF144B		
RNF213		
RNF215		
RNF34		
RNF8		
ROBO1		
RORA		
RORA		
RP4-691N24.1		
RPL32		
RPL32		
RPS6KA2		
RRM2		
RUFY1		
RUNDC3A		
RUNX2		
RYBP		
S100PBP		
SAMD7		
SAR1A		
SAV1		
SBK1		
SC5DL		
SCAMP5		
SCIN		
SDAD1		
SDC4		
SDHC		
SDSL		
SEMA3A		
SEMA4G		
SEMG2		
SERPINB3		
SERPINE2		
SETD2		
SETD4		
SFMBT1		
SGK3		
SGSM1		
SH3BP5		
SH3GLB1		
SIDT1		
SIRT6		
SLBP		
SLC16A7		
SLC19A2		
SLC22A8		
SLC28A2		
SLC2A9		

miR-17 (Jurkat DE + predicted target genes of	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
SLC34A2		
SLC43A3		
SLC4A4		
SLK		
SMAD7		
SMOX		
SMYD2		
SNCA		
SND1		
SNIP		
SNTB2		
SNX22		
SNX7		
SOBP		
SOCS4		
SOS2		
SPATA2		
SPCS2		
SPRR2F		
SRBD1		
SRR		
SS18		
SSH1		
SSTR2		
SSU72		
ST6GAL1		
ST8SIA5		
STAR		
STARD10		
STAT5A		
STC2		
STIM1		
STYXL1		
SUPT4H1		
SYN1		
SYN2		
TAF12		
TAGAP		
TBC1D19		
TBC1D2		
TBC1D3C		
TCF7L2		
TCTEX1D1		
TEK		
TFE3		
TFEB		
TFPI2		
TGIF2		
TGOLN2		
THYN1		
TICAM2		
TIPARP		
TLR8		
TMEM14C		
TMEM167B		
TMEM22		
TMEM62		
TMEM64		
TMEM71		
TMEM87A		

miR-17 (Jurkat DE + predicted target genes of	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
TMEM9		
TMEM98		
TMEM9B		
TMTC4		
TMUB2		
TNFAIP8L1		
TNFRSF17		
TNFSF14		
TNFSF4		
TOPORS		
TP53INP1		
TPD52		
TPK1		
TPM3		
TPMT		
TPP2		
TRAFD1		
TRAK1		
TRHDE		
TRIB3		
TRIM4		
TRNP1		
TRPC1		
TSC22D1		
TSC22D1		
TSEN34		
TSPYL6		
TXNIP		
UBAP1		
UBE2A		
UBE2G2		
UBL3		
UBOX5		
UGT2B15		
UGT2B17		
ULK3		
UNC13C		
UPF3B		
UQCR		
USP21		
USP35		
USP48		
USP53		
VANGL1		
VAPB		
VPS25		
VPS33B		
VPS45		
WDR31		
WDR33		
WNK3		
XBP1		
XKR6		
XKR8		
XPC		
XPR1		
YWHAH		
ZBTB41		
ZBTB46		
ZCCHC14		

miR-17 (Jurkat DE + predicted target genes of ZDHHC11	MS DE mRNA + miR-17 Jurkat	MS DE mRNA + miR-17 (Jurkat DE +
ZFAND3		
ZFP82		
ZHX2		
ZKSCAN1		
ZMAT5		
ZNF177		
ZNF211		
ZNF213		
ZNF256		
ZNF323		
ZNF395		
ZNF419		
ZNF517		
ZNF546		
ZNF615		
ZNF620		
ZNF680		
ZNF695		
ZNF740		
ZNF750		
ZNF761		
ZNF773		
ZNF823		
ZSWIM4		
ZZZ3		

DE - differential expression; MS - Multiple Sclerosis; red – up-regulated in MS; black – down-regulated in MS

Table S3. Genes dysregulated in miR-20a knock-in and knock-down Jurkat transformants.

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
SEPT2	CASC3	ACTR2
SEPT2	CEBPB	ARHGAP30
SEPT7	CREBBP	BID
MARCH8	DPF2	BMP2K
AASDHPPT	FLI1	CASC3
ABAT	FTL	CEBPB
ABCC10	H3F3A	CREBBP
ABCD3	HIST1H2BD	EIF4EBP2
ABTB1	HIST1H2BK	H3F3A
ACAD11	IDS	IDS
ACADM	IFNA7	IFNA7
ACAT2	LIMS1	KRAS
ACTR10	NUAK2	LPCAT1
ACTR1A	OSBPL2	MAP4K4
ACTR2	PCTP	OSBPL2
ADAM17	PRSS7	PCTP
ADAMTSL2	RASSF2	RASSF2
ADM2	RHOB	RXRA
ADRA2B	RXRA	STK40
AGK	STK40	TRIOBP
AHNAK	TRIOBP	TSC22D1
AIPL1	UBE2D3	ZFP36
AKAP13	WIPI1	ACAD11
AKIRIN1	ZFP36	CDKN2C
ALDOC	ACTB	COQ5
ALG9	ACTR2	IL23A
ALKBH1	ARHGAP30	MFHAS1
ALPK2	ARMET	NAPB
AMACR	BID	WDR33
AMPD2	BMP2K	C13orf27
ANGPT1	EIF4EBP2	C20orf20
ANKRD40	ETS2	
ANXA13	HLA-DQB1	
AP2M1	KRAS	
AP3M1	LPCAT1	
ARAP2	MAP4K4	
ARCN1	SLBP	
ARHGAP30	SNX15	
ARHGEF9	TSC22D1	
ARIH2	ACAD11	
ARL4A	BOLA3	
ARL6IP5	BXDC1	
ARL6IP6	C6orf48	
ARMC10	CCDC53	
ARMC2	CCDC59	
ARMCX1	CCNB1IP1	
ARPP-21	CDKN2C	
ARRB2	COQ5	
ARRDC1	DRG1	
ARSD	EIF2B4	
ASL	ERP27	
ASPH	HEMGN	
ASS1	HSPE1	
ATAD3B	IL23A	
ATF5	LGALS1	
ATG12	MCART1	
ATP1B4	MFHAS1	

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
BAGE	MRPS21	
BBS7	NAPB	
BCL2L11	NAT5	
BECN1	NUCKS1	
BID	NUP37	
BMF	PIGF	
BMP2K	RIOK2	
BMPR1A	RPL12	
BRP44L	RPL24	
BTBD7	RPL36AL	
C10orf46	RPL39	
C10orf47	SFRS16	
C11orf63	STK16	
C12orf44	TIMM23	
C12orf48	TMEM126A	
C12orf66	WDR33	
C12orf67	ANXA1	
C12orf68	C13orf27	
C13orf27	C20orf20	
C14orf102	C21orf33	
C14orf147	C22orf28	
C15orf44	EIF3E	
C15orf54	HIST1H4C	
C16orf58	KLRB1	
C17orf37	MRPL55	
C1orf161	NAE1	
C1orf21	NME1	
C1orf52	RPL11	
C1orf95	RPL13A	
C20orf111	RPL41	
C20orf117	RPL6	
C20orf20	RPS15A	
C21orf34	RPS27	
C2orf66	RPS27A	
C3orf15	RPS29	
C4orf3	TAF15	
C4orf30		
C4orf32		
C4orf34		
C4orf49		
C5AR1		
C5orf41		
C5orf51		
C6orf162		
C6orf182		
C6orf224		
C7orf41		
C7orf43		
C7orf53		
C8orf33		
C9orf102		
C9orf47		
C9orf97		
CACNA1E		
CACNA2D3		
CADM2		
CAMK2N1		
CAMKK1		
CAPN3		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
CASC1		
CASC3		
CASP7		
CBFA2T2		
CBFA2T3		
CBL		
CC2D1A		
CCDC112		
CCDC132		
CCDC3		
CCDC52		
CCDC62		
CCDC86		
CCDC90B		
CCND3		
CCNG1		
CCNL1		
CCRK		
CCRL1		
CD163		
CD164		
CD24		
CD36		
CD5		
CD83		
CDC2L5		
CDC42SE2		
CDH26		
CDK2		
CDK8		
CDKN2C		
CDT1		
CEBPB		
CEBPD		
CES3		
CHCHD7		
CHD6		
CHIC1		
CHIC2		
CHM		
CHN2		
CHRDL1		
CHRNB1		
CLCC1		
CLDN16		
CLDN18		
CLDND1		
CLEC2B		
CLEC4A		
CMKLR1		
CMTM3		
CMTM4		
CNOT6		
COBL		
COG3		
COL11A1		
COL13A1		
COL17A1		
COL2A1		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
COMMD5		
COPA		
COPS2		
COPS7A		
COPS8		
COQ10B		
COQ5		
CORO2B		
CPA4		
CPEB3		
CR1		
CREBBP		
CRISPLD2		
CRK		
CRMP1		
CSNK1E		
CSNK2A2		
CST9L		
CSTF1		
CTDSPL		
CTSL1		
CUGBP2		
CUL4A		
CXorf38		
CYP19A1		
CYP26B1		
CYP4F3		
CYP4X1		
DACH1		
DAG1		
DAND5		
DAZL		
DBN1		
DCDC2		
DCHS1		
DCLRE1C		
DDR2		
DDX47		
DECR2		
DEDD		
DENND5A		
DGUOK		
DHCR24		
DHRS2		
DHX35		
DHX9		
DIAPH2		
DICER1		
DIP2B		
DKK2		
DLEC1		
DNA2		
DNAJB5		
DNAJB7		
DNAJB9		
DNAJC27		
DNAJC30		
DNALI1		
DNASE2		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
DOCK3		
DPP3		
DPY19L4		
DPYD		
DPYSL5		
DSEL		
DTNA		
DTX4		
DUOX1		
DUSP10		
DUSP10		
DUSP4		
DUSP6		
DYNC1I1		
DZIP3		
ECHDC1		
ECOP		
EDA		
EDNRB		
EEF1A1		
EFHC2		
EFNA4		
EFTUD1		
EIF1AX		
EIF2B2		
EIF4A2		
EIF4B		
EIF4EBP2		
EIF4H		
EMR2		
ENDOD1		
ENPP6		
ENTPD1		
EPB41L2		
ERBB2		
ERO1L		
ERO1LB		
ERP29		
ERP44		
ESR1		
ETS1		
ETV5		
EVI1		
EXD1		
EZH2		
FADS1		
FAM110A		
FAM129A		
FAM12B		
FAM175A		
FAM177A1		
FAM38B2		
FAM62A		
FAM65A		
FAM81A		
FAM83C		
FBXO11		
FBXO41		
FBXW7		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
FCGR3B		
FEN1		
FGF2		
FGFR1		
FKBP14		
FLJ20184		
FMNL3		
FNTB		
FOXK1		
FOXP4		
FOXRED2		
FRMD6		
FUT2		
FUT4		
FXN		
FXR1		
G3BP2		
GABPA		
GALK2		
GALNT12		
GALR2		
GAS8		
GATA3		
GATAD2A		
GBF1		
GCOM1		
GDA		
GDF6		
GFPT1		
GGA1		
GHRH		
GIMAP4		
GIPC1		
GLIPR2		
GM2A		
GNE		
GORAB		
GPC3		
GPM6A		
GPR135		
GPR81		
GPR83		
GRLF1		
GRM7		
GTF2H1		
GTPBP1		
H1F0		
H3F3A		
HADH		
HARS		
HBEGF		
HDAC9		
HEMK1		
HERC6		
HERPUD1		
HERPUD2		
HIAT1		
HIST1H2AG		
HLA-A		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
HLA-DRA		
HMGB1		
HMGCS1		
HMGXB4		
HNRNPC		
HNRNPF		
HNRNPR		
HORMAD2		
HOXC6		
HOXD13		
HP1BP3		
HPRT1		
HSD17B1		
HSD17B6		
HSPA8		
HSPB3		
HSPD1		
HTN1		
HVCN1		
HYOU1		
HYPK		
ICA1L		
IDH1		
IDI2		
IDS		
IFNA17		
IFNA7		
IFT81		
IGF2BP1		
IL23A		
IL8		
ILF3		
IMPA1		
IMPDH1		
INCENP		
ING2		
INPP5F		
INSIG1		
INSM2		
IP6K1		
IQGAP2		
IRF8		
IRF9		
ISOC1		
ITGA2		
ITIH2		
ITIH5		
ITM2C		
JTB		
KANK1		
KBTBD3		
KCMF1		
KCNH4		
KCNH5		
KCNMB2		
KCNMB2		
KCNQ1		
KIAA0232		
KIAA1024		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
KIAA1370		
KIAA1715		
KIAA1804		
KIF20B		
KIF21B		
KIF5B		
KLF10		
KLHL24		
KLHL25		
KLHL28		
KRAS		
KRIT1		
KRIT1		
KRT10		
LAIR1		
LBR		
LCORL		
LDLR		
LDLRAP1		
LEPROTL1		
LIF		
LIPH		
LLGL1		
LMNB1		
LOC153328		
LOC643684		
LPCAT1		
LPIN1		
LRRC1		
LRRC31		
LRRC40		
LRRC47		
LRRN2		
LSS		
LY6H		
LYST		
LYZ		
MAEA		
MAN1C1		
MANBA		
MAP4K4		
MAPT		
MAPT		
MASP1		
MCM4		
MCM7		
MDH1B		
MED23		
MEF2D		
MEST		
MFGE8		
MFHAS1		
MFN1		
MFSD11		
MGLL		
MIER1		
MLF2		
MMAB		
MMGT1		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
MORF4L2		
MPP7		
MRE11A		
MRPL15		
MRPL47		
MST150		
MSTO1		
MTFR1		
MUC17		
MUDENG		
MUT		
MVD		
MXRA7		
MYH9		
MYO18A		
MYOM1		
MYOM2		
MYPN		
NAP5		
NAPB		
NAT12		
NBPF14		
NCBP2		
NDFIP2		
NELF		
NFAT5		
NFATC1		
NFATC2IP		
NFYA		
NHLRC3		
NIP30		
NMNAT2		
NMT1		
NONO		
NOP2		
NOTCH1		
NOVA2		
NP		
NPY5R		
NQO1		
NTM		
NUDT21		
NUDT9		
NUFIP1		
NUP153		
NUP54		
NUPL1		
OAZ1		
OGN		
OPN3		
OPTN		
ORC5L		
OSBPL2		
OSBPL6		
OSTCL		
OTUD6B		
OTUD7A		
PANK1		
PAPD5		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
PAPPA		
PAX2		
PBOV1		
PCDH11X		
PCDHA6		
PCDHA6		
PCF11		
PCGF6		
PCTP		
PDCD6		
PDE1A		
PDS5B		
PDZD11		
PEG3		
PFN2		
PGM2L1		
PHACTR4		
PHF17		
PHF21A		
PHKA1		
PHYHIP		
PI4K2B		
PIF1		
PIGA		
PIGH		
PIGY		
PIK3IP1		
PIK3R1		
PIP4K2A		
PIP4K2C		
PISD		
PITPNB		
PLA2G12B		
PLK4		
PNMAL1		
PODXL		
POLDIP2		
POLE		
POLR3H		
POU3F2		
PPAP2B		
PPM1B		
PPM1K		
PPP1R3B		
PPP1R3F		
PPP1R9B		
PPP2R2C		
PPP2R3A		
PPP4R4		
PPTC7		
PRDM1		
PRDM16		
PRDM2		
PRDM8		
PRDM9		
PRDX3		
PRKAR1A		
PRKAR1A		
PRKCB		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
PRKDC		
PRNP		
PROK2		
PRR15		
PRR16		
PRRG4		
PRX		
PSAT1		
PSD3		
PSD3		
PSMD5		
PTGER3		
PTN		
PTPRO		
PUM1		
PUS7L		
PVRL2		
RAB11A		
RAB11FIP4		
RAB27A		
RAB35		
RAB37		
RAB3IP		
RAB43		
RABGEF1		
RAD21		
RAD23B		
RAD54B		
RALB		
RAN		
RANBP17		
RAPGEF1		
RARA		
RARRES1		
RASSF2		
RB1		
RBBP7		
RBM12		
RBM16		
RCBTB1		
RCOR2		
RDBP		
REM2		
RFC2		
RFFL		
RFFL		
RFXAP		
RG9MTD3		
RGS17		
RHOBTB3		
RHOT1		
RIMS3		
RNASE6		
RNASEL		
RNF19A		
RNF215		
RNF8		
RNMTL1		
ROBO1		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
RP2		
RP6-213H19.1		
RPA1		
RPL18		
RPP30		
RPS6KA1		
RPS6KA2		
RRAS2		
RTN1		
RUFY1		
RUNDC3A		
RXRA		
RYK		
SAFB2		
SAMD7		
SAR1A		
SC4MOL		
SC5DL		
SC65		
SCAMP1		
SCARB2		
SCD		
SCGB2A1		
SCN9A		
SCRIB		
SCRN3		
SCYL2		
SDAD1		
SDC4		
SDSL		
SEC23A		
SEC61A2		
SEL1L		
SELI		
SELL		
SELM		
SELT		
SEMA4G		
SEPHS1		
SERPINB9		
SERPINE2		
SETD2		
SF1		
SFRS3		
SGK269		
SGOL1		
SHISA2		
SIRT5		
SKAP2		
SLC1A3		
SLC22A2		
SLC22A8		
SLC25A10		
SLC25A24		
SLC25A34		
SLC25A38		
SLC26A1		
SLC26A7		
SLC27A6		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
SLC2A6		
SLC2A9		
SLC35B1		
SLC37A1		
SLC39A14		
SLC48A1		
SLC4A7		
SLC7A1		
SLC7A11		
SLC7A5		
SLMO2		
SMAD6		
SMCHD1		
SMOC1		
SMOX		
SNAPIN		
SNIP		
SNN		
SNRPD3		
SNTB2		
SNW1		
SORBS2		
SORD		
SOX17		
SOX7		
SP1		
SPCS3		
SPINK7		
SPIRE1		
SPNS1		
SPRED2		
SPRN		
SPRY4		
SPSB4		
SPTLC1		
SR140		
SRGAP2		
SRR		
SSR1		
SSTR2		
ST8SIA5		
STAG1		
STAG2		
STAU2		
STBD1		
STIM1		
STK40		
STMN1		
STX16		
STYXL1		
SURF6		
SUV420H1		
SYN1		
SYNPO2L		
SYT13		
SYT15		
SYVN1		
TADA1L		
TAF12		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
TAX1BP1		
TBC1D17		
TBC1D19		
TBC1D2		
TCEA1		
TCEAL1		
TCERG1		
TCF20		
TCF7L2		
TCHH		
TCTEX1D1		
TDG		
TDP1		
TEC		
TECPR1		
TFG		
TGFBR1		
THAP10		
THAP8		
THSD4		
THUMPD1		
TIAM2		
TICAM2		
TIMP2		
TIPIN		
TM4SF19		
TMEM106B		
TMEM128		
TMEM135		
TMEM151A		
TMEM168		
TMEM188		
TMEM20		
TMEM214		
TMEM217		
TMPO		
TMSB4X		
TMTC4		
TMX1		
TNFAIP8		
TNFRSF1A		
TNFSF14		
TNPO3		
TOM1		
TPD52		
TPD52L2		
TPD52L2		
TPK1		
TPM3		
TPRG1		
TRAF7		
TRAPPC2		
TRDMT1		
TRIB3		
TRIM58		
TRIM68		
TRIOBP		
TRIP11		
TRNP1		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
TRPC1		
TSC22D1		
TSC22D1		
TSEN15		
TSLP		
TSPAN17		
TSPYL4		
TTC39A		
TTN		
TUBGCP4		
TXLNB		
UBC		
UBE2D2		
UBE2E2		
UBE2K		
UBE2L3		
UBQLN1		
UBTF		
UBXN4		
UGT2B15		
UGT2B17		
UNC13A		
UNKL		
UQCR		
USP2		
USP33		
USP35		
USP48		
UTP14A		
VANGL1		
VASH1		
VAT1L		
VAV3		
VEGFA		
VENTX		
VKORC1L1		
VPS13B		
VPS41		
WDR1		
WDR16		
WDR31		
WDR33		
WHSC1L1		
WNK3		
WWOX		
XBP1		
XBP1		
XKR4		
XPR1		
XRN2		
YIPF2		
ZBTB7A		
ZC3H13		
ZC3H14		
ZDHHC11		
ZDHHC4		
ZFAND3		
ZFAT		
ZFP36		

miR-20a (Jurkat DE + predicted target genes of miRNA)	MS DE mRNA + miR-20a Jurkat DE	MS DE mRNA + miR-20a (Jurkat DE + Target)
ZFYVE9		
ZKSCAN1		
ZMAT3		
ZNF177		
ZNF211		
ZNF292		
ZNF317		
ZNF323		
ZNF329		
ZNF37A		
ZNF468		
ZNF491		
ZNF510		
ZNF530		
ZNF584		
ZNF587		
ZNF607		
ZNF626		
ZNF669		
ZNF672		
ZNF681		
ZNF682		
ZNF691		
ZNF695		
ZNF750		
ZNF761		
ZNF766		
ZNF800		
ZNF823		
ZRANB2		
ZSCAN10		

DE - differential expression; MS - Multiple Sclerosis; red – up-regulated in MS; black – down-regulated in MS

Table S4. Gene networks implicated in Multiple Sclerosis (MS) pathogenesis from miR-17 knock-in and -down experiments and from mRNA expression in whole blood

Pathway	P Value	No. Genes Represented	No. Genes on Pathway	No. Genes in miRNA list
miR-17 Jurkat differential expression, miR-17 target genes, MS mRNA differential expression				**34**
Immune response_IL-17 signalling pathways	7.82E-03	2	205	
miR-17 Jurkat differential expression, MS mRNA differential expression				**82**
Vitamin B7 (biotin) metabolism	3.78E-10	7	99	
Signal transduction_Activin A signalling regulation	8.54E-08	7	216	
Translation _Regulation of translation initiation	3.48E-06	7	376	
Translation_(L)-selenoaminoacids incorporation in proteins during translation	3.11E-04	5	349	
miR-17 Jurkat differential expression, miR-17 target genes				**683**
Development_Transactivation of PDGFR in non-neuronal cells by Dopamine D2 receptor	3.64E-04	8	143	
Development_A2B receptor: action via G-protein alpha s	3.80E-04	9	181	
Aspartate and asparagine metabolism	9.18E-04	5	62	
Chemotaxis_CXCR4 signalling pathway	9.35E-04	8	165	
Cholesterol Biosynthesis	9.88E-04	5	63	
Membrane-bound ESR1: interaction with G-proteins signalling	1.34E-03	9	216	
Development_Angiopoietin - Tie2 signalling	1.55E-03	7	139	
Development_A3 receptor signalling	1.71E-03	9	224	
Transcription_CREB pathway	2.90E-03	10	289	
Development_IGF-1 receptor signalling	3.32E-03	8	202	
Cytoskeleton remodeling_FAK signalling	3.42E-03	8	203	
HIV-1 signalling via CCR5 in macrophages and T lymphocytes	3.57E-03	6	121	
Development_EGFR signalling pathway	3.96E-03	8	208	
Immune response_Role of integrins in NK cells cytotoxicity	4.70E-03	6	128	
Thiamine metabolism	4.93E-03	2	9	
miR-17 Jurkat differential expression				**2187**
Development_Flt3 signalling	1.47E-04	14	157	
Development_A2B receptor: action via G-protein alpha s	1.95E-04	15	181	
Immune response_CD137 signalling in immune cell	1.98E-04	11	106	
Development_EGFR signalling pathway	2.84E-04	16	208	
Apoptosis and survival_Endoplasmic reticulum stress response pathway	1.14E-03	13	171	
Cholesterol Biosynthesis	1.89E-03	7	63	
Development_Transactivation of PDGFR in non-neuronal cells by Dopamine D2 receptor	2.41E-03	11	143	
Immune response_Role of DAP12 receptors in NK cells	3.35E-03	12	171	
G-protein signaling_Ras family GTPases in kinase cascades (scheme)	3.81E-03	8	90	

Table S5. Gene networks implicated in Multiple Sclerosis (MS) pathogenesis from miR-20a knock-in and -down experiments and from mRNA expression in whole blood

Pathway	P Value	No. Genes Represented	No. Genes on Pathway	No. Genes in miRNA list
miR-20a Jurkat differential expression, miR-20a target genes, and MS mRNA differential expression				**31**
Transcription_Sin3 and NuRD in transcription regulation	3.11E-06	4	150	
Immune response_Antiviral actions of Interferons	3.46E-06	4	154	
Immune response_TLR3 and TLR4 induce TICAM1-specific signaling pathway	2.85E-05	3	88	
Immune response_IFN alpha/beta signaling pathway	3.36E-05	3	93	
Immune response_Innate immunity response to RNA viral infection	7.92E-05	3	124	
Signal transduction_Activin A signaling regulation	4.06E-04	3	216	
G-protein signaling_Ras family GTPases in kinase cascades (scheme)	1.66E-03	2	90	
Cell cycle_Chromosome condensation in prometaphase	1.84E-03	2	95	
Apoptosis and survival_Cytoplasmic/mitochondrial transport of proapoptotic proteins Bid, Bmf and Bim	1.92E-03	2	97	
Cell cycle_Sister chromatid cohesion	2.08E-03	2	101	
Regulation of lipid metabolism_Regulation of lipid metabolism via LXR, NF-Y and SREBP	2.25E-03	2	105	
Development_Glucocorticoid receptor signaling	2.78E-03	2	117	
Transcription_Ligand-dependent activation of the ESR1/SP pathway	3.62E-03	2	134	
Transcription_Role of heterochromatin protein 1 (HP1) family in transcriptional silencing	3.67E-03	2	135	
Cell cycle_Initiation of mitosis	4.00E-03	2	141	
Cell cycle_Role of Nek in cell cycle regulation	5.24E-03	2	162	
miR-20a Jurkat differential expression and MS mRNA differential expression				**93**
Translation _Regulation of translation initiation	6.16E-14	14	376	
Signal transduction_Activin A signaling regulation	1.15E-12	11	216	
Translation_(L)-selenoaminoacids incorporation in proteins during translation	1.05E-11	12	349	
Vitamin B7 (biotin) metabolism	1.69E-09	7	99	
Immune response_Antiviral actions of Interferons	8.89E-07	6	154	
Transcription_Sin3 and NuRD in transcription regulation	1.60E-05	5	150	
Immune response_TLR3 and TLR4 induce TICAM1-specific signaling pathway	8.28E-04	3	88	
Immune response_IFN alpha/beta signaling pathway	9.72E-04	3	93	
Immune response_Innate immunity response to RNA viral infection	2.22E-03	3	124	
Transcription_Ligand-dependent activation of the ESR1/SP pathway	2.76E-03	3	134	

Immune response _Immunological synapse formation	3.32E-03	4	294	
Development_NOTCH1-mediated pathway for NF-KB activity modulation	5.03E-03	3	166	
Development_Notch Signaling Pathway	6.10E-03	3	178	
Transport_Macropinocytosis regulation by growth factors	8.50E-03	3	201	
miR-20a Jurkat differential expression and miR-20a target genes				**914**
Cholesterol Biosynthesis	7.23E-05	7	63	
Regulation of lipid metabolism_RXR-dependent regulation of lipid metabolism via PPAR, RAR and VDR	7.10E-04	6	66	
DNA damage_NHEJ mechanisms of DSBs repair	1.05E-03	6	71	
Transcription_Ligand-dependent activation of the ESR1/SP pathway	1.61E-03	8	134	
Regulation of lipid metabolism_Regulation of lipid metabolism via LXR, NF-Y and SREBP	1.66E-03	7	105	
Cell cycle_Start of DNA replication in early S phase	4.04E-03	7	123	
Apoptosis and survival_Cytoplasmic/mitochondrial transport of proapoptotic proteins Bid, Bmf and Bim	5.07E-03	6	97	
miR-20a Jurkat differential expression				**2741**
Cholesterol Biosynthesis	1.14E-05	11	63	
Translation _Regulation of translation initiation	1.90E-04	28	376	
Regulation of lipid metabolism_Regulation of lipid metabolism via LXR, NF-Y and SREBP	1.21E-03	11	105	
Translation_Insulin regulation of translation	2.21E-03	14	165	
Cell cycle_Spindle assembly and chromosome separation	2.56E-03	19	262	
Translation_(L)-selenoaminoacids incorporation in proteins during translation	3.30E-03	23	349	
Development_Flt3 signalling	3.84E-03	13	157	

Appendix Three: Supplementary material for Publication Three

Common genetic variants in the plasminogen activation pathway are not associated with multiple sclerosis

Supplementary table 1: Gene expression-genotype association analysis. Bonferroni corrected p value threshold = 0.00098

		MS		IFN-B		Control	
Gene	SNP	Genotype comparison	p value	Genotype comparison	p value	Genotype comparison	p value
MMP-9	rs13969	AA vs AC vs CC	0.7341	AA vs AC vs CC	0.5399	AA vs AC vs CC	0.6112
MMP-9	rs2274756	AA vs AG vs GG	0.5708	AG vs GG	0.7758	AA vs AG vs GG	0.8832
MMP-9	rs13925	AA vs AG vs GG	0.5708	AG vs GG	0.8333	AA vs AG vs GG	0.7791
MMP-9	rs17576	AA vs AG vs GG	0.7341	AA vs AG vs GG	0.4565	AA vs AG vs GG	0.7997
MMP-9	rs3787268	AG vs GG	0.7809	AG vs GG	0.1833	AA vs AG vs GG	0.7669
MMP-9	rs3918253	CC vs CT vs TT	0.9878	CC vs CT vs TT	0.158	CC vs CT vs TT	0.2806
MMP-9	rs3918241	AA vs AT vs TT	0.5357	AT vs TT	0.7758	AA vs AT vs TT	0.8946
PLAU	rs2227564	CC vs CT vs TT	0.6034	CC vs CT	0.4121	CC vs CT vs TT	0.0205
PLAU	rs2227562	AG vs GG	0.5711	-		AA vs AG vs GG	0.6827
PLAU	rs4065	CC vs CT vs TT	0.4708	CT vs TT	0.2333	CC vs CT vs TT	0.158
PLAU	rs2227551	GG vs GT vs TT	0.9194	GT vs TT	0.4121	GG vs GT vs TT	0.0278
PLAU	rs2227566	CC vs CT vs TT	0.407	CC vs CT vs TT	0.4534	CC vs CT vs TT	0.1093
PLAUR	rs4760	AA vs AG	0.1912	AA vs AG vs GG	0.1623	AA vs AG	0.7366
PLAUR	rs344787	AA vs AT vs TT	0.9565	AA vs AT vs TT	0.2557	AA vs AT vs TT	0.1163
PLAUR	rs2302524	CT vs TT	0.1153	CT vs TT	0.6623	CC vs CT vs TT	0.4485
PLAUR	rs4251854	AA vs AC	0.4884	-		AA vs AC vs CC	0.5871
SERPINB2	rs6104	CC vs CG vs GG	0.8799	CC vs CG	0.6485	CC vs CG vs GG	0.3857