Gene Expression and Variation in Multiple Sclerosis

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Abstract

Neurological diseases are complex and difficult diseases to study. Although new approaches and therapies have been developed for other disorders such as Alzheimer’s, Epilepsy, and Parkinson’s disease, Multiple Sclerosis (MS) still remains seemingly inexorable. MS is the archetypal complex disease; it is a paradigm of debilitating neuropathology. Discounting trauma, MS is the most common chronic neurological condition resulting in disability affecting young adults. In its typical form it primarily affects the brain, spinal cord, and optic nerves and is characterised by lesions in the central nervous system (CNS) separated in space and time. Chronic inflammatory cells have been shown to be involved and axons and myelin are ultimately damaged. The changes observed in the MS brain are not uniformly distributed with foci of inflammation termed lesions or plaques being spread throughout the CNS. Inflammatory demyelination of the CNS leads to aberrant conduction of nerve signals resulting in anomalous impulse trafficking. This aberration results in a diverse array of neurological signs and clinical features. The MS plaque evolves with time; in the early stages there is local breakdown of the blood-brain barrier (BBB), then inflammation and oedema, loss of myelin, and finally the deposition of the CNS equivalent of scar tissue; gliosis. The resulting shrunken area of sclerosis may or may not be associated with clinical deficits depending on the localisation and extent of the lesion. The CNS shows some potential for remyelination, and patients often experience remissions characterised by return of normal function.
The underlying pathological sequence corresponds to the clinical patterns seen in sufferers, with characteristic phases of relapses and remissions. Several clinical courses of the disease are recognised and serve to distinguish different stages of the disease. Approximately 80% of patients present with the relapsing-remitting form of the disease (RRMS). This phase sees the patient suffering paroxysmal symptomatic episodes with near-complete remissions. Residual disability may accumulate with time and this leads to the next stage, the secondary-progressive form (SPMS). The SPMS course is similar to the RRMS phase but for the steady accumulation of neurological deficits and disability. The third and most severe phenotype of the disease is the primary-progressive form (PPMS). The PPMS course is characterised by progressive disability, although not necessarily uniformly increasing with time, and does not generally involve remissions. The natural history of MS is highly variable and patients show variable expressivity with some progressing through all stages swiftly while others do not.

Amalgamating numerous lines of evidence, the foremost hypothesis is that MS is the result of an environmental trigger in genetically susceptible individuals. The role of immune mechanisms in MS is most strongly supported by the presence of chronic inflammatory cells, and findings that genes in the major histocompatibility complex (MHC) are specifically involved. The MHC findings in conjunction with twin concordance studies lend strong support to the notion that MS involves a significant genetic component. MS is more common in temperate regions than
the tropics and this geographical variation is thought to suggest an environmental factor (e.g. virus) may trigger the condition in susceptible individuals. Epidemiological studies have taught us that MS risk can be transferred from location to location if the sufferer relocates, however the risk appears to be correlated with where the first 15 or so years of life were spent. Not discounting other possible causes or multiple triggers, these two lines of evidence together suggest that if an environmental factor does in fact trigger the disease, it is most likely to be active in the first few decades of life.

MS genetics research strategies have involved attempts at distinguishing the exact genes involved in susceptibility, and a great deal of energy has also been expended attempting to unravel the complexities associated with the heterogeneity amongst cases. Indeed, the heterogeneity of the disorder presents some of the greatest challenges, and to date only the MHC gene region (located on chromosome 6p21.3) has been clearly implicated. However, the MHC region alone cannot explain the complexity seen in MS and therefore other genes are also thought be involved. A powerful approach in molecular genetics entails the determination of gene expression. Particular gene expression patterns can provide investigators with a ‘fingerprint’ of gene expression in healthy tissues as a baseline, which is then compared to disease tissues. In MS research, obtaining large numbers of well-defined brain samples remains challenging; however the importance of tissue samples as repositories of genetic information cannot be over-emphasized. Previous research has used smaller numbers of tissues with
technologies such as microarrays in an attempt to detect specific genes that may be acting differently in the MS affected brain compared to healthy controls.

In the first part of this thesis, we undertook gene expression investigations in normal-appearing white matter (NAWM) which is predominantly comprised of neuroglia, investigating MS versus healthy matched control brain tissues. Gene expression alterations in distinct brain regions may underlie the heterogeneity of idiopathic neuroinflammatory disorders. Variations in expression patterns between groups and within clinical sub-types of MS may suggest distinct molecular processes. We undertook quantitative determination of gene expression in 7 secondary-progressive multiple sclerosis (SPMS) affected brains, comparing expression to matched normal appearing white matter tissues from healthy neuropathology free controls, to assess gene expression of the claudin11 oligodendrocyte transmembrane protein (CLDN11), glial cell derived neurotrophic factor (GDNF), neuregulin 1 (NRG1), protein tyrosine phosphatase receptor type C (PTPRC), and the toll-like receptor 3 (TLR3) genes using a sensitive real-time expression technique, relative quantitative real-time PCR (qRT-PCR). We detected significantly increased expression of NRG1 and TLR3 in MS frontal white matter compared to healthy matched controls. Immunoblots were carried out as a qualitative check to confirm the presence of the investigated proteins of interest. We detected increased expression of NRG1 in the NAWM tissues which represents a gene expression pattern consistent with gliosis and attempted remyelination. Given that NRG1 plays an important role during neuronal and glial
growth and differentiation future studies of subsequent isoforms of this gene may prove rewarding. We also detected increased expression of TLR3 which represents T-cell activity and possibly transcriptional activation of proinflammatory genes leading to the expression of proinflammatory cytokines. These observations suggest that further studies in TLR3 in MS may also yield additional important findings. Although expression changes were also seen in CLDN11 in frontal and temporal, GDNF frontal and temporal, NRG1 temporal, PTPRC frontal and temporal samples; those results did not reach statistical significance.

We also undertook gene expression studies in the normal-appearing grey matter (NAGM) which is primarily comprised of neurons, of MS brain tissues versus healthy matched controls. Our findings include statistically significant increased gene expression of GDNF in frontal, NRG1 overall, PTPRC overall and frontal, and TLR3 overall brain tissues compared to healthy matched neuropathology free controls. Increased expression of GDNF, NRG1, PTPRC, and TLR3 suggest gene expression patterns primarily indicative of astrocyte mediated defence of dopaminergic neurons (↑GDNF), attempts at remyelination (↑NRG1), autoreactive lymphocyte mediated inflammation (↑PTPRC), and attempted axonal repair (↑TLR3). The upregulation of these genes may also represent other pathological processes in MS and their entire contributions still remain to be thoroughly investigated. Future studies may explain the role of these genes in MS pathogenesis. We detected no statistically significant gene expression
differences for CLDN11 overall, frontal, or temporal, NRG1 frontal, PTPRC temporal, or TLR3 frontal tissue samples.

Characterisation and profiling of genes involved immune pathways, neuronal differentiation, and myelination, may explain the link between the pathogenesis and the molecular mechanisms underlying the development of MS. In the second part of this thesis we undertook genetic variation studies to investigate whether particular candidate genetic variations are associated with MS in an Australian population.

Firstly, we undertook genotyping studies in the genes MTHFR and MTRR. Genetic variations within these genes have been reported to result in elevated intracellular pHcy levels leading to CNS dysfunction, neurodegenerative, and cerebrovascular diseases. Our investigations entailed the genotyping of a cohort of 140 cases and matched controls for MTRR and MTHFR, by restriction length polymorphism (RFLP) techniques. Two polymorphisms: MTRR A66G and MTHFR A1298C were investigated in an Australian age and gender matched case-control study. No significant allelic frequency difference was observed between cases and controls. Our findings suggest no evidence for an association between the MTRR A66G and MTHFR A1298C polymorphisms and MS in the tested population.
Secondly, we undertook genotyping in the gene PTPRC. Autoreactive T lymphocytes and their associated antigens have long been presumed important features of MS pathogenesis. The Protein tyrosine phosphatase receptor type C gene (PTPRC) encodes the common human leukocyte antigen receptor CD45. Variations within PTPRC have been previously associated with diseases of autoimmune origin such as type 1 diabetes mellitus and Graves’ disease. We set out to investigate two variants within the PTPRC gene, C77G and C772T in subjects with MS, and matched healthy controls to determine whether significant differences exist in these markers in our case-control population. We employed high resolution melt analysis (HRM) and restriction length polymorphism (RFLP) techniques to determine genotypic and allelic frequencies. Our study found no significant difference between frequencies for PTPRC C77G (155 cases versus 171 controls). Similarly, we did not find evidence to suggest an association between PTPRC C277T and MS (181 cases versus 180 controls). Linkage disequilibrium (LD) analysis was carried out between C77G and C772T, which showed that strong linkage disequilibrium exists between the two tested loci (D’=0.9970, SD=0.0385). Although the PTPRC gene plays a significant role in regulating CD4+ and CD8+ autoreactive T-cells, interferon-beta responsiveness, and other potentially important processes, our study did not support a role for the two tested variants of this gene with MS susceptibility in the tested population.

Thirdly, we undertook genotyping in the gene TLR3. Autoimmunity appears to be a key aspect of MS with autoreactive T-cells thought to mediate central nervous
system inflammation. Toll-like receptors are known to mediate cellular recognition of pathogens via entities known as pathogen associated molecular patterns. Toll-like receptor 3 is encoded by the gene TLR3 and is recognised as an important factor in dsRNA virus recognition. Furthermore, the gene is known to be involved in the expression of neuroprotective mediators. We set out to investigate 2 variations within the TLR3 gene, an 8bp insertion-deletion [-/A]₈ and a single base-pair variation C1236T, in a multiple sclerosis affected population versus healthy matched controls, to determine whether significant differences exist in these markers in our population.

We used capillary gel electrophoresis (GeneScan) and TaqMan genotyping assay techniques to resolve genotypes for each marker, respectively. Our study found no significant difference between frequencies for TLR3 [-/A]₈ (205 cases versus 196 controls). Similarly, we found no evidence for association for TLR3 C1236T with MS (199 cases versus 198 controls). Although the role of TLR3 and the toll-like receptor family remain significant in neurological and central nervous inflammatory disorders, our study did not suggest an association with MS for the two tested variants in this gene within the tested population.

This research employed genomic techniques and gene expression studies to examine the role of several candidate genes in an attempt to better understand their possible associations with MS susceptibility. The significant gene expression findings of this research have identified several genes that may
potentially be used for further investigation of the complex genetics of multiple sclerosis.
Acknowledgements

This work would not have been possible without the enduring support of many individuals, some of which have reformed my understanding of the scientific process completely. The pursuit of scientific truth is paved with numerous complexities (and much caffeine) and the complete volume and breadth of molecular biology is now insurmountable by a single individual (regardless of his brilliance).

I would first and foremost like to express my deepest gratitude to Professor Lyn Griffiths, under whose watchful eye I have studied for the better part of this last decade in an effort to produce results worthy of her generosities. The years I have spent in her laboratory represent my formal training in the discipline of Molecular Genetics and I do not think I could have been more privileged then to spend those years in the Genomics Research Centre.

Secondly, I would like to convey my sincerest appreciation to the Trish Multiple Sclerosis Research Foundation, its directors Carol and Roy Langsford, and its eminent research committee, for awarding me postgraduate funding to carry out this research.

Several collaborators have participated in making these investigations possible. Patient samples for the genetic variation studies were kindly provided by
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Many “scientists-in-waiting” have contributed to my efforts in the lab and I would like to acknowledge their assistance here collectively. As it is well recognised in neuroscience that memory can be treacherous, I refrain from listing them individually lest I forget to mention somebody resulting in a shemozzle.

I dedicate my efforts (as humble as they may be) to the MS sufferers and their families who endure this curse in its various manifestations, and pause for a moment to remember my mother, who I continue to miss more and more each day.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

____________________
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# Abbreviations

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<tr>
<td>α</td>
<td>Probability of type I error</td>
</tr>
<tr>
<td>ACVR2</td>
<td>Activin type 2 receptor</td>
</tr>
<tr>
<td>ACTN3</td>
<td>Actinin alpha 3</td>
</tr>
<tr>
<td>ADEM</td>
<td>Acute disseminated encephalomyelitis</td>
</tr>
<tr>
<td>AGE</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMV</td>
<td>Recombinant avian myeloblastosis virus</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMS</td>
<td>Benign multiple sclerosis</td>
</tr>
<tr>
<td>bp</td>
<td>base pair/s</td>
</tr>
<tr>
<td>CA</td>
<td>Chronic active</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>CCR5</td>
<td>Chemokine (C-C motif) receptor 5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
</tbody>
</table>
C1QB Complement 1-q-subcomponent-B chain
CI Chronic inactive
CIS Clinically isolated syndrome
CJD Crutzfeld-Jakob disease
CLDN11 Claudin 11
CNS Central nervous system
COX Cyclo-oxygenase
CPMS Chronic progressive/Clinically probably multiple sclerosis
CSF Cerebrospinal fluid
CT Cycling threshold/Computerised tomography
CTLA4 Cytotoxic T-lymphocyte-associated protein 4
D’ Lewontin’s linkage disequilibrium measure
DNA Deoxyribonucleic acid
DEPC Diethylpyrocarbonate
DHPLC Denaturing high-pressure liquid chromatography
dNTP Dinucleotide triphosphate
dsDNA Double-stranded DNA
dsRNA Double-stranded RNA
DTT Dithiothreitol
DZ Dizygotic
EAE Experimental allergic encephalomyelitis
EBNA2 Epstein-Barr virus nuclear antigen 2
EBV Epstein-Barr virus
EDSS  Expanded disability status scale
EDTA  Ethylenediaminetetraacetic acid
EGF   Epidermal growth factor
EP    Evoked potential
ERCC5 Excision repair cross-complementing 5
EtBr  Ethidium bromide
EtOH  Ethanol
FA    Formaldehyde agarose
FDA   Food and drug administration
GAMES Genetic analysis of multiple sclerosis in Europeans
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
Gd    Gadolinium
gDNA  Genomic deoxyribonucleic acid
GDNF  Glial cell derived neurotrophic factor
GFAP  Glial fibrillary acidic protein
GGF   Glial growth factor
GGF2  Glial growth factor 2
GRN   Granulin
GSTM1 Glutathione S-transferase mu-1
GWA   Genome-wide association
H     Histidine
HCl   Hydrochloric
HCV   Hepatitis C virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hcy</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin stain</td>
</tr>
<tr>
<td>HERV</td>
<td>Human endogenous retrovirus</td>
</tr>
<tr>
<td>HHV</td>
<td>Human herpesvirus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>(^1)H-MRS</td>
<td>Proton magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>HPC</td>
<td>High performance computer</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSP70</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human T-cell lymphocytotrophic virus</td>
</tr>
<tr>
<td>HWE</td>
<td>Hardy-Weinberg equilibrium</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes melitus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IFN-α</td>
<td>Interferon-alpha</td>
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<tr>
<td>IFN-β</td>
<td>Interferon-beta</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin-γ</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL7R</td>
<td>Interleukin-7 receptor</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>IPMDC</td>
<td>International panel on MS diagnostic criteria</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-γ–inducible protein of 10 kDa</td>
</tr>
<tr>
<td>IRF1</td>
<td>Interferon regulatory factor 1</td>
</tr>
<tr>
<td>IRF5</td>
<td>Interferon regulatory factor 5</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IVIg</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KIF1B</td>
<td>Kinesin family member 1B</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma associated herpesvirus</td>
</tr>
<tr>
<td>LCA</td>
<td>Leukocyte common antigen (CD45)</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>LFB</td>
<td>Luxol fast blue stain</td>
</tr>
<tr>
<td>LKA4</td>
<td>Leukotriene A-4 hydrolase</td>
</tr>
<tr>
<td>LOD</td>
<td>Log of odds for genetic linkage</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
<td>MboII</td>
<td>Restriction enzyme MboII</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MGB</td>
<td>Minor-groove binder</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Mig</td>
<td>Monokine induced by IFN-γ</td>
</tr>
<tr>
<td>min</td>
<td>minute/s</td>
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<tr>
<td>Acronym</td>
<td>Full Name</td>
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</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MMS</td>
<td>Malignant multiple sclerosis</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MOPS</td>
<td>4-Morpholinepropanesulfonic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MTHFR</td>
<td>5,10-Methylenetetrahydrofolate reductase</td>
</tr>
<tr>
<td>MTRR</td>
<td>5-Methyltetrahydrofolate-homocysteine-methyltransferase reductase</td>
</tr>
<tr>
<td>MYT1</td>
<td>Myelin transcription factor 1</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygotic</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NAGM</td>
<td>Normal appearing grey matter</td>
</tr>
<tr>
<td>NAWM</td>
<td>Normal appearing white matter</td>
</tr>
<tr>
<td>NCBI</td>
<td>National centre for biotechnology information</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa light chain enhancer of activated B-cells</td>
</tr>
<tr>
<td>NINJ1</td>
<td>Ninjurin 1</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NRG1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>NspI</td>
<td>Restriction enzyme NspI</td>
</tr>
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</table>
NPL  Non-parametric linkage
NTC  No-template control
OCB  Oligoclonal band
ON   Optic neuritis
OPC  Oligodendrocyte precursor cell
OPN  Osteopontin
ORF  Open reading frame
PAGE Polyacrylamide gel electrophoresis
PAMP Pathogen-associated molecular pattern
PBMC Peripheral blood mononuclear cell
PBP  Prostatic binding protein
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
pH   Pondus hydrogenii
pHcy Plasma homocysteine
p.i.  Post-infection
PLP1 Proteolipid protein 1
PML  Progressive multifocal leukoencephalopathy
PP   Primary progressive
PPMS Primary progressive multiple sclerosis
PRKCB1 Protein kinase C-beta-1
PRMS Progressive relapsing multiple sclerosis
PROCR Endothelial protein C receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PTGDS</td>
<td>Prostaglandin D2 synthase</td>
</tr>
<tr>
<td>PTPRC</td>
<td>Protein tyrosine phosphatase receptor type C</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation normal T-cell expressed and secreted</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rhGGF2</td>
<td>Recombinant human glial growth factor 2</td>
</tr>
<tr>
<td>RPL17</td>
<td>Ribosomal protein L17</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPS4X</td>
<td>Ribosomal protein S4 X-linked</td>
</tr>
<tr>
<td>RPTP</td>
<td>Receptor-type protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk/Relapsing-remitting</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapsing remitting multiple sclerosis</td>
</tr>
<tr>
<td>Rsal</td>
<td>Restriction enzyme Rsal</td>
</tr>
<tr>
<td>rt-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SEP</td>
<td>Somatosensory evoked potential</td>
</tr>
<tr>
<td>SJL</td>
<td>Swiss Jim Lambert</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SMDF</td>
<td>Sensory-motor derived factor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>Secondary progressive</td>
</tr>
<tr>
<td>SPMS</td>
<td>Secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>SPP1</td>
<td>Secreted phosphoprotein 1</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistics package for the social sciences</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single-stranded conformational polymorphism</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline with tween 20</td>
</tr>
<tr>
<td>TDT</td>
<td>Transmission disequilibrium test</td>
</tr>
<tr>
<td>TEN</td>
<td>Toxic epidermal necrolysis</td>
</tr>
<tr>
<td>T$_{H1}$</td>
<td>T-helper cell type 1</td>
</tr>
<tr>
<td>T$_{H2}$</td>
<td>T-helper cell type 2</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-interleukin receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>Toll-interleukin receptor domain containing adaptor protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melt temperature</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler's murine encephalomyelitis virus</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TPMS</td>
<td>Transitional progressive multiple sclerosis</td>
</tr>
<tr>
<td>T-reg</td>
<td>T-regulatory lymphocyte</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VEP</td>
<td>Visual evoked potential</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen 4</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number tandem repeat</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w</td>
<td>Measure of effect size</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>XRCC9</td>
<td>X-ray repair complementing defective repair 9</td>
</tr>
</tbody>
</table>
Publications Arising from Work Described in this Thesis


Related Publications


Other Publications


Conference Presentations


Chapter 1

Introduction
1.1 Significance

Multiple Sclerosis (MS) is the most common idiopathic inflammatory disease of the central nervous system (Page, Kurtzke et al. 1993; Poser 1994; Weinshenker 1994; Rothwell and Charlton 1998). It is the leading cause of neurological disability in early to middle adulthood in the Caucasian population, only surpassed by trauma (Al-Omaishi, Bashir et al. 1999). MS predominantly affects Caucasians between the ages of 18 and 40 years with females accounting for approximately 2/3 of all cases. MS diagnosis currently depends on clinical observations and the interpretation of supportive radiological and laboratory investigations. The precise aetiology of the disease is unclear; however it is known that both genetic and environmental factors are involved (Ebers and Sadovnick 1994; Keegan and Noseworthy 2002; Giovannoni and Ebers 2007; Ebers 2008).

This research intends to contribute to our understanding of the molecular genetics of MS by investigating genes implicated in the pathogenesis of the disease. Novel candidate genes involved in the neuropathology of MS will be investigated. The ultimate benefit derived from a clear explanation of the genetics of MS would be the possible opportunity to develop new strategies for diagnosis and treatment.
1.2 Rationale

Multiple Sclerosis (MS) is a neurological disease that has been considered a discrete clinical entity since the early 1830s. It is the most common cause of acquired neurological disability affecting young adults, other than trauma. MS Pathology is characterised by chronic inflammation in the central nervous system (CNS) which results in selective axonal damage and myelin destruction. Myelin damage leads to numerous and varied neurological signs and symptoms owing to aberrant nerve impulse trafficking. The aetiology of MS is thought to be autoimmune with circumstantial evidence suggesting that an environmental agent triggers the condition in genetically susceptible individuals. Onset typically occurs in early to middle adulthood with a female preponderance of approximately 2:1 which varies minimally between populations. Twin-studies demonstrate higher concordance rates in monozygotic (MZ) compared to dizygotic (DZ) twins, and siblings and the general population. These observations are suggestive of an underlying genetic component.

The natural history of MS has been elucidated over the past few decades however the exact causes of the disease remain somewhat undefined. MS is a paradigm of complex disease showing widely variable clinical expression with unpredictable course. Despite concerted efforts, the genetic aspects of MS are still not comprehensively understood. Early diagnosis of MS has proven problematic and current treatments demonstrate variable efficacy. A better
understanding of how MS develops may lead to the development of improved clinical diagnosis and more efficacious therapeutic interventions.

1.3 Aims & Hypotheses

This research is based on the premise that particular genetic variations and gene expression patterns underlie the molecular pathology of MS. This research will investigate candidate genes for gene expression patterns that characterise MS, and test for association between numerous candidate gene variations and susceptibility to MS.

This research encompasses 2 discrete aims. The specific aims are to:

1. Accurately quantify novel candidate gene expression in MS.

The first aim of this research involves investigation of post-mortem brain tissue messenger ribonucleic acid (mRNA) from MS sufferers (brain samples) versus health matched controls by relative quantitative real-time polymerase chain reaction (qRT-PCR), investigating transcripts of interest from novel candidate genes thought to be involved in MS pathogenesis.
2. Examine genomic variations that may be associated with MS.

The second aim of this research involves the investigation of genetic variations in candidate genes thought to be involved in MS susceptibility. The source of genetic material for these studies is peripheral blood-derived lymphocytic deoxyribonucleic acid (DNA) from MS sufferers versus age, gender, and ethnicity matched healthy controls. Association between particular genotypes of interest in candidate susceptibility genes and MS are to be investigated.

The ultimate goal of MS research is to determine the processes leading to the pathologic state. An enhanced understanding of the underlying pathological processes leading to the disease would offer opportunities to improve current clinical approaches to the disease. Genetic research offers evolving technologies for investigating the clinical heterogeneity seen in MS, and may help elucidate the molecular mechanisms underpinning the disease. The investigations of this research may contribute new findings to our current understanding of the genetics associated with MS susceptibility.

1.4 Thesis Structure

This research will investigate the gene expression of some potential candidate genes thought to be involved in the neuropathology of MS. Furthermore, genetic variation studies will be undertaken to test for association with MS in an attempt
to identify gene variants that may be contributing to MS susceptibility. The candidate genes for this research have been chosen based on their known involvement with neurobiological processes, particularly central nervous system inflammation, myelination, and neurogenesis, and their suggested involvement from previous MS susceptibility investigations. Five main studies will be undertaken. The first two studies will investigate the gene expression of five genes (CLDN11, GDNF, NRG1, PTPRC, and TLR3) in the normal appearing white matter (NAWM) and normal appearing grey matter (NAGM) of the MS brain compared to healthy controls. The remaining three studies will investigate genetic variations in four candidate genes in MS (MTHFR & MTRR, PTPRC, and TLR3). The investigations comprise five standalone chapters of the thesis in two broad sections; gene expression studies (Chapter 5 NAWM, Chapter 6 NAGM), and genetic variations studies (Chapter 7 MTHFR & MTRR, Chapter 8 PTPRC, and Chapter 9 TLR3). Those five chapters of the thesis represent independent scientific articles.
Chapter 2

Clinical Background
2.1 Aetiology and Pathogenesis of MS

The natural course of MS is unpredictable and constitutes an acute burden for most sufferers. Relapses are a cardinal hallmark of the disease that occurs with irregular frequency. It is now known that particular molecular changes tend to precede relapses. Understanding those molecular changes may be the first step towards consistently predicting the clinical course in MS.

The precise aetiology of MS is currently unknown and the pathogenesis of the disease continues to be refined and updated. It is recognised that MS is a complex disease with distinct clinical and pathological phenotypes. It is thought that those phenotypes most likely reflect different pathways to tissue injury. The natural history of MS is not easily prognosticated due to its variable expressivity. The cardinal hallmarks of the disease have been revised many times throughout history and continue to be refined as both clinical and molecular knowledge advance. There have been many revisions to the diagnostic criteria of MS over the years (Kurtzke, Beebe et al. 1977; Poser 1978; Weinshenker, Bass et al. 1989; Phadke 1990; Weinshenker, Rice et al. 1991; Weinshenker, Rice et al. 1991; Runmarker and Andersen 1993; Poser and Brinar 2004). Recent studies report that immunomodulatory treatments (such as Interferon and monoclonal antibody therapies) can alleviate the symptoms of the disease, suggesting that the core process leading to tissue injury is immunological in origin. It is recognised that axonal and myelin injury result from a complex set of events
involving many immunological mediators (Weiner 2004). Prominent theories regarding the events leading to the tissue injury seen in MS implicate the increased expression of interleukin-12 (IL-12), interferon-gamma (IFN-γ) and T\textsubscript{H}1-mediated cytokines, with decreases in interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor beta (TGF-β). Investigations of MS cerebrospinal fluid (CSF) during attacks have shown the elevation of 3 particular chemokines that act toward T-cells and mononuclear phagocytes: IFN-γ–inducible protein of 10 kDa (IP-10); monokine induced by IFN-γ (Mig); and regulated on activation normal T-cell expressed and secreted (RANTES) (Sorensen, Tani et al. 1999; Weiner and Cohen 2002). In a past study, Yong and colleagues demonstrated that interferon-beta (INF-β) can reduce activated T-cell migration by inhibiting the activity of T-cell matrix metalloproteinases (MMPs), which is significant because activated and autoreactive T-cells are though to be prominent contributors to MS pathogenesis (Yong, Chabot et al. 1998). Identifying and unravelling the molecular events which culminate to form the clinical manifestations of the disease is an important objective of MS research which may give rise to novel therapeutic approaches.

It is hypothesised that an environmental mediator such as a latent virus or infection triggers MS in genetically susceptible individuals (Poser, Benedikz et al. 1992). One of the cardinal manifestations of MS is inflammatory demyelination of CNS tracts, which leads to a reduction in conduction velocity with distortion and loss of impulse traffic along those pathways. The peripheral nervous system
remains predominantly unaffected (Ziemssen 2005). Several practical constraints have limited effective long-term follow-up studies of MS. Lag-time between onset of the disease and confirmed diagnosis has been reported to have impeded research efforts in the past (Lublin 2005). Initial studies in MS appeared to demonstrate bias towards the more severe cases that accumulate within the clinical setting (McAlpine 1961; McAlpine 1964; Bonduelle 1967).

The ideal sample size for an MS study is not known (because effect size cannot be accurately predicted until susceptibility factors are defined) and there has been debate regarding whether a true inception cohort is possible due to confounding factors such as the length of time to diagnosis (Polman 2008). Numerous studies involving the prospective collection of patient information have demonstrated the heterogeneous nature of the duration of illness and disability experienced by sufferers. Those studies reiterate that MS is a complex neurological disorder (Allison 1950; Gruber 1962; Panelius 1969; Gudmundsson 1971; Percy, Nobrega et al. 1971; Broman, Andersen et al. 1981; Clark, Detels et al. 1982; Detels, Clark et al. 1982; Patzold and Pocklington 1982; Visscher, Liu et al. 1984; Bronnum-Hansen, Koch-Henriksen et al. 1995).
Although the clinical course is individually variable, most patients eventually develop severe neurological disability. In 85% of MS sufferers onset is with acute or sub-acute episodes of neurological disturbance termed a clinically isolated syndrome (CIS) (Miller, Barkhof et al. 2005). Several factors predict a more favourable clinical course: Optic neuritis (ON) (Confavreux, Vukusic et al. 2003; Nilsson, Larsson et al. 2005), isolated sensory symptoms (Eriksson, Andersen et al. 2003), long interval to second relapse and no disability after 5 years from onset (Miller, Hornabrook et al. 1992; Franklin and Nelson 2003), and normal magnetic resonance imaging (MRI) results (Jacobs, Kaba et al. 1997; Brex, Ciccarelli et al. 2002; Beck, Trobe et al. 2003; Minneboo, Barkhof et al. 2004). Factors that have been associated with poor prognosis include: multifocal CIS (Comi, Filippi et al. 2001), afferent system involvement, high relapse rate and/or substantial disability within the first 5 years (Eriksson, Andersen et al. 2003), and abnormal MRI with high lesion load (Frederiksen, Larsson et al. 1991; Morrissey, Miller et al. 1993; Jacobs, Kaba et al. 1997; Soderstrom, Ya-Ping et al. 1998; Comi, Filippi et al. 2001; Brex, Ciccarelli et al. 2002; Beck, Trobe et al. 2003; Minneboo, Barkhof et al. 2004). It is generally accepted that the key demographics that predict unfavourable outcome are age of onset after 40 years, male gender, motor or cerebellar symptoms, a primary-progressive course, and a high number of attacks from the outset of clinical presentation (Kantarci and Weinshenker 2005).
The expanded disability status scale (EDSS) is a multivariate model used to predict long-term outcome in MS. The baseline EDSS remains the most accurate predictor of deterioration in classical MS (Weinshenker, Rice et al. 1991). Unfortunately, the EDSS is not uniformly useful for determining outcome in all patients. Uncertainty still exists regarding the underlying causes and significance of associated viral infections (Sibley, Bamford et al. 1985), pregnancy (increased frequency of attacks postpartum) (Birk, Ford et al. 1990), and climatic influence (Clark, Detels et al. 1982; Detels, Clark et al. 1982; Hammond, English et al. 1988).

MS predominantly affects women of childbearing age. The influence of pregnancy on MS has been repeatedly examined (Birk, Ford et al. 1990; Confavreux, Hutchinson et al. 1998; Ghezzi and Zaffaroni 2008). A summary of those findings shows that relapses are reduced in the later stages of pregnancy and then increased postpartum (up to approximately 3 months duration). Studies indicate that there is no increase in birth anomalies (defects, stillbirths) among females with MS and there appears to be no ill effect in general on the foetus (Damek and Shuster 1997). These observations (in MS) are similar to several other autoimmune diseases which also predominantly affect women during the reproductive years (e.g. rheumatoid arthritis, systemic lupus erythematous, and Sjögren’s syndrome) (Cutolo, Sulli et al. 1995; Geenen, Perrier de Huterive et al. 2002).
Interestingly, MS tends to become clinically silent during pregnancy, which also implies a significant immune component (Buyon 1998). Buyon and colleagues hypothesise that those remittance periods could be attributed to the downregulation of immunological processes that recognise and eliminate non-self molecules during the pregnancy period.

Additional to the role of $T_{H1}$ (pro-inflammatory) and $T_{H2}$ (anti-inflammatory) cytokines, studies have also investigated the role of progesterone and prolactin as potential therapeutic immunoregulators. To date, one of the most significant findings in this regard is that in the presence of progesterone, lymphocytes in pregnant women produce protein factors capable of stimulating a $T_{H1}$ to $T_{H2}$ shift (Szekeres-Bartho and Wegmann 1996). That immunogenic shift causes proinflammatory cytokines to be downregulated and anti-inflammatory cytokines to be upregulated, tilting the balance in favour of suppression of inflammatory events. Some investigations continue to pursue hormonal phenomena however the observed changes appear to be only part of a complex interplay between MS biology and the environment. Both the aetiology and pathogenesis of the disease remain important areas of MS research.
2.2 Incidence and Prevalence of MS

Prevalence of MS increases with distance from the equator (see Figure 2-1 below) (Kurtzke 1980). Northern Europe, North America, and Australia have a similar prevalence of approximately 0.1% in the general population. Incidence is much reduced at the Arabian Peninsula, Asia, and South Americas. Seasonal variations and exacerbations have been described in epidemiological studies, noting higher incidence at temperate climates (Goodkin and Hertsgaard 1989; Jin, de Pedro-Cuesta et al. 2000). Several studies have concluded that migration occurring before the age of 15 years confers the susceptibility of the new region (Dean and Elian 1997; Hammond, McLeod et al. 2000). If migration occurs after the age of 15, the person appears to retain the susceptibility of their country of origin (Franklin and Nelson 2003). MS mainly affects Caucasians. The disease is approximately 20-fold less in the Inuit of Canada, the Native American tribes of North America, the Australian Aborigines, and the Maori tribes of New Zealand (Cabre, Signate et al. 2005). Numerous studies have also supported the finding that incidence is higher in Northern Europeans than in Asians, Africans, Maoris, and North American Indians (McFarlin and Lachmann 1989). Clusters of MS cases have been reported in Finland (Wikstrom 1975; Wikstrom and Palo 1975), North and South New Zealand (Skegg, Corwin et al. 1987), the Faroe Islands (Benedikz, Magnusson et al. 1994), and North Sweden (Binzer, Forsgren et al. 1994). Whilst in themselves these reports are interesting, the overall relevance of these observations to MS in general remains unknown. Studies of genetic
isolates and familial clusters of MS cases have led to widespread support for the belief that polygenic mechanisms are involved (Ebers, Sadovnick et al. 1995). Genetic studies have shown that adopted first-degree relatives share a relative risk (RR) of 1.0 with the general population, half-siblings approximately 6.5, first-degree relatives 15-25, dizygotic twins similarly 15-25, offspring of conjugal MS 147.5, and monozygotic twins approximately 190 (Sadovnick, Yee et al. 2005). Although informative, familial aggregation of MS cannot be solely explained by environmental factors (Ebers, Sadovnick et al. 1995). Efforts to explain these findings have shown that maternal effects such as intrauterine and perinatal factors, breastfeeding, and genomic imprinting, have no appreciable effect on familial risk (Sadovnick, Ebers et al. 1996). Seasonal variations have been reported in several studies demonstrating changes in onset of exacerbations but it is uncertain if those findings are relevant to all populations (Goodkin and Hertsgaard 1989; Jin, de Pedro-Cuesta et al. 2000; Jin, Yang et al. 2008). While incredibly informative, it is sometimes difficult to discern true effects in epidemiological studies as cases and controls are usually exposed to the same environmental factors. Still, epidemiological studies have contributed valuable findings to the MS literature.
Figure 2-1 World Distribution of Multiple Sclerosis

Figure 2-1 shows the world distribution of MS demonstrating the equatorial gradient whereby a higher incidence of cases occurs at high and low latitudes. (Source: Spencer S. Eccles Health Sciences Library, University of Utah Sciences Centre, Salt Lake City, Utah, USA; accessed at: http://library.med.utah.edu/kw/ms/mml/ms_worldmap.html).
2.3 Pathological Hallmarks of MS

The brain and spinal cord consist of grey and white matter and accommodate numerous highly specialised cell types including neurons and microglia (see Figure 2-2 below). Neurons are primarily responsible for brain function while microglia are special supportive cells of the central nervous system (CNS). A prominent feature of MS pathology is the demyelination of nerve axons in the CNS (see Figure 2-3 below). Demyelination results in improper nerve conduction and subsequently nervous system function. The characteristic demyelination seen in the MS brain and spinal cord may be clearly demonstrated by histopathology; staining techniques (as seen in Figure 2-4 below). Destruction of oligodendroglia (the cells responsible for myelinating nerve axons), and perivascular inflammation are well recognised in MS (Kanda 2003). Demyelinated regions (termed lesions) are spatially and temporally diverse and have been correlated with varied clinical presentation of the disease (Prineas, Kwon et al. 2001; Kremenchutzky, Rice et al. 2006). Studies have correlated alterations in disease activity with structural changes by magnetic resonance imaging (MRI) (Killestein, Rep et al. 2002). The underlying tissue injury in MS may be both anatomically discrete and multifocal. The lateral and posterior columns, cervical and dorsal regions, optic nerves, and periventricular areas of the brain are principally affected. Tracts of the midbrain, pons variolii, and cerebellum may also become demyelinated. Injury occurs at the grey matter in both the cerebellum and the spinal cord. Imaging techniques are used to
demonstrate demyelinating lesions (also referred to as plaques) by techniques such as computerised tomography (CT scan) and more increasingly variants of magnetic resonance imaging (MRI) (see Figure 2-5 below). MRI techniques are now delivering increasingly enhanced scans of the MS brain. In typical MS the cellular bodies and nerve axons are generally preserved in early lesions. Progressed, chronic lesions differ in that they show axon destruction localised mainly to the long tracts. Fibrous gliosis (the CNS equivalent of scar tissue deposition) gives the long tracts a sclerotic appearance, and hence the term sclerosis has been widely adopted. Early and late lesions are often found simultaneously and chemical changes in both lipid and protein constituents of myelin have been documented (Holley, Gveric et al. 2003). We continue to expand our understanding of MS pathology as molecular, histochemical, and neuroimaging methods evolve. These technologies are helping to define the MS plaque at the microscopic level and are central to assessing tissue changes at different stages of the pathology. Pathological studies have shown that immune factors associated with multiple different effector mechanisms contribute to the inflammation, demyelination, and tissue injury observed in MS (Lucchinetti 2008). Although current therapeutic regimes benefit most sufferers to some extent, specific preventative or curative treatments do not yet exist.
Figure 2-2 shows the diversity of specialised cells of the brain in the grey and white matter tissues (Source: Instructor’s Resource CD-ROMs: Images, Animations, & Lecture Presentation for Fundamentals of Anatomy & Physiology, Sixth Edition, by Frederic H. Martini, Ph.D., Pearson Education, Inc. publishing as Benjamin Cummings).
Figure 2-3 Saltatory Nerve Signal Dysfunction

Figure 2-3 shows nerve conduction in myelinated and demyelinated axons. (A) Saltatory nerve conduction in myelinated axons occurs with the nerve impulse jumping from one node of Ranvier to the next. Sodium channels (shown as breaks in the solid black line) are concentrated at the nodes where axonal depolarisation occurs. (B) Following demyelination, additional sodium channels are redistributed along the axon itself, thereby allowing continuous propagation of the nerve action potential despite the absence of myelin (Source: Fauci, A.S., Kasper, D.L., Braunwald, E., Hauser, S.L., Longo, D.L., Jameson, J.L., Loscalzo, J.: Harrison’s Principles of Internal Medicine, 17th Edition, accessed at: http://www.accessmedicine.com).
Figure 2-4 shows example MS histopathology (H&E stain on the left, Loyez stain on the right) demonstrating pale-stained demyelinating areas (D) adjacent to normal-staining white matter (W) (Source: Burkitt, H.G., Stevens, A., Lowe, J.S., Young, B.: Wheater’s Basic Histopathology, 3rd Edition, p270, Churchill Livingston, Pearson Professional Limited).
2.4 Signs and Symptoms of MS

The onset of MS may be insidious or alternately abrupt and the initial symptoms may vary from trivial to severe (Kremenchutzky, Rice et al. 2006). CNS dysfunction with persistent remissions and exacerbations characterises MS. Individual patients may present with a broad spectrum of neurological impairments at different times (see Figure 2-6 below). The most common symptoms are: paresthesia in one or more extremity, trunk, or face; weakness or clumsiness of an extremity; and/or visual disturbances. Visual disturbances include partial blindness and pain in one eye (due to retrobulbar optic neuritis), diplopia, dimness of vision, or scotomas. Other acute signs include: fleeting ocular palsy, transient weakness of an extremity, stiffness or fatigability of a limb, gait disturbances, difficulties with bladder control, and vertigo. A wide variety of signs are evidence of widespread CNS involvement. Exposure to heat may accentuate the signs and symptoms; a phenomenon known as Uthoff’s syndrome. Dysarthria, ataxia, and tremor, together comprise Charcot’s triad, the classical MS symptom picture. MS patients often report debilitating fatigue. Fatigue is attributable to sympathetic vasomotor involvement (Flachenecker, Rufer et al. 2003). Both motor and sensory involvements are significant. Apathy, lack of judgment, and inattention may occur with emotional labiality being relatively common. Many patients report euphoria, and/or reactive depression. Severe changes such as dementia and mania are relatively uncommon, but sometimes occur during the later stages of the disease.
Figure 2-6 shows a pictorial summary of the typical MS symptom picture; (Clinical Feature of MS) dysarthria, ataxia, tremor, ocular dysfunction, mood alterations, and loss of motor control are prominent clinical features of the disease (Source: Ginsberg, L.: Lecture Notes on Neurology 7th Edition, p142, Blackwell Science Limited).
2.5 Findings on Physical Examination

Weakness of the limbs may manifest as loss of strength or dexterity, fatigue, or a gait disturbance. Exercise-induced weakness is a common characteristic of MS. Spasticity is often associated with spontaneous muscle spasms. Ocular signs may be present. Sensory symptoms are varied and include both paresthesias and hypesthesia (e.g. reduced sensations). There is often corticobulbar pathway involvement. Convulsive seizures may occur in some patients. Scanning speech (slow enunciation with a tendency to hesitate at the beginning of a word or syllable) is common in advanced stages of the disease. Several ocular signs are generally present. Changes in visual fields, transient ophthalmoplegia with diplopia (indicating brainstem, and 3rd, 4th, and 6th cranial nerve involvement), and nystagmus are common findings. Total blindness as a direct result appears to be rare. Deafness is also rare. Unilateral facial numbness is sometimes present, resembling trigeminal neuralgia. Deep reflexes are generally increased (e.g. knee and ankle jerks). Superficial reflexes, particularly upper and lower abdominal are diminished or absent. Tremors (from cerebellar lesions) are common, with continued purposeful effort accentuating the sign. Motion is ataxic: shaky, irregular, tremulous, and generally ineffective. A static tremor (especially when the head is unsupported) is common (Ginsberg 1999).
2.6 Clinical Course of MS

The clinical course of MS can be primarily progressive, or relapsing. Months or years may separate episodes. Relapses become more frequent with time and usually result in permanent disability (Redelings, McCoy et al. 2006). Frequent attacks appear to correlate with rapid progression of the disease. Some studies report that early age of onset may be a predictor of less rapid and severe subsequent course in some cases (Visscher, Liu et al. 1984). There are several generally accepted classifications of courses of the disease defined by degree of debilitation over time (see Figure 2-7 below):

2.6.1 Relapsing Remitting MS (RRMS)

RRMS is characterised by relapses during which new symptoms may present or past symptoms may reappear and worsen. Relapses are followed by periods of remission during which the patient may partially or fully recover. Episodes may last for days, weeks, or months, and recovery may be gradual or instantaneous. Most sufferers are first diagnosed with RRMS. Depression has been noted in these patients and has been primarily attributed to functional disabilities (Mendes, Tilbery et al. 2003).
2.6.2 Secondary Progressive MS (SPMS)

SPMS is an advanced form of the disease that demonstrates worsening of signs and symptoms between relapses. During SPMS the patient may experience numerous relapses but these eventually merge into an overall progressive course. There tends to be little to no recovery from disabilities acquired during this phase of the disease. It is not known what causes the transition from RRMS to the secondary progressive phase.

2.6.3 Primary Progressive MS (PPMS)

PPMS manifests and then progresses with virtually no remissions. PPMS differs from RRMS and SPMS mainly in that onset is typically in the late thirties or early forties.

2.6.4 Progressive Relapsing MS (PRMS)

PRMS exhibits progressive course from onset and is punctuated by relapses. There is significant recovery immediately following a relapse, but general worsening of symptoms occurs between relapses.
Figure 2-7 Clinical Courses of MS in Current Clinical Practice

Figure 2-7 shows the 4 clinical courses of MS in use in current clinical practice:
Clinical sub-groupings that have been used in the past, but are generally no longer in use include:

2.6.5 Benign MS (BMS)

BMS is a sub-group of RRMS, which has been used to describe the clinical course of patients who have suffered MS for fifteen or more years without experiencing any serious or enduring disability.

2.6.6 Malignant MS (MMS)

MMS is a classification pertaining to cases that progress quickly and cause severe disability within short periods of time.

2.6.7 Chronic Progressive MS (CPMS)

CPMS is a classification that is no longer in use. PPMS and SPMS were once collectively called Chronic Progressive MS. Chronic progressive MS is easily confused with Clinically Probable MS, a terminology which is however still in use. Clinically probable MS refers to the presentation of a patient with an attack and lesion that cannot yet be definitively diagnosed as MS (first attack) although the
most likely diagnosis would be MS discounting other differential diagnoses.

2.6.8 Transitional Progressive MS (TPMS)

TPMS is a form of the disease that has been infrequently referred to. TPMS is defined by a progressive course commencing many years after an isolated neurological incident.

2.7 Current Diagnosis of MS

Because no distinct clinical feature or diagnostic test is sufficient for the diagnosis of MS, diagnostic criteria have included a combination of both clinical and para-clinical studies (Poser and Brinar 2004). The International Panel on MS Diagnostic Criteria (IPMDC) with the support of the United States National Multiple Sclerosis Society, and the International Federation of Multiple Sclerosis, periodically revise diagnostic recommendations. The IPMDC asserts that the diagnosis of MS remains a partly objective and partly subjective process. Diagnosis of MS is best made by an expert who is familiar with the disease (usually a consultant neurologist with MS expertise), its differential diagnoses, and the interpretation of para-clinical assessments such as imaging, cerebrospinal fluid (CSF) analysis, and evoked potentials (Polman, Reingold et
A definitive diagnosis is not possible following an initial attack. Remissions and exacerbations, and clinical evidence of disseminated CNS lesions in several anatomically discrete locations are highly suggestive of MS. Exclusion of pertinent differential diagnoses is essential. Laboratory findings generally show abnormal CSF in ≥55% of cases. Total Immunoglobulin-γ (IgG) antibodies may be ≥13%, and lymphocytes and protein titres may be elevated. Oligoclonal bands (OCBs) indicating intra-blood brain barrier (BBB) synthesis of IgG may be demonstrated upon agarose gel electrophoresis (AGE) of the CSF in approximately 90% of cases. Oligoclonal bands represent excess antibody produced by plasma cells. In some disease such as sclerosing panencephalitis, the OCBs represent antibodies directed against the causative agent (measles virus) (Burgoon, Caldas et al. 2006), however in MS no such disease specific causative antigen has yet been indentified. Interestingly, some patients with proven MS do not demonstrate oligoclonal bands (Nakashima, Fujihara et al. 2003), and conversely clinical deficits do not always coincide with the presence of OCBs (Hafler, Duby et al. 1988). Studies have however reported the presence of antibodies against myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) in both the human (Reindl, Linnington et al. 1999; Berger, Rubner et al. 2003), animal experimental (Linnington, Bradl et al. 1988; Brunner,
Lassmann et al. 1989), and cell culture forms (Kerlero de Rosbo, Honegger et al. 1990) of the disease.

CSF concentrations of myelin basic protein (MBP) may be elevated during active demyelination. Contrast and non-contrast magnetic resonance imaging (MRI) reveals plaques. MRI scans are often then compared with contrast-enhanced computerised tomography (CT) scans for confirmation. Double-dose delayed CT-scan may be employed to give fine resolution films. Recorded electrical responses known as evoked potentials (EP) are tested to ascertain sensory and motor involvement. Patter-shift visual evoked potential (VEP), brainstem auditory, and somatosensory evoked potentials (SEP), are usually delayed (Mitsui and Ohno 2003; Nakashima, Fujihara et al. 2003). Recently, proton magnetic resonance spectroscopy ($^1$H-MRS) has been used to provide biochemical activity profiles for both disease and normal tissue (Watanabe, Fukatsu et al. 2003). It has been reported that gliomas and acute MS plaques have indistinguishable chemical resonance spectra by traditional magnetic resonance techniques. The differentiation of different plaques (glioma/tumefacative) has been reported using a more recent MRI method (Butteriss, Ismail et al. 2003). MRI is an evolving technology that promises to contribute significantly to the future of MS diagnosis.
2.8 Differential Diagnoses for MS

MS is the most common idiopathic inflammatory condition of the central nervous system. The distinction between MS and other benign or fulminant inflammatory demyelinating conditions is primarily based on the chronicity and severity of signs and symptoms (Kantarci and Weinshenker 2005). Differential diagnosis considerations include; small cerebral infarctions, syringomyelia, amyotrophic lateral sclerosis (ALS), acute disseminated encephalomyelitis (ADEM), progressive multifocal leukoencepalopathy (PML), syphilis, pernicious anaemia, spinal arthritis, ruptured intravertebral disks, basilar impression, systemic lupus erythematosus (SLE), and hereditary ataxias. CNS tumours, abscesses, mass lesions, and vascular malformations of the brain and/or spinal cord must also be excluded (Compston, McDonald et al. 2005). Particular attention should be directed to the area about the foramen magnum, since treatable lesions at the junction of the spinal cord and medulla (e.g. subarachnoid cyst, and foramen magnum tumours) occasionally cause a variable and fluctuating spectrum of motor and sensory signs that mimics MS. The full spectrum of differential diagnoses that must be excluded complicate the timely diagnosis and delivery of early therapeutic interventions in MS (Ginsberg 1999).
2.9 Current Treatment of MS

Immunomodulatory drugs have been the primary choice for the treatment of MS. Three forms of interferon (IFN-β1a; Avonex, IFN-β1a; Rebif, and IFN-β1b; Betaferon/Betaseron), along with Glatiramer acetate (Copaxone) have been shown to reduce both relapse rates (by approximately 30%) and rate of formation of focal inflammatory lesions as measured by MRI (Ochi 2003). Intravenous immunoglobulin (IVIg) is another therapeutic possibility, but is not currently United States Food and Drug Administration (FDA) approved.

Interferon therapy is a common therapeutic approach especially in newly diagnosed sufferers. The action of interferon is thought to reduce the burden of autoreactive T-cells in MS patients (Zafranskaya, Oschmann et al. 2007). Interferons may be administered as once weekly intramuscular (IM) injection (IFN-β1a; Avonex), or on alternate days (IFN-β1b; Betaferon/Betaseron), or three times weekly subcutaneous (SC) injection (IFN-β1a; Rebif). Once weekly injections are termed “low-dose regimes”, whilst more frequent injections are designated as “high-dose regimes” (Rovaris, Capra et al. 1999). Interferon preparations that are administered multiple times weekly (Rebif and Betaferon/Betaseron) appear to have slightly increased efficacy compared to once weekly regimes (Avonex) but are also more likely to induce neutralising antibodies that reduce the clinical benefit. Interferons act by inhibiting proinflammatory T-cell responses and reduce the capacity of activated T-cells to
enter the nervous system (Namaka, Pollitt-Smith et al. 2006). Side effects of interferon therapy include flu-like symptoms, injection site reactions, altered liver function, lymphopenia, and hepatotoxicity.

Glatiramer acetate (Copaxone) is classified as an immunomodulatory protein. Its mode of action is thought to be induction of regulatory T-cells that reduce inflammation in the central nervous system (Iarlori, Gambi et al. 2008). Copaxone is administered SC daily. Side effects of treatment include flushing, chest-tightness, shortness of breath, palpitations, and increased anxiety.

Natalizumab (Tysabri) is a recombinant humanised monoclonal antibody to the cellular adhesion molecule α-4 integrin. Initially, Natalizumab was approved by the FDA after clinical studies found that it was approximately twice as effective as other disease-modifying therapies at preventing acute MS attacks (Miller, Khan et al. 2003). Although the exact mode of action of this therapy is unknown it is thought to inhibit leukocyte migration from the blood into CSF and hence reduce inflammation and demyelination. Natalizumab was originally approved by the United States food and drug administration in 2004 only to be withdrawn from the market in March of 2005 after two patients treated in combination with IFN-β1a developed the viral infection progressive multifocal leukoencepalopathy (PML) (Langer-Gould, Atlas et al. 2005). Following safety reviews, Natalizumab was returned to the US and European markets (as well as Australia) in 2006 under special prescription conditions (Tenser 2006). It is currently indicated for the
reduction of frequency of relapses in relapsing-remitting MS only (Kappos, Bates et al. 2007).

Alemtuzumab (Campath) is also a humanised monoclonal antibody, which binds to the CD52 antigen (a low molecular weight glycoprotein), which exists on the surface of most T and B lymphocytes, monocytes, macrophages, and some granulocytes. The drug acts by activating complement mediated lysis of CD52-positive cells (Moreau, Coles et al. 1996). Alemtuzumab is a relatively new MS therapy and whilst it shows some promise at reducing relapse rates, it is only currently indicated for use in the aggressive relapsing-remitting form of the disease (mainly due to side-effects) (Hirst, Pace et al. 2008). Recent studies show that once tissue injury is established, suppression of inflammation does not limit brain injury or protect from clinical progression and therefore it is hoped that patients receiving anti-inflammatory therapy before the cascade of events leading to uncontrolled destruction of axons and neuroglia will not progress to the secondary-progressive stage of the disease (Coles, Deans et al. 2004). These observations and rationale has led to much interest in the application of Campath in MS.

Recommended routine acute treatment of relapses of MS is by a short course of intravenous (IV) methylprednisolone followed by 2 weeks of the oral for of the drug (Sorensen, Haas et al. 2004). Plasma exchange regimes have also been of some benefit to patients with fulminant attacks of demyelination, which have
been unresponsive to glucocorticoids (Schilling, Linker et al. 2006). Whilst these therapies target some potentially important molecular phenomena, distinct targeted therapies are yet to be developed. A specific beneficial therapy will ultimately rely on a clear understanding of the underlying molecular mechanism of the disease, which is yet to be clearly defined.

2.10 Prognosis for MS Sufferers

Most MS patients experience progressive neurological disability (Poser 1981). Fifteen years after onset, only 20% of patients have no functional limitation. Twenty-five years after onset >80% will have reached the secondary progressive stage. With three or more T2-weighted lesions upon MRI, the risk of developing MS within 10 years is approximately 70-80%. Two or more Gadolinium (Gd) enhancing lesions are highly predictive of MS and disability has been correlated with T2 weighted lesion load (Fisniku, Brex et al. 2008). Typical abnormalities on somatosensory or visual-evoked potentials and CSF examination are thought to be of similar prognostic value, although those relationships are not yet completely explained. Mortality as a direct consequence of MS is uncommon. The 25-year survival rate is estimated to be approximately 85% of expected. Death most commonly occurs as a result of a secondary complication such as pneumonia or suicide (Feinstein 1997).
2.11 Immunology of MS

Previous studies have reported that MS is primarily an autoimmune disorder (Berger, Rubner et al. 2003; Gaertner, de Graaf et al. 2004; Weiner 2004). Indeed, a family history of MS or other related autoimmune disorder is often helpful with regards to establishing a confirmed diagnosis (Sadovnick, Baird et al. 1988; Sadovnick and Ebers 1993). Previous investigations demonstrate abnormal B-cell response evidenced by IgG oligoclonal bands in the CSF in many patients (Hafler, Duby et al. 1988). In addition, cells in the CSF secreting myelin basic protein MBP antibodies, phospholipid protein (PLP) antibodies, and myelin oligodendrocyte glycoprotein (MOG) antibodies have been documented (Sellebjerg, Jensen et al. 2000). However, not all patients with confirmed MS have oligoclonal bands in the CSF, which confounds an already complicated diagnosis. The exact cause of the immune response in MS is currently unknown. The dominant theory is that abnormal T-helper cells that are autoreactive against myelin antigens are formed. These cells cross the blood-brain barrier with the help of adhesion molecules (such as very late antigen 4; VLA-4), which are then stimulated by myelin antigens presented by local antigen presenting cells (APCs) to differentiate into $T_{H1}$ proinflammatory cytokine secreting cells. The proinflammatory cytokines interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) are thought to be key contributors to neuroinflammation. This process, along with activation of B-cells, cytotoxic T-cells, and macrophages, is
hypothesised to result in inflammatory demyelination (Steinman 1996; Steinman 2001) (see Figure 2-8 below).

Both innate (predominantly phagocytic cells; macrophages and polymorphonuclear cells) and adaptive (lymphocytes; B-cells and T-cells) immune responses are though to be involved in MS. Lymphocytes can generate a huge repertoire of high-affinity antigen receptors and are responsible for the development of immunological memory. The innate system cells express cell surface antigen receptors termed toll-like receptors (TLRs) that are invariant and only recognise conserved pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, peptidoglycan, CpG DNA motifs, dsRNA, and bacterial flagella (Schnare, Barton et al. 2001; Barton and Medzhitov 2002).

Cells of the adaptive immune system show marked variation in their antigen receptors recognising many different antigens. Recognition of antigens by these receptors leads to clonal expansion of the sensing cell, which then establishes immunological specificity and memory. The adaptive system is subdivided into humoral and cellular components (B-cells and T-cells, respectively). Antigen recognition by B-cells (humoral recognition) induces B-cell proliferation and differentiation resulting in generation of antibody secreting plasma cells (Winges, Gilden et al. 2007). T-cells cannot recognise soluble antigen, these cells instead recognise peptide antigen bound and presented by a major histocompatibility complex (MHC). Two main types of T-cell exist: CD4$^+$ T-helper cells (which
require MHC class II presentation of antigens) and CD8\(^+\) cytotoxic T-cells (which require MHC class I presentation of antigens). CD4\(^+\) T-helper cells activate the innate immune system and B-cells via cytokine release, while CD8\(^+\) cytotoxic T-cells kill virally infected cells (Choi and Splitter 1994). Classically, it was believed that the adaptive system could innately recognise self and non-self, however it is now thought that the immune system must learn to identify self antigens (Rennie 1990).

Autoimmune diseases such as MS are thought to develop when self-tolerance is misdirected or lost and numerous mechanisms have been proposed for the maintenance of tolerance to self-antigen (Walker and Abbas 2002). One such mechanism is T-cell anergy, whereby T-lymphocytes are functionally inactivated following an antigen encounter, but remain viable for an extended period of time in a hypo-responsive state (Schwartz 2003). T-cell anergy is initiated by T-cell receptor in the absence of the co-stimulatory molecule CD28. During this process, the expression of cytotoxic T-lymphocyte associated antigen 4 (CTLA4) is required. Alegre and colleagues have shown CTLA4 expression to be up-regulated directly after T-cell activation (Alegre, Frauwirth et al. 2001). Furthermore, mice that are deficient in CTLA4 develop lethal inflammatory autoimmune diseases characterised by severe inflammatory infiltrates in multiple organs (Waterhouse, Penninger et al. 1995; Waterhouse, Marengere et al. 1996). Blocking CTLA4 and B7 (B7 being another co-stimulatory molecule) interaction in experimental allergic encephalomyelitis (EAE), has been shown to
exacerbate the severity of that model (Hurwitz, Sullivan et al. 1997). Unfortunately, genomic investigations into CTLA4 in humans have reported mixed findings with both positive (Kantarci, Hebrink et al. 2003) and negative (Dyment, Steckley et al. 2002) results, therefore the implications of this mechanism in MS remain unsure. Although currently incomplete, the immunology of MS continues to generate much interest and numerous research efforts are underway to define the exact nature of this element of the disease.

There is evidence to suggest a temporal relationship between infectious triggers, such as increased Epstein-Barr virus (EBV) titres, and the onset of neurological symptoms (Levin, Munger et al. 2005). Elevated antibody titres to Epstein-Barr virus (EBV) have been found prior to the onset of MS and EBV has been found in serum of patients with MS exacerbations (Wagner, Munger et al. 2004). Detailed studies advocate the systemic activation of myelin-reactive T-cells that secrete proinflammatory \( T_{H1} \) cytokines in MS. T-cell activation has been correlated with inflammatory activity on Gadolinium-enhanced MRI (Jensen, Langkilde et al. 2004) and chemokine and chemokine receptor expression in the CSF of patients remains topical. Several chemokines and their ligands have been implicated as part of a specific MS immune response (Sorensen, Roed et al. 2004).

Although potential candidates exist, a validated immunological marker capable of predicting predisposition or clinical course remains elusive (Bielekova and Martin 2004). The development of such biomarkers could have a significant impact on
the diagnosis of MS. The characterisation of reliable biomarkers involved in MS-specific neuroinflammation, demyelination, and gliosis could prove valuable to our understanding of the immunology of the disease (Bielekova and Martin 2004).

Immunological changes reported to date cannot entirely explain the pathology of MS (Chaudhuri and Behan 2004). The existence of disease-discordant monozygotic twins suggests an important role for environmental factors in the aetiology of the disease. Animal models have clearly demonstrated that infections can trigger autoimmune diseases (von Herrath, Homann et al. 1997; Bach 2005). But the question whether an infectious trigger precedes the development of MS is still not completely answered. A significant issue regarding the incidence of infection and then the development of the disease is the possibility that the two events are separated quite substantially in time (Sullivan, Visscher et al. 1984). Studies have reported that childhood infections could result in increased susceptibility to developing the disease later in life (James 1988). Overall, the immunology of MS remains an important part of research efforts as investigators strive to better understand the immunological mechanisms that underlie development of the disease.
Figure 2-8 shows a current model of MS pathogenesis as summarised by Baranzini and colleagues (Baranzini and Hauser 2002). The model shows the relationships between cells of the immune system and the molecular mechanisms thought to contribute to CNS demyelination and damage.
2.12 Infectious Triggers of MS

The underlying rationale in the search for infectious triggers in MS is that the discovery of a definite MS pathogen would prove fundamentally significant, and to that end many aspects of viral infection have been investigated. A number of studies have been based on the premise that an infectious entity (such as a virus) may be associated with the development of multiple sclerosis (Cirone, Cuomo et al. 2002; Ransohoff, Wei et al. 2002; Mattson and Taub 2004; Stuve, Racke et al. 2004), however none have been conclusively linked with the disease (Hernan, Zhang et al. 2001).

Evidence for infectious triggers in neurological conditions include reports of oligoclonal bands (OCBs) in the CSF directed against human T-lymphotropic virus 1 (HTLV-1); which has been shown to result in myelopathy (Muraro, Wandinger et al. 2003), and intrathecal synthesis of oligoclonal IgG in neurosyphilis patients (which is directed against Treponema pallidum; a gram-negative spirochaete bacterium) (Vartdal, Vandvik et al. 1982). Additionally, progressive multifocal leukoencepalopathy (PML), an exclusively human demyelinating neuropathy, has been linked to viral triggers (Padgett, Walker et al. 1971; Padgett, Rogers et al. 1977). Various microorganisms have been loosely associated with MS over the years, but none have yet been clearly linked to the disease. Studies have reported increased coincidence of MS and infection with Chlamydia pneumoniae (a gram-negative intracellular pathogen) (Munger,
Peeling et al. 2003; Munger, DeLorenze et al. 2004), however other studies have found no association (Tsai and Gilden 2001). Two human herpes viruses have also generated some interest in MS research; human herpesvirus 6 (HHV-6), and the human herpesvirus 4; Epstein-Barr virus (EBV). First contact with both of these viruses is usually in the first decades of life. Studies of HHV-6 have shown that the virus exists not only in the MS brain but also in normal, healthy brains (Cuomo, Trivedi et al. 2001). This finding (amongst others) has led to the widespread notion that viral reactivation must be involved if these viruses really do play a role in the development of MS.

Some of the most credible reports of a link with infection (to date) implicate the Epstein-Barr virus (EBV), which is known to cause infectious mononucleosis. Associations between EBV and MS are supported by observations that there is an increased risk of developing MS after infectious mononucleosis (Marrie, Wolfson et al. 2000). Furthermore, other studies report that cases of MS are rare amongst patients without serum anti-EBV antibodies (Wandinger, Jabs et al. 2000). Serological studies have found significant elevated anti-EBV antibodies before the onset of MS, in particular the elevation of EBV nuclear antigen 2 (EBNA2) (Ascherio, Munger et al. 2001). Unfortunately, EBV infection cannot explain all instances of MS susceptibility as EBV infection is both relatively common and widespread, and yet only a small proportion of people infected with the virus go on to develop MS (Hernan, Zhang et al. 2001). Furthermore, in
studies by Hilton and colleagues, no EBV-specific RNA was detected in MS brains by *in situ* hybridisation (Hilton, Love et al. 1994).

Epidemiological studies report that childhood exposure to viruses correlates with increased incidence, and MS ‘epidemics’ have been reported, most notably in the Faroe Islands (Benedikz, Magnusson et al. 1994). Animal models have established that viruses can cause diseases with prolonged incubation periods. Remitting and relapsing courses, and myelin destruction mediated by a variety of mechanisms has also been discussed (Seay and Wolinsky 1982; Seay and Wolinsky 1983; Fazakerley, Khalili-Shirazi et al. 1988; Zurbriggen, Yamawaki et al. 1993; Johnson 1994; Jakob and Roos 1996; Skundric 2005). MS patients occasionally exhibit elevated levels of antibody against Measles, Parainfluenza 3, Influenza C, Varicella, Herpes Simplex, Rubella, Epstein-Barr, HTLV-I, HTLV-II, Corona-, Adeno-, and Simian 5 viruses in serum and CSF (Salmi, Reunanen et al. 1983; Larsen, Bloomer et al. 1985; Goswami, Randall et al. 1987; Ohta, Saida et al. 1988). The pathology of certain experimental models of virus-induced inflammatory demyelinating diseases resemble that of MS. Intermittent detection of viral RNA using *in situ* hybridisation has also been reported (Njenga, Marques et al. 2004). Animal models support the hypothesis that viruses can initiate the immunopathogenic events leading to demyelination (Murray, Brown et al. 1992; Murray, Cai et al. 1992).
Given that so many infectious triggers could potentially lead to increased susceptibility, a logical approach to this dilemma is to study the biological receptors that become activated during infection. One important group of receptors is the toll-like family. Toll-like receptors (TLRs) function as pathogen pattern recognition molecules that sense and initiate innate and adaptive immune responses against infectious triggers (Wang, Miyahara et al. 2008). Previous studies have shown that toll-like receptor 3 (TLR3) in particular can act as a potent negative regulator of axonal growth in mammals (Cameron, Alexopoulou et al. 2007), control activation of adaptive immune responses (Schnare, Barton et al. 2001), and can induce astrocytes to produce neuroprotective mediators under certain circumstances (Bsibsi, Persoon-Deen et al. 2006). Given these various findings, further investigations of toll-like receptors (TLRs), particularly genetic studies of the genes that code for the receptors, could yield interesting results. The notion of an infectious trigger in MS remains intriguing, and warrants further research.
Chapter 3
Genetic Background
3.1 Defining Complex Disorders

Idiopathic inflammatory demyelinating diseases consist of a broad spectrum of disorders that vary in their clinical course, regional distribution, and pathology. MS is a complex disorder that shows marked heterogeneity both clinically and pathologically. Genetic approaches to investigating complex disorders may yield a better understanding of disease processes which in turn may lead to more efficacious treatments.

3.1.1 The Nature of Complex Disorders

The term complex disorder refers to a disease phenotype that does not exhibit classic Mendelian inheritance attributable to a single gene. Complex disorders may exhibit familial tendencies (such as clustering and concordance amongst relatives) and are in essence polygenic. Other hallmarks of complex disease include environmental risk factors, seasonal variations, birth order influences, cohort effects, variable ages of onset, and irregular disease progression (clinical course). Complex disorders are usually both polygenic and multifactorial; examples include cardiovascular disease, diabetes mellitus, Alzheimer’s disease, cancers, and autoimmune disorders (such as systemic lupus erythematosus and Graves’ disease) (Mahmood, Kureshi et al. 2004). In complex disorders, the interaction of multiple genetic loci and their resulting effect on phenotypic
expression and disease risk is far more complex than that of monogenic disorders (Flintoft 2004). Complex disorders more often than not result from mutations that create subtle changes in the function of a spectrum of gene products (proteins), which makes discerning all the genes involved in a particular disorder challenging (Lander and Schork 1994). The genes involved in a complex disorder are commonly referred to as susceptibility genes because they predispose the individual to an increased risk of developing a disease, as a contrast to causative genes (distinct variations that lead to a particular phenotype) as seen in most Mendelian disorders. Genetic variations that underlie Mendelian diseases are generally single-nucleotide changes that have a strong effect on protein function, consistent with the fact that those diseases involve single mutations with significant phenotypic effects. In contrast, complex diseases are caused by multiple variations with each variant contributing only a small effect individually (Thomas and Kejariwal 2004). The ultimate goal of studying complex disorders is to establish the genetic components of a particular disease and to attempt to explain the heterogeneity seen within disorders. To this end several approaches have been developed in an effort to investigate complex disorders.

Classical genetic studies encompass investigations into the role of inheritance in disease. These approaches have identified important risk factors for several other disorders in the past, such as diabetes mellitus (Bell, Horita et al. 1984) and Alzheimer’s disease (Corder, Saunders et al. 1993). Traditionally, linkage
approaches have attempted to implicate likely regions of association with particular phenotypes. These investigations have focused on establishing genetic maps of regions associated with disease. Complex diseases remain difficult to analyse partly owing to the number of loci involved, so in an attempt to discern multiple contributing loci, genome-wide investigations have been developed. Genome-wide association studies investigate genetic variation across the entire human genome and are designed to identify genetic associations with observable traits or the presence or absence of disease (Pearson and Manolio 2008). A follow on approach in genetics focuses on what is termed genomic convergence. This technique assembles data gathered from different methods to help identify candidate genes involved in complex traits. This approach has been used successfully to identify candidate susceptibility genes for Parkinson’s and Alzheimer’s disease (Li, Oliveira et al. 2003). Once likely genomic regions are implicated, candidate gene studies may be carried out in an attempt to determine whether there is association (positive correlation) with the disease being investigated. Forms of these investigations include case-control association studies (examining unrelated affected and unaffected individuals) and familial inheritance studies examining disease allele transmission (Lewis 2002). A significant challenge to the efficient correlation of genotype with phenotype in MS is the effect of widespread heterogeneity.
### 3.1.2 Heterogeneity of MS

MS is an example of a complex disease that is most likely polygenic by nature. Although Mendelian patterns of inheritance sometimes exist in complex disorders (Goate, Chartier-Harlin et al. 1991), findings for MS to date most convincingly support a polygenic non-Mendelian mode of inheritance (Sadovnick and Ebers 1993; Sawcer, Jones et al. 1996). MS involves environmental factors (increased geographical incidence at high and low latitudes), twin heritability (increased concordance rates with degree of relatedness), some degree of variable age of onset, and clearly irregular disease progression (clinical course) and expressivity. Interestingly, the epidemiological observations cannot be explained wholly by either environment or genetics alone. Lucchinetti and colleagues have reported on the pathological heterogeneity of MS several times, citing that their pathological and clinical observations of a large series of MS cases continues to support pathogenic heterogeneity in immune effector mechanisms involved in MS lesion formation (Lucchinetti, Bruck et al. 2004). The investigators explain that CNS lesion formation may not occur identically in all patients. Furthermore, these authors also recently discussed the pathology of MS in relation to stages of lesion activity, phases of the disease, and the observed clinical course of sufferers which all show marked heterogeneity (Lucchinetti 2008).

Genetic heterogeneity in MS is also well established and has been reported numerous times by several groups. For example, studies by Haines and
colleagues have reported linkage to chromosomes 6p21, 6q27, and 19q13 with LOD scores >3.0, as well as regions on chromosomes 12q23-24 and 16p13, and analysis taking into account HLA-DR2 also identified two additional potential linkage regions on chromosomes 7q21-22 and 13q33-34 (Haines, Bradford et al. 2002). In recent times, it has become evident that studying genetic heterogeneity and gene-gene interactions (epistasis) is an important consideration in attempting to explain complex disorders. A recent study by Brassat and colleagues explains that it is conceivable that distinct gene-gene interactions contribute to the MS phenotype in different populations (Brassat, Motsinger et al. 2006). In their studies using locus association models to predict MS risk, they found that they could ascertain 75-76% accuracy (with P<0.01) within their tested population. These results demonstrate the importance of exploring both main effects and gene-gene interactions in the study of complex diseases. Discerning valuable results from complex interactions remains one of the major challenges in research involving the genetics of MS, but understanding those interactions may provide valuable insights into pathogenesis in individual populations.
3.2 Genetic Studies of MS

Epidemiological and clinical observations have led to the realisation that MS susceptibility is most likely genetically determined. To investigate genetic contribution to the overall phenotype the role of inheritance in MS has been investigated using traditional methods involving ethnic, familial, and twin studies as well as special (unique) genetic populations. Understanding the role and extent of inheritance in MS forms the basis of further genetic testing in unrelated individuals as seen in case-control association studies.

3.2.1 The Role of Inheritance in MS

Compelling evidence from separate lines of investigation now exists to suggest that susceptibility to MS is inherited, at least to some extent. The MS-prone genotype is thought to result from either independent or possibly interacting polymorphic genes, with each contributing only a modest effect to the overall phenotype (Hauser 2005). A complication of inheritance in MS is that the genes conferring susceptibility may not be consistent between individuals. Geographical studies examining longitudinal associations have reported that familial aggregation in MS is genetically determined; however the degree of complexity associated with that inheritance remains unclear (Ebers, Sadovnick et al. 1995). The recurrence risk in offspring of conjugal pairs has provided some reliable
insights into the measure of this complexity (Ebers and Sadovnick 1994; Ebers and Sadovnick 1994). Investigations have shown that it is unlikely that a shared environment during childhood or adolescence plays a significant part in the familial aggregation of MS (Sadovnick, Ebers et al. 1996). Although susceptibility appears to be mainly genetically determined, there is no simple discernable Mendelian pattern of inheritance in MS (Hauser 2005). Recent advances in biotechnology, investigation of candidate genes by new genomic techniques, and gene expression studies by new sensitive technologies now prove promising approaches for research into the genetic aspects of the role of inheritance in MS (Dyment, Ebers et al. 2004). To date, approaches to determining the extent of the role of inheritance in MS have included studies investigating ethnic susceptibility to MS, the effects of migration on the risk of developing the disease, the degree of familial aggregation in different populations, the epidemiological distribution of MS cases, studies on the involvement of environmental factors on MS susceptibility, twin, half-twin, and adoptee studies, parent-child concordance investigations, conjugal MS studies (albeit somewhat rare), and investigations into special genetic populations (such as the Sardinian Island isolate located in the Mediterranean Sea). These traditional genetic approaches have been previously used in an attempt to clarify the exact modes and extent of inheritance in MS.

So far, no effect of shared environment has been proven and little is certain about the potential environmental triggers that may lead to the disease (e.g. viral
infections, including those by the Epstein-Barr virus). Some studies have supported the role of Epstein-Barr virus (EBV) in MS (Ascherio, Munger et al. 2001; Levin, Munger et al. 2003), although it is unlikely that EBV is involved in primary pathogenesis as it cannot be detected in MS brains (Rodriguez Carnero, Martinez-Vazquez et al. 2002) and asymptomatic EBV infection is common in healthy individuals (Walling, Brown et al. 2003).

Approximately 25% to 30% of monozygotic twins of an affected individual develop MS versus only approximately 3% to 5% of dizygotic twins and other siblings (Kenealy, Pericak-Vance et al. 2003). Studies have repeatedly shown that the incidence of MS increases with degree of relatedness between individuals. Monozygotic twins show the highest concordance rates and unrelated individuals have the least (see Figure 3-1 below) (Compston and Coles 2008). The trend of increasing susceptibility has been an important factor in the basis of genetic approaches to studying MS. Discerning the extent and effect of heritability in MS remains an important focus of research efforts because explaining the hereditary component could not only determine the susceptibility loci involved in development of the disease but also potentially help explain the contribution of non-genetic factors to the overall phenotype (which appear to vary between populations).
Figure 3-1 Estimated Lifetime Risk of MS with Relatedness

Figure 3-1 shows the currently estimated lifetime risk (risk percentage) associated with developing MS based on the degree of relatedness between individuals as summarised by Compston et al. (Compston and Coles 2008). The highest risk is seen amongst monozygotic twins (25-35%). The risk for developing MS increases with increasing degree of relatedness, suggesting a significant genetic component.
3.2.2 Ethnic Susceptibility to MS

MS is particularly prevalent in people of Northern European descent, including those living in Australia, New Zealand, and North America. Studies in the past have suggested that the most frequent occurrence of MS is in geographical areas settled by the Vikings, Vandals, and the Goths. It is hypothesised that migrants from those areas carry genes conferring susceptibility to MS throughout the New World, Europe, South Africa, Australia, and New Zealand (Poser 1994). Northern European Caucasians show the highest susceptibility to the disease, whilst native black Africans demonstrate the lowest rates, and furthermore seem to harbour a natural resistance to disease as shown by migration studies (Dean, Bhigjee et al. 1994). The disease is very rare in the Japanese (Kuroiwa, Shibasaki et al. 1983) as well as in the Chinese (Yu, Woo et al. 1989). To date, MS has not been reported in ethnically pure Eskimos, Inuits, North and South Amerindians, Australian aborigines, New Zealand Maoris, Pacific Islanders, or Lapps (Poser 1994).

3.2.3 Migration and Susceptibility to MS

Migration studies in MS support a role for genetic factors in the disease. North American Japanese appear to have a higher susceptibility than native Japanese and other Asians, whilst African (Kurtzke, Delasnerie-Lauppre et al. 1998) and
Asian migrants (Detels, Brody et al. 1972) generally retain their low susceptibility rate when relocating. Other studies have also shown that Caribbeans in the UK demonstrate increased rates of MS, and that native South Africans of English decent have a lower susceptibility than more recent English immigrants (Dean and Elian 1997). In general, studies have shown that if migration occurs after the age of 15 years, the susceptibility rate of the country of origin is retained. Hammond and colleagues report that in the Australian population, prevalence of MS in Australian born individuals is strongly correlated with latitude. MS is more prevalent with increasing southern latitude and the highest rates in Australia are seen in Hobart, Tasmania (Hammond, English et al. 2000).

3.2.4 Familial Aggregation in MS

Family studies have provided strong evidence for the involvement of genetic factors in MS. The risk of developing MS increases in relatives of affected individuals with first-degree relatives being higher than subsequent generations (Ebers, Sadovnick et al. 1995). The lifetime risk for siblings with one affected parent is approximately 10 to 20-fold increased, and with two affected parents approximately 20 to 30-fold increased, as compared to unaffected individuals (Compston and Coles 2008). A recent study by Hensiek and colleagues found that familial factors do not significantly affect eventual disease severity; however they increase the probability of a progressive clinical course, either from onset or after a phase of relapsing remitting disease (Hensiek, Seaman et al. 2007). The
authors concluded that the familial effect is more likely to reflect genetic rather than environmental conditions. The findings of familial studies have paved the way for investigations in MS regarding the significance of genetic epidemiology, twin, half-sibling, and adoptee studies, as well as investigations into parent-child concordance rates. The premise of those studies is that if MS harbours a significant genetic component then the initial findings of investigations involving families would be corroborated. Indeed those studies continue to find that MS does entail a significant genetic contribution, particularly in susceptibility to the disease.

3.2.5 Genetic Epidemiology of MS

Studying the genetic epidemiology of MS is expected to yield a variety of benefits including the extent of risk and susceptibility in particular populations and possible identification of clinical subtypes of the disease. Several studies have demonstrated a racial preponderance to the disease, whilst others have reported some degree of resistance to MS (for example in some Native groups). Genetic epidemiological studies have enriched these ethnicity studies by showing that the racial differences persist even in areas of high MS incidence. Conversely, it has also been shown that high risk individuals retain their expressivity in geographical areas of low MS incidence (Sadovnick 1995). Whilst not entirely attributable to genetics alone, epidemiological analyses have contributed valuable information regarding the world distribution of MS. In the Australian population, ethnic
stratification appears to be low with prevalence rates increasing uniformly North to South (approximately 4-fold increased risk in Hobart, Tasmania as compared to Brisbane, Queensland) (Sadovnick and Ebers 1993).

3.2.6 Genetic and Environmental Factors

Originally, MS was thought to be caused by an environmental factor such as a virus or other infectious agent; however that hypothesis has been frustrated over the years by a lack of evidence for a direct causative link. Since the advancement of modern genetics, the focus has shifted from a causative infectious agent to polygenic susceptibility that may be triggered by environmental variables. The relative contribution of genetic versus environmental factors has been mainly studied using twin and half-sibling approaches. Both multiple and twin birth rates are high enough in the general population to allow for these types of studies (approximately 1% overall). Extrapolating the effect of inheritance allows for the estimation of other contributing factors, such as environment (Sadovnick, Dyment et al. 1997). Although concordance rates (the rate at which both siblings are affected by the disease) are shown to be increased by degree of genetic relatedness, they only approach approximately 30% (Ebers 2008). Genetic studies to date have failed to prove a definitive association between MS and specific genes. Furthermore, individuals from the same genetic background at different latitudes (for example between North and South Australia) have different risk for the disease. Together,
these lines of evidence suggest that while the genetic contribution is substantial, there must be other factors at play. Environmental factors refer not only to the influence of geography and climate, but also the influence those factors might have on behaviour. Some other factors which may be involved in MS susceptibility include geochemical factors, dietary intake, and ecological associations (Irvine, Schiefer et al. 1989; Lauer 1995; Lauer 1997). The idea that sunlight and Vitamin D may be linked to MS has been around since at least the early 1970s (Goldberg 1974). The role of Vitamin D remains topical and investigations continue into its role in MS. Some studies have reported that high levels of Vitamin D protect against MS (Munger, Levin et al. 2006) and there is optimism about its possible role in disease modification (Broadley 2007). However, more research is required to explain its exact role and biological action. A recent study by Ebers and colleagues reports that ecological data accommodate more than one type of environmental effect and that geographical patterns in Australia imply that modifiable environmental factors hold the key to preventing some 80% of cases (Ebers 2008). The authors explain that genetic epidemiology provides overwhelming evidence that genetic background has an important complementary role in that if genetic factors are held constant, the environment sets the disease threshold, and although these could be independent additive risk factors, it seems more likely that susceptibility is mediated by direct interactions between the environment and genes.
3.2.7 Twin Studies in MS

A number of studies have investigated the occurrence of MS in twins (concordance rate). While the concordance rates tend to vary from population to population, the trend of increased concordance in monozygotic twins (MZ) compared to dizygotic twins (DZ) persists consistently around the world. Estimates for concordance in MZ twins range from approximately 21%-40% and DZ twins from approximately 3%-5% (Heltberg, Kalland et al. 1985; Ebers, Bulman et al. 1986; Willer, Dyment et al. 2003). An increased rate of heritability in susceptible individuals correlated with increased incidence of MS suggests significant genetic factors contribute to MS pathogenesis. The premise underlying twin studies is that twins are more likely to be exposed to similar factors at similar times than non-twins. A long-term follow-up study of twins by Ebers and colleagues, investigating 26 MZ and 43 DZ twin pairs (where at least 1 twin suffered MS) showed a trend of increased susceptibility with degree of genetic relatedness, with MZ twins having the highest incidence rates (approximately 31%) followed by DZ twins (approximately 5%), compared to non-twin siblings (approximately 4%) (Ebers, Bulman et al. 1986). In general the trend seen in twin studies alludes to the fact that genetic factors are involved in MS and that MS susceptibility cannot be explained by any single gene or locus owing to the observations of the sharp decline by decreasing degree of relatedness. To further investigate this phenomenon researchers have also proceeded to investigate half-siblings. Half-siblings are distinct in that they do not share their
entire genetic complement (but rather only half) with their counterparts. The underpinning motive in half-sibling studies is that if genetic relatedness influences MS susceptibility, then half-siblings should be less susceptible to the disease than full-siblings and twins.

3.2.8 Half-Sibling Studies in MS

Half-sibling studies aim to complement twin studies by explaining the relative contribution of genetic versus environmental influences in MS. Full-siblings share 50% of their genomic content, whilst half-siblings only share 25%. Furthermore, half-siblings can be either maternal (share the same mother) or paternal (share the same father). These studies can lend interesting insights into the genetics of MS, and can test an added dimension of complexity depending on whether the siblings were raised together or apart. These studies aim to determine concordance rates in half-siblings as compared to full-siblings, the effects of maternal versus paternal heredity, and the effects and extent of environment based on whether the siblings were raised together or apart. The parent-of-origin effect has been investigated in other diseases, such as Alzheimer’s, as it can be important to discern whether susceptibility is inherited from the mother of the father (Bassett, Avramopoulos et al. 2006). In recent studies, familial autoimmunity has been linked to maternal parent-of-origin effects (Zeft, Shear et al. 2008). This effect may have important implications on linkage analysis of complex traits (Strauch, Fimmers et al. 1999). The parent-of-origin effect has
also been under scrutiny in MS research, and half-sibling studies by Ebers et al have shown that the risk of MS is significantly lower in that group (approximately 2%) compared to full-siblings. Furthermore, the authors observed that the parent-of-origin effect (as discerned by half-sibling studies) appears to be maternal in nature for MS susceptibility (Ebers, Sadovnick et al. 2004).

3.2.9 Adoptee Studies in MS

Adoptee studies primarily attempt to tease valuable information from adopted and mixed (adopted and non-adopted combination of children in the family) families in order to shed light on factors that affect or alter the familial aggregation seen in MS. These studies allow for the thorough investigation of specific genetic versus environmental factors by controlling for degree of relatedness. For the purposes of these studies, adopted children are considered first-degree non-related familial counterparts and are classified as non-biological relatives (not sharing genetic information).

Thus far, adoptee studies have shown that the incidence of MS is not significantly different in adoptees when compared to the general population (Mumford, Wood et al. 1994). This finding implies that familial aggregation of MS is to a large degree genetically determined. Furthermore, the findings of adoptee studies have previously suggested that essentially no effect of shared environment can be detected (Thorpe, Mumford et al. 1994). Adoptee studies,
while relatively rare, contribute valuable information to investigation of the extent of genetic effects in MS research.

3.2.10 Conjugal MS

An interesting (albeit rare) approach to investigating the heredity of MS is to study conjugal pairs with MS (Kaufman 1992). Conjugal pairs are partners who both suffer MS who in turn produce children that then may or may not have the disease. Conjugal studies can offer valuable insights into the inheritance of susceptibility to MS. Conjugal studies have been used to determine observed versus expected rates of occurrence, finding that susceptibility alleles are shared by unrelated individuals with the disease (Ebers, Yee et al. 2000). A recent study published by Maghzi et al suggests that observed conjugal rates suggest that an increased risk of developing MS exists in some environments attributes the increased risk to environmental variables (Maghzi, Etemadifar et al. 2007).

Conjugal studies have also investigated immunogenetic mechanisms in MS. Fredrikson and colleagues report that in their studies, two families with conjugal MS show a dominant T-cell response against the same MBP peptide within the family both in MS-affected parents and unaffected children, and explain that this T-cell response seems to be independent of the HLA class II phenotypes of the family members (Fredrikson, Michelsberg et al. 1992). Although inherently difficult to conduct (due to the low rate of occurrence) conjugal studies continue
to generate interest particularly in investigations pursuing the age-corrected recurrence risk for developing the disease, and may provide insights into complicated inheritance patterns in future studies.

### 3.2.11 Special Populations in MS

The notion of a special population in MS research mainly alludes to the degree of informativeness of the tested cohort. These populations also generally demonstrate some degree of uniqueness in terms of isolation or stratification. A special population that has been used in investigating the genetics of MS is the Sardinian Island population (located in the Mediterranean Sea). The Sardinian population is distinct in that it shows a high incidence of genetic disorders and also a distinctive HLA haplotype (Pugliatti, Solinas et al. 2002). Marrosu and colleagues undertook studies to estimate the presence of familial aggregation and determine the contribution of genetic factors to familial clustering of MS in patients coming from Sardinia, a Mediterranean island considered a genetically homogeneous, isolated area having high genetic disease incidence and prevalence (Marrosu, Lai et al. 2002). The authors found that MS prevalence was dramatically greater than the regional average and 1.5 times greater than that observed in siblings of affected cases, reiterating the importance of heredity in susceptibility to MS.
Another approach has involved the study of reported MS epidemics. Reports of a sharp increase in the number of MS cases in the Faroe Islands between 1943 and 1973 have been of particular interest. The initial reports of new cases of MS correlated well with the placement of British soldiers on the Islands during the Second World War, and was initially published by Kurtzke et al in 1979 (Kurtzke and Hyllested 1979). Much controversy has surrounded those initial studies and debate still exists as to the meaning of the association (Benedikz, Magnusson et al. 1994). While special populations often lend valuable findings to the literature, there remains a distinct need to ascertain the applicability of those findings to the general population within each complex disease that is studied.

3.3 Genome-Wide Screens in MS

Genome-wide screening studies have been undertaken in MS in an attempt to define the genetic loci contributing to disease susceptibility. Genome-wide studies interrogate the entire genome to varying degrees of resolution in order to generate candidate regions of interest for follow-up investigations such as case-control association studies.
3.3.1 Genome-Wide Approaches

Genome-wide studies use high-throughput genotyping techniques to assay many DNA markers such as microsatellites or single-nucleotide polymorphisms (SNPs) and relate them to clinical conditions and measurable traits. To date, nearly 100 loci for as many as 40 common diseases and traits have been identified and replicated in genome scan linkage or association studies, many in genes not previously suspected of having a role in the disease under study, with some located in genomic regions containing no known genes (Pearson and Manolio 2008). Genome scan studies represent an important advance in genetic discovery. Genome scanning studies do not suffer some of the limitations of other genetic approaches such as requiring a thorough understanding of the disease mechanism to discern appropriate loci for study. The more general genome scanning approach can identify and associate susceptibility loci that otherwise would not have been easily recognised. Large cohorts of multiplexed families are normally used for genome scan linkage studies, whilst large case-control populations are used for genome-wide association studies, and it is generally necessary to conduct simulations during analysis to determine results (Evans and Cardon 2004).

Approaches using single and two-trait loci, sibling-pairs, affected pedigree members, and transmission disequilibrium all have their respective strengths and drawbacks (Risch 1990; Risch 1990; Risch 1990). A full genome search was
undertaken by Ebers and colleagues in 1996 and found that the human leukocyte antigen (HLA) coding region on chromosome 6p21 may predispose people to MS susceptibility (Ebers, Kukay et al. 1996). Since that time, other groups have confirmed these findings and also implicated the 17q22 locus (Sawcer, Jones et al. 1996). The most repeatedly implicated regions in MS are located at chromosomes 6p21 and 17q22, although some degree of heterogeneity appears to exist between populations. One important application of a genome scanning approach is that it may help define the heterogeneity in MS susceptibility between populations as more scans are done in distinct populations.

While these studies are clearly several steps removed from actual clinical application, the genome scan approach is revolutionary because it permits interrogation of the entire human genome at levels of resolution previously unattainable, in thousands of unrelated individuals, unconstrained by prior hypotheses regarding genetic associations with disease (Hirschhorn and Daly 2005).

### 3.3.2 MS Susceptibility Loci in Australians

A number of studies have been conducted using the Australian population. Ban and colleagues performed a genome wide screen of linkage in the Australian population using a panel of 397 microsatellite markers in 54 affected sibling-pairs in 2002 (Ban, Stewart et al. 2002). The authors report that multipoint linkage
analysis revealed four regions of suggestive linkage (on chromosomes 2p13, 4q26-28, 6q26 and Xp11) and 18 additional susceptibility regions (at 1q43-44, 3q13-24, 4q24, 4q31-34, 5q11-13, 6q27, 7q33-35, 8p23-21, 9q21, 13q31-32, 16p13, 16p11, 16q23-24, 17p13, 18p11, 20p12-11, Xp21-11 and Xq23-28). As a follow on from those studies, the authors also investigated linkage disequilibrium in Australian DRB1*1501 positive MS patients. In that study, the authors found linkage with four genomic regions (12q15, 16p13, 18p11 and 19q13) previously identified in linkage genome screens.

Three additional regions of novel linkage were also identified (11q12, 11q23 and 14q21) (see Figure 3-2 below for susceptibility regions identified in the Australian population). The authors concluded that further analysis of those regions is required to establish whether the linkages observed are due to epistatic interaction with the HLA locus (previously implicated). It must be emphasized that although many susceptibility regions have been implicated, it is still unclear which regions harbour the most significant markers, or how they are contributing to the phenotype.
Figure 3-2 shows the 2 reported Non-MHC susceptibility regions in the Australian population; located at chromosomes 1q42 and 19q13. Genes in these regions (amongst others that remain as yet undefined) may be harbouring important genetic susceptibility loci. Chromosome 1 is the largest of all the human chromosomes and harbours the most genes in the human genome. The human protein tyrosine phosphatase receptor C gene (PTPRC), which is central to immune modulation (a gene in this thesis) resides on the long arm of chromosome 1 (1q31) close to the susceptibility region.
3.3.3 MS Susceptibility Loci in Non-Australians

Genome screens using non-Australians have been undertaken in British (Coraddu, Sawcer et al. 1998; Sawcer, Maranian et al. 2002), American (Haines, Bradford et al. 2002; Vitale, Cook et al. 2002), Canadian (Dyment, Cader et al. 2008), the Canary Islands (Coraddu, Reyes-Yanez et al. 1998), Italian (Broadley, Sawcer et al. 2001), Finnish (Kuokkanen, Gschwend et al. 1997), Scandinavian (Akesson, Oturai et al. 2002), and German (Goedde, Sawcer et al. 2002) populations. Following these initial studies a collaborative project was established in 2001; the Trans-Atlantic Multiple Sclerosis Genetic Cooperative. The aim of the collaborative was to combine the raw genotyping data from three large multiple sclerosis genome screens and perform a global meta-analysis in order to compare and summarise the linkage results from the different studies (TMSGC 2001). The collaborative found that the highest non-parametric linkage (NPL) score in the meta-analysis was on chromosome 7q (NPL=2.58) while a second region; 6p21 was also implicated (NPL=2.20). In an adjunct collaborative meeting, the Genetic Analysis of Multiple Sclerosis in Europeans (GAMES) consortium was established in 2003. The main objective of GAMES has been to collaboratively identify susceptibility genes using genome wide association screening methods. The results of the GAMES analyses have been published since 2003 in a series of papers (Alizadeh, Genin et al. 2003; Bielecki, Mycko et al. 2003; Eraksoy, Hensiek et al. 2003; Eraksoy, Kurtuncu et al. 2003; Goertsches, Villoslada et al. 2003; Goris, Sawcer et al. 2003; Harbo, Datta et al.
2003; Heggarty, Sawcer et al. 2003; Hensiek, Roxburgh et al. 2003; Liguori, Sawcer et al. 2003; Rajda, Bencsik et al. 2003; Santos, Pinto-Basto et al. 2003; Weber, Infante-Duarte et al. 2003; Yeo, Roxburgh et al. 2003). Thousands of genetic markers were used for the studies and then analysed for similarities across populations. A consistent finding across all the studies was that the regions: 6p21, 7q, 19p, 19q, 5p, 5q, and Xq appear to be playing some (as yet undefined) role in MS susceptibility.

3.4 Genes and Genetic Loci Implicated in MS

Identifying the genes that play a role in MS has proved challenging owing mainly to the undefined pathogenesis and low incidence of aggregation in the disease; however several classes of loci have been repeatedly implicated in MS susceptibility. These include the human leukocyte antigen (HLA) loci, the T-cell receptor (TCR) loci, and the tumour necrosis factor alpha and beta (TNF-α and TNF-β) loci. These loci are thought to harbour susceptibility genes and alleles that may be contributing to the disease phenotype.
3.4.1 Candidate Gene Studies of MS

Candidate gene studies follow on from general screening approaches in an attempt to define the genes and subsequent variations within those genes that contribute to the disease phenotype. Many candidate gene studies have been undertaken in MS with varying degrees of success. Owing to its polygenic nature no single candidate gene has been implicated in MS as yet, however as technologies and techniques evolve, candidate gene studies may begin to explain which genes are key to susceptibility to the disease. A broad list of genes has been investigated in MS and it is most practical to classify these according to categories based on the most significant findings. To date these have been major histocompatibility (MHC) related, non-MHC related, and immune system genes.

3.4.2 MHC Associations

Traditionally, candidate genes for MS investigations have been selected based on molecular or pathological reasoning such as involvement in particular biological processes or pathways. Numerous investigations have reported positive association results that have subsequently proven difficult to replicate. A review by Colhoun and colleagues reports that up to 95% of initial claims of genetic association cannot be replicated due to a high incidence of type I error (Colhoun, McKeigue et al. 2003). It must be mentioned though, that the
candidate gene approach has also yielded some valuable findings. One such
discovery has been the association of the major histocompatibility complex
(MHC) region (see Figure 3-3 below) with MS susceptibility. The link between
age of MS onset and HLA type has been studied and findings indicate that the
HLA-DR15 and DR17 genotypes are positively associated with earlier age of
onset in some populations (Masterman, Ligers et al. 2000; Masterman and Hillert
2002). The MHC is now thought to be clearly associated with MS and studies
continue to find association with the genomic region and the MS phenotype
(Masterman, Ligers et al. 2000; Masterman and Hillert 2002).

However, the MHC region (which codes for the human leukocyte antigen; HLA,
located on chromosome 6p21.3) comprises some 200 known genes over a
distance of approximately 4 megabases, demonstrating enormous genetic
diversity (including pseudogenes) and a specific causative marker is yet to be
identified. Many of the genes located in the MHC region regulate the
development, maturation, and composition of T-cell repertoire (amongst other
immunological processes). Needless to say, efforts to clearly delineate the
involvement of the MHC in MS have proven challenging. The ability to respond to
a foreign antigen and the exact nature of that response is to a large extent
determined by the HLA system. The alleles that code for the HLA determine the
individuals’ particular response to foreign antigens, and furthermore variations
within the area may also contribute to the development of autoimmunity which is
known to be a key factor in MS (McFarland and Martin 2007). In addition to a
highly polymorphic nature, the HLA harbours strong linkage disequilibrium (LD) across its entire region further complicating analysis. These factors contribute to complex gene interactions.

Different HLA alleles have been associated with MS in different populations, for example Hillert and colleagues report an association of the DR15 allele (Hillert 1994) in MS Caucasians, Coraddu and colleagues report a DR4 allele association in the Canary Islands (Coraddu, Reyes-Yanez et al. 1998) and additionally DR51 association in the United Kingdom (Coraddu, Sawcer et al. 1998), while Saruhan-Direskeneli and colleagues report the Dw2 (DRB1*1501, DQA1*0102, and DQB1*0602) haplotype as associated with MS in Caucasians in a Turkish population (Saruhan-Direskeneli, Esin et al. 1997). The MHC remains an interesting and complex susceptibility region in MS research efforts.
Figure 3-3 shows the relative locations of the HLA Class I and HLA Class II regions within the MHC HLA region on (the human) chromosome 6. The MHC HLA region has been implicated in numerous immune disorders and harbours a high degree of genetic complexity (Source: The Major Histocompatibility Complex database (dbMHC) within the National Center for Biotechnology Information (NCBI) located at: http://www.ncbi.nlm.nih.gov/gv/mhc/).
3.4.3 The HLA Locus in MS

The human leukocyte antigen locus (HLA) has generated much interest in recent years. HLA Class II antigens are comprised of two chains; α and β, and are products of rearrangements of genes within the HLA locus located on chromosome 6. There are three known class II loci in humans; HLA-DP, -DQ, and –DR. The different loci include different arrangements of α and β chains (DP=2 α and 2β, DQ=1α and 1β, and DR=1α and 3β). Each of these regions (DP, DQ, and DR) are high polymorphic. The HLA antigens are involved in presentation of exogenous antigenic peptides by specialised antigen-presenting cells (APCs). Antigens are internalised by APCs, digested, and then short fragments are expressed in conjunction with the HLA antigen on the surface of the cell. Each HLA antigen can present a unique subset of fragments and the presentation repertoire overlaps extensively between HLA Class II antigens. MS research has been primarily concerned with HLA alleles because it is thought that a misdirected immune response (as seen in autoimmune disorders) may be mediated by this system (Ho, Tiwari et al. 1982). Involvement of the HLA system in MS has been studied from numerous perspectives. Initially, Poser and colleagues investigated the effects of HLA type with prognosis of MS (Poser, Ritter et al. 1981). In 2000, Masterman and colleagues catalogued clinicodemographic data for 816 patients with definite MS and investigated the impact of the MS-associated HLA class II DR15 on aspects of the disease phenotype. The authors demonstrated that carriage of DR15 is positively...
associated with lower age of onset of MS (Masterman, Ligers et al. 2000). More recently, studies by Harbo and colleagues have attempted to explain the Class II risk alleles in association with variations in Class I HLA (Harbo, Lie et al. 2004). In a recent study, Greer and colleagues report that carriage of HLA-DR4, -DR7, or -DR13 molecules by MS patients correlated with increased blood T-cell immunoreactivity to proteolipoprotein (PLP), as well as the development of lesions in the brainstem and cerebellum (Greer, Csurhes et al. 2008). The authors also reported that levels of PLP-specific antibodies in the blood also correlated with the presence of cerebellar lesions. Those findings suggest that circulating T-cells and reactive antibodies against specific myelin antigens can correlate with lesion distribution in MS. Furthermore, these findings reiterate the importance of HLA regulation in MS pathogenesis.

3.4.4 TCR Loci and MS

The T-cell receptor (TCR) is an important element of the immune system, regulating several aspects of immune response and function. The TCR is known to interact with peptide fragments that are presented by human leukocyte antigens (HLAs). Immune responses are established following TCR activation. Two forms of TCR are known to date; those that express αβ-TCR (and require that antigens be presented in the context of HLA antigens) and another subset known to express γδ-TCR (which are not restricted to only recognising HLA presented antigens). The TCRs are encoded by three distinct gene regions on
chromosomes 7 (the TCR-β locus), 14 (the TCR-αβ locus), and another 7 region (the TCR-γ region). A large degree of complexity accompanies the constant, joining, diversity, and variable recombination of these loci to code for a wide-array of diverse TCR recognition capabilities. The four loci mentioned undergo rearrangement during development in order to facilitate the assembly of functional TCR genes. The underlying premise regarding investigations into TCR related targets in MS research is that allelic variation in TCR related genes influences disease susceptibility. Studies have previously outlined the role TCRs may have in causing inappropriate autoantigen mediated immune responses (Weiner 2004). Other studies have examined the role of variations within genes that code for the T-cell receptor, and their association with MS susceptibility (Jacobsen, Hoffmann et al. 2002). Another approach regarding T-cells has been to examine T-cell regulation by co-stimulatory molecules such as CD28 (Alegre, Frauwirth et al. 2001). The role of TCRs remains an interesting topic in MS research, and studies continue to investigate the potential role of both T-cells and their associated receptors in MS susceptibility.

### 3.4.5 TNF-α and β and MS

Tumour necrosis factors alpha (α) and beta (β) (TNF-α and β) are pro-inflammatory cytokines that are known to be involved in the pathogenesis of MS (Comabella, Julia et al. 2008). Proinflammatary cytokines initiate and sustain inflammatory processes and may be the trigger for cellular responses that result
in demyelination. Both TNF-α and TNF-β have been detected in demyelinating plaques and are known to be toxic to oligodendrocytes as shown in animal models (Akassoglou, Bauer et al. 1998). In studies by Roth and colleagues, the TNF-C1, -A11, and -B4 microsatellite alleles have been associated with MS, possibly because of their association with the HLA DRB1*1501 haplotypes (Roth, Nogueira et al. 1994). Several negative associations between TNF expression and MS have been published (Sotgiu, Pugliatti et al. 2000), however it is uncertain if TNF polymorphisms affect cytokine production similarly in different populations (de Jong, Westendorp et al. 2002). Although TNF variations may contribute to MS susceptibility it is still unclear what role variations within the genes coding for those genes contribute to the pathogenesis of the disease. More specific future investigations are required to draw valid conclusions for association between TNF variants and MS susceptibility.
3.4.6 Other Non-MHC Associations

Non-MHC genes of interest to MS also exist, and they include intercellular adhesion molecules (ICAMs), which are accessory molecules expressed on the surface of antigen presenting cells (APCs), and several other immune candidates including the T-cell regulator gene SH2D2A, the cellular adhesion molecule gene ICAM-1 (Mycko, Kwinkowski et al. 1998; Dai, Harbo et al. 2001; Nejentsev, Laaksonen et al. 2003). Genomic investigations involving genes encoding the T-cell receptor, cytokines, and the myelin basic protein have been reported by Oksenberg (Oksenberg, Sherritt et al. 1989) and Hashimoto and colleagues (Hashimoto, Mak et al. 1992), but then failed to be confirmed by others (Rose, Gerken et al. 1993; He, Xu et al. 1998). A recent paper by Gregory and colleagues reported both allelic and functional association of MS within the Interleukin 7 receptor alpha chain (IL7R) (Gregory, Schmidt et al. 2007). The likely causal single-nucleotide polymorphism (SNP) in IL7R is described by the authors as being located within the 6th alternatively spliced exon of the gene. Several studies have confirmed the IL7R association and the marker is now being investigated for association in additional populations (Lundmark, Duvefelt et al. 2007; Lundmark, Duvefelt et al. 2007; O'Doherty, Kantarci et al. 2008). Numerous negative findings have also been published (T-cell receptor-alpha, interleukin-1 receptor agonist, and the chemokine (C-C motif) receptor 5 gene; CCR5) (Dyment, Sadovnick et al. 1997) however these findings may not necessarily be applicable to all populations. Further investigation of astrocyte and
oligodendrocyte specific markers, immune system genes, and factors associated with viral response may yield valuable findings. Gene expression studies by sensitive RT-PCR techniques could also be implemented to accurately discern the involvement of previous and novel candidates (Whitney, Ludwin et al. 2001; Tajouri, Mellick et al. 2005).

A study by Vandenbroeck et al found that age of onset may be linked to a variable number tandem repeat (VNTR) in the interleukin-4 gene (IL-4) (Vandenbroeck, Martino et al. 1997). The same study by Vandenbroeck and colleagues went on to analyse the relationship between age of onset and disease severity but found no positive association for that parameter. Following these studies, Barcellos and colleagues reported that a 32bp deletion in the chemokine (C-C motif) receptor 5 gene (CCR5) delays the age of onset (Barcellos, Schito et al. 2000). CCR5 promoter polymorphisms have also been reported to influence interferon (IFN) responsiveness (Konishi, Horiike et al. 2004). Studies into the interferon-gamma (IFN-γ) gene found that Germany, Northern Italian, Sardinian, and Swedish populations show some association with MS (Goris, Epplen et al. 1999). Subsequent studies, involving Nordic populations have however failed to replicate those findings (Dai, Masterman et al. 2001).

A large degree of genetic heterogeneity exists in MS phenotypes and studies have implicated numerous candidates. One example of a gene which seems to have varied effect in different populations is apolipoprotein E (ApoE; which is
located on chromosome 1q21), with both positive (Kantarci, Atkinson et al. 2000) and negative (Ferri, Sciacca et al. 1999) associations reported. Kantarci and colleagues went on to attempt to summarise these types of effects in a review of numerous studies in 2002, in the process highlighting the complexity of the heterogeneity seen in MS (Kantarci, de Andrade et al. 2002).

The vitamin D (1,25-dihydroxyvitamin D3) receptor gene (VDR; located on chromosome12q13.11) has also been the focus of various studies. It is believed that the geographical gradient observed in MS distribution may be explained by variations within this gene owing to its involvement in immune regulation with respect to sunlight exposure (Smolders, Damoiseaux et al. 2008). Studies have associated VDR polymorphisms with Japanese (Fukazawa, Yabe et al. 1999) and Australian (Tajouri, Ovcarić et al. 2005) populations but have not been replicated in some other studies (Steckley, Dyment et al. 2000). Several aspects of VDR and its associated ligands remain interesting topics of research, with studies encompassing both associated genes (such as the vitamin D binding protein gene) (Niino, Kikuchi et al. 2002), as well as serum markers (Munger, Levin et al. 2006). Recent studies have investigated the effects of vitamin D supplementation during childhood and pregnancy with a view to decreasing MS susceptibility (Chaudhuri 2005) and animal studies have investigated the effects that vitamin D deficiency on the brain and immune system (Yang, Smith et al. 1993; Eyles, Smith et al. 2005; Almeras, Eyles et al. 2007; Eyles, Almeras et al. 2007). These studies found that vitamin D deficiency is detrimental to both brain
and immune function. Although vitamin D and the genes coding for its related functions, such as VDR are interesting targets more research needs to be carried out to discern the relevance of those variants and their effects in different populations (Broadley 2007).

The human genome project has sparked renewed interest in the development of new genomic techniques (Consortium 2003). DNA pooling approaches, where a large number of samples are screened simultaneously with relatively modest resolution, are now becoming available (Craig, Huentelman et al. 2005). These approaches provide reduced cost and throughput (Chi, Lou et al. 2008), but the technique still requires refinement (Rosenthal and Schisterman 2008; Zhao and Wang 2009). Genome-wide scans in MS have received much attention over the years. Studies have repeatedly implicated the 6p21 region, which harbours the major histocompatibility complex (MHC) with some linkage also to the 17q22 area (Sawcer, Jones et al. 1996). Recently, Aulchenko et al. reported association between MS and a variant in the kinesin family member 1B gene (KIF1B) in a genome-wide association (GWA) study of 2,679 cases and 3,125 controls (Aulchenko, Hoppenbrouwers et al. 2008). They concluded that KIF1B is a gene expressed by neurons that is plausibly involved with irreversible axonal loss in MS. Indeed, the insights provided by genome-wide analyses, such as those carried out by the International Multiple Sclerosis Genetics Consortium, seem valuable and future studies may reveal even more pertinent findings as the technique is refined (Sawcer, Ban et al. 2005; Sawcer 2006). A thorough review
of both classical genomic and emerging techniques, detailing both the benefits and pitfalls of these techniques, was recently reviewed by Sawcer and colleagues (Sawcer 2008). The authors express that we cannot know \textit{a priori} what effects on risk will be conferred by susceptibility alleles (nor can we know their frequencies or modes of inheritance), but go further to explain that emerging genomic techniques have recently given us tools analogous to the electron microscope for the detection of loci possibly involved in susceptibility. Investigations into the genetic variations in MS continue to catalogue contributing variants which will eventually lead to a better understanding of the disease.

\textbf{3.4.7 Genes Involved in Immunity}

An area of MS genetics which demands particular attention is genes involved in immunity. The action of immune cells (such as T and B-cells) is coupled to secretion of cytokines and interleukins, which act as chemical messengers that mediate immune responses. Genetic variations within the genes that code for immunological mechanisms have generated much interest in MS research. Variations within the T-cell receptor genes (TCRs) can be assessed using several techniques to identify changes in the number, diversity, or DNA sequence of these regions. To date, the genes involved with coding for $\alpha$ and $\beta$ loci (coding for TCR-$\alpha$ and $\beta$ subtypes) have been predominantly investigated. Several population based germline polymorphism investigations have been performed, however the results for association in different populations have been
somewhat conflicting. Biddison and colleagues reported a positive association with TCR-Vβ region and MS in DR2⁺ sufferers (Biddison, Beall et al. 1989). However, these findings have proved difficult to reproduce in subsequent follow-up studies (Fugger, Sandberg-Wollheim et al. 1990; Vandevyver, Buyse et al. 1994). In a Spanish study, the TCR-Vβ haplotype Cβ/Dβ appeared to be associated with MS susceptibility, however only in HLA-DR2 patients (Martinez-Naves, Victoria-Gutierrez et al. 1993). One difficulty with traditional polymorphism methods is that it is unclear what functional significance associated variants have biologically when the variants lie outside of known coding regions. Familial linkage approaches can circumvent this shortcoming. Seboun and colleagues undertook studies to investigate the inheritance of TCR-β genes using 40 sibling pairs concordant for the relapsing-remitting form of MS (Seboun, Robinson et al. 1989). The investigators found that one particular allelic form of the TCR-β variable region gene segment was overrepresented on MS chromosomes compared with those parental chromosomes not transmitted to MS offspring both in the MS sibling pair families and in a second group of families containing only one individual affected with MS. The authors concluded that a gene within the TCR-β complex or a closely linked locus seems to influence susceptibility to MS. Investigations into haplotype sharing and the TCR-α locus have also been performed. Martell and colleagues found a significant difference between cases and controls in DR2 individuals (Martell, Marcadet et al. 1987), implying that TCR-α loci may also been implicated in MS susceptibility. However, studies by several other groups failed to reproduce the same associations (Hillert, Leng et
al. 1992; Lynch, Rose et al. 1992; Eoli, Wood et al. 1994). The main difficulty in resolving conflicting findings in this region stems from the inherent complexity of the TCR locus.

Other immune genes and markers also remain of interest and include interleukin-2 (IL-2). IL-2 is known to partake in an autocrine feedback loop that can trigger T-cell activation and has been shown to be an important factor in regulating T-cell suppressor function (Thornton, Donovan et al. 2004). T-cell activation is a crucial step in the predominant MS pathogenesis theory whereby activated T-cells traverse the blood-brain barrier (BBB) which ultimately results in myelin breakdown in the CNS. Furthermore, IL-2 has been shown to mediate MHC Class II antigen presentation on astrocytes and macrophages and microglial interferon-gamma (IFN-γ) response (Martino, Moiola et al. 1995; Grau, Herbst et al. 1997). Studies have also explored the role of complement (which is a primary humoral immunity mechanism) and complement receptors in MS. Complement is known to induce microglia to secrete IL-1 and IL-6 as well as TNF-α (Rajan, Klein et al. 1998). Infiltration of the CNS by activated T-cells also stimulates macrophages (Hulkower, Brosnan et al. 1993). Macrophages have been shown to be able to ingest myelin in the animal models of MS (Rinner, Bauer et al. 1995). The genes involved with these various immune mechanisms continue to be of interest in the pursuit for causative genetic variations in MS research.
3.5 Experimental Models of MS

Animal models have contributed extensively to our understanding of certain aspects of the pathogenesis of MS. They have helped clarify some basic aspects of neuroimmunology, clarified processes of central nervous system inflammation, and helped explain fundamental aspects of immune-mediated CNS damage. Induction of inflammatory demyelinating lesions after active immunisation of monkeys using brain tissue was first reported by Rivers and colleagues in 1933 (Rivers, Sprunt et al. 1933). Induced models of neuroinflammation demonstrate a complex pathogenesis involving both humoral and cell-mediated responses (Lassmann, Stemberger et al. 1983). Although experimental models have been induced in several different animal species (mainly murine models; EAE and TMEV), there remain pathological features of MS for which no valid animal models exist. Such examples include MS lesions with T-cell and macrophage-dominated inflammation in the absence of immunoglobulin and complement deposition. MS lesions with complete destruction and loss of oligodendrocyte progenitor cells also cannot be reproduced. Although animal models mimic aspects of MS disease pathology (and thus aid in the study of systems pathology) no animal model succinctly encompasses the complicated neuropathology seen in the human version of the disease.
3.5.1 TMEV Model

Theiler's murine encephalomyelitis virus (TMEV) was first reported in 1937 (Theiler 1937). TMEV is a single-stranded, positive polarity RNA Cardiovirus that belongs to the Picornaviridae family. TMEV has been widely used in experiments to induce neurological disease in mice (Dal Canto, Kim et al. 1996; Tsunoda and Fujinami 1996; Lipton and Jelachich 1997; Monteyne, Bureau et al. 1997; Oleszak, Chang et al. 2004). Infection of susceptible strains of mice (e.g. SJL strains) with TMEV results in biphasic disease of the CNS, consisting of early acute disease followed by late chronic demyelination 30-40 days post infection (p.i.). TMEV induced late chronic demyelination closely resembles that seen in MS. Susceptibility of both mice to TMEV-induced demyelination, and humans to MS appears to be major histocompatibility (MHC) dependent. Mouse susceptibility has been associated with the H-2D class I s,q,r,p,f haplotypes (Lipton and Melvold 1984; Clatch, Melvold et al. 1985; Rodriguez, Nickerson et al. 1991; Altintas, Cai et al. 1993; Rodriguez, Dunkel et al. 1993), whilst susceptibility in humans has been associated with the DRB1*1501, DQA1*0102, and DQB1*0602 haplotypes. Those haplotypes correspond to the cellular types DR2 and Dw2. Approximately half of Caucasian patients are DR2+/Dw2+ whereas healthy populations only express that cell type at approximately 30% (Ho, Tiwari et al. 1982; Vartdal, Sollid et al. 1989; Steinman, Miller et al. 1994). These findings suggest that further susceptibility factors must exist. TMEV
models clearly demonstrate uncomplicated, induced CNS demyelination, in mice, but only mimic the full spectrum of pathology seen in patients with MS.

3.5.2 EAE Model

The experimental allergic encephalomyelitis model is the most widely adopted animal model of MS. Whilst EAE is the most widely used animal model of autoimmune disease it could also be argues that it is not one of the best understood. Whilst the EAE model may suggest processes involved in the human form of the disease the model is also limited by a number of factors, not the least of which is that it is induced (as opposed to occurring spontaneously) and that the animal form only shows 2 distinct clinical courses as opposed to the 4 seen in humans. These factors aside, EAE has taught us several lessons in MS pathology and continues to be the cornerstone of animal work in MS. There are several forms of the disease which can be induced in specific ways.

3.5.2.1 MBP-Induced EAE

Myelin basic protein (MBP) induced EAE is well described by Sundvall and colleagues (Sundvall, Jirholt et al. 1995). The MBP form uses B10.RIII mice to induce a relapsing-remitting form of the disease using MBP 90-101 peptides. The chief finding of studies using the MBP-induced EAE model is that susceptibility in
those animals was determined primarily by the H-2 locus on chromosome 17. However, several strains of mice share the H-2 haplotype and it is still uncertain as to what extent the H-2 chromosome 17 association is applicable to the overall pathogenesis of the disease.

3.5.2.2 MOG-Induced EAE

The chronic variant of the myelin oligodendrocyte glycoprotein (MOG) experimental allergic encephalomyelitis model is also a widely used animal model of MS. MOG EAE is a T-cell mediated autoimmune disease induced in rodents. Immunisation with myelin proteins and their fragments produces an autoimmune paralytic disease resembling MS in the human (Kerlero de Rosbo, Mendel et al. 1995; Mendel, Kerlero de Rosbo et al. 1995). Chronic MOG EAE shares several pathological hallmarks with MS including activated T-cell proliferation, perivascular cuffing, demyelination of axons, and axonal dystrophy (Steinman 2001). The peptides used to induce MOG EAE are MOG protein fragments (Brocke, Quigley et al. 1996). An important finding of MOG EAE studies has been that the neurological symptoms of MOG EAE can be adoptively transferred to naïve mice following injection of myelin protein reactive T-cells derived from an immunised littermate. This indicates that autoreactive T-cells play a critical role in the development of MOG EAE. However, pathology is not seen following sole transfer of autoreactive T-cells. Thus it is postulated that
pathogenic T-cells act in unison with autoantibodies to breach the BBB to incite neuroinflammation (Genain, Cannella et al. 1999; Lalive, Menge et al. 2006).

3.5.2.3 PLP-Induced EAE

Proteolipoprotein-induced (PLP) EAE is well described by Encinas and colleagues (Encinas, Lees et al. 2001). H-2\textsuperscript{+} SJL/J and congenic B10.S mice are used to induce EAE in these susceptible strains which then mimic the T-cell response also seen in humans. The T-cell response in PLP-EAE is strongly directed against encephalitogenic antigens (PLP 139-151 peptides) (Encinas, Lees et al. 1996). The most significant findings to date involving PLP-induced EAE have been an association between chromosome 8 and CNS inflammation (Encinas, Lees et al. 2001) and the definition of two susceptibility loci in these animals on chromosomes 3 and 4. The authors attribute those linkages to the incidence of inflammation, which could be an artefact of the induction of the model. Recent studies by Rosenbluth et al have found that PLP is an important factor in maintaining proper myelin structure (Rosenbluth, Nave et al. 2006). The investigators report that regulation of PLP appears to play an important role in maintenance of myelin compaction along the external surfaces of the lamellae and to a limited extent, along the cytoplasmic surfaces of axons.
3.5.2.4 Spinal Cord Homogenate-Induced EAE

Spinal cord homogenate-induced EAE is yet another form of the animal model of MS. The model has been used for some time with reports of the method dating back to at least 1975 (Yasuda, Tsumita et al. 1975). In a study by Baker et al the investigators induced EAE in highly susceptible AB/H mice as well as low susceptibility NOD mice in an effort to ascertain susceptibility regions (Baker, Rosenwasser et al. 1995). The investigators found significant linkage to chromosomes 7, 11, and 18 in the mouse model. A recent study by Liu and colleagues has shown that vaccination with dendritic cells pulsed with spinal cord homogenate protein can promote the functional recovery of mice with spinal cord injury (Liu, Zhao et al. 2008). Furthermore, linkage analyses have been carried out using spinal cord homogenates in rats to investigate antibody responses to myelin oligodendrocyte glycoprotein showing that the main region implicated in response is the MHC (Yang, Bergsteinsdottir et al. 2001). EAE continues to be used in animal model investigations of MS and is also principally used for targeted studies investigating phases of inflammation, and the testing of new anti-inflammatory drugs. Another valuable utility of the EAE model has been the ability to study epistasis in the simplified animal model which would otherwise be immensely complex in the human form of the disease.
3.5.3 Investigating Epistasis

Typical EAE expression is limited to two forms of the disease as opposed to the human variant which has four (clinical courses). It has been previously postulated that this limitation in the EAE model is due to limited gene-gene interactions (epistasis). In 2006, Gregersen and colleagues reported that characterisation of two multiple-sclerosis-associated HLA-DR alleles at separate loci by a functional assay in mice indicate that the linkage disequilibrium between the two alleles may be due to a functional epistatic interaction (Gregersen, Kranc et al. 2006). The authors go on to describe that one allele appears to modify the T-cell response activated by a second allele through activation-induced cell death. Such epistatic interaction might prove to be an important general mechanism for modifying exuberant immune responses that are deleterious to the host and could also help to explain the strong linkage disequilibrium in this and perhaps other HLA haplotypes. Animal models could contribute significantly to our understanding of the complex gene-gene interactions of MS once most of the major candidate regions have been identified.
3.6 Gene Expression Studies of MS

3.6.1 Approaches to Gene Expression

The study of gene expression classically encompasses techniques of Northern-blotting, real-time PCR, and microarray based techniques. The specificity and sensitivity for detection of gene expression by real-time PCR is currently unsurpassed (Bustin, Benes et al. 2005; Nolan, Hands et al. 2006). The comparison of disease and non-disease derived gene products allows for comparative gene expression between samples of interest. The technique has been used in other fields of research to successfully determine cytokine levels, derive receptor expression, measure hormone receptor levels, and quantify levels of specific messenger RNA (mRNA) from candidate genes (King, Jokhi et al. 1995; Lewohl, Crane et al. 1997; Rose'Meyer, Mellick et al. 2003). Precise determination of relative gene expression has also been demonstrated (Kivisakk, Mahad et al. 2004; Mandel, Gurevich et al. 2004). Candidate genes for MS, including T-cell specific antigen, cytokines from whole blood samples, and chemokine receptors, have been studied; however other potentially valuable targets still remain uninvestigated (Kahl, Kruse et al. 2002; Sindern, Patzold et al. 2002; Muraro, Wandinger et al. 2003). The major drawbacks of real-time PCR methods have traditionally been limited throughput and the need for gene-specific optimisation. These issues have proved costly in the past; however increased interest in this field has led to investments in both instrumentation and
chemistry which now allow for increased throughput and improved analysis. Studies using array hybridisation analysis have previously been used to investigate gene expression in MS in a broad sense. This approach can be used to determine gene activity across many thousands of candidate genes simultaneously with modest resolution (Oksenberg, Baranzini et al. 2001). In other research, this technology has been applied to examine gene expression patterns in a variety of pathways, for example in embryogenesis and cancer (Perou, Sorlie et al. 2000; Ridley 2000; Chung, Bernard et al. 2002; Weigelt, Hu et al. 2005; Hu, Fan et al. 2006). Array-based approaches usually precede finer resolution methods such as quantitative real-time PCR.
Expression analysis has been recently used to investigate oligodendrocyte differentiation in MS with some success (John, Shankar et al. 2002). New forms of the technique are continually evolving. Specific detection of viral nucleic acids is just now becoming possible (Ghedin, Pumfery et al. 2004). Application of this technology to MS research may prove rewarding. The main disadvantages of these tools have been access to sufficient suitable tissue samples, the limited reproducibility of results between experiments, debate over the best method for analysis, and the lack of resolution that certain variants of these assays can provide (Oertelt, Selmi et al. 2005; Stevens and Doerge 2005).

A review of the gene expression literature implicates several genes in MS. Whitney and colleagues report increased expression of leukotriene A(4) hydrolase (LKA4), tumour necrosis factor-alpha 2 (TNF-α2), interferon regulatory factor 2 (IRF-2), activin type 2 receptor (ACVR2), protein kinase C-beta 1 (PRKCB1), and myelin transcription factor 1 (MYT1), amongst others (Whitney, Becker et al. 1999). Further studies by others have also implicated prostaglandin D2 synthase 21kDa (brain) (PTGDS), prostatic binding protein (PBP), ribosomal protein L17 (RPL17), secreted phosphoprotein 1 (SPP1), heat shock protein 70 (HSP70), myelin basic protein (MBP), and glial fibrillary acidic protein (GFAP) (Mycko, Papoian et al. 2003). Mycko and colleagues reported increased expression of HSP 70 and correlated this finding with the presence of chronic
active (CA) plaques in the MS brain citing that HSP70 has been found to promote myelin autoantigen presentation amongst antigen presenting cells, however other studies by Bomprezzi et al. and Lock and colleagues observed the opposite (Lock, Hermans et al. 2002; Bomprezzi, Ringner et al. 2003).

In an effort to explain the enzymatic involvement of 5-lipoxygenase and leukotriene A(4) hydrolase in the production of proinflammatory molecules in MS, Chabas and colleagues carried out experiments investigating the influence of the proinflammatory cytokine osteopontin (OPN) on autoimmunity (Chabas, Baranzini et al. 2001). Their study found that OPN appears to regulate T\textsubscript{H}1-mediated demyelinating disease and concluded that in conjunction with the mediators cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2), OPN may offer a potential target in blocking development of progressive MS. Additionally, other investigators have also reported findings implicating the cyclooxygenase pathway (Paintlia, Paintlia et al. 2004). Recent follow-up studies by the same investigators (Paintlia et al.) have demonstrated that combined therapy with Lovastatin and Rolipram (drugs targeting the COX pathway) suppresses the degree of severity in EAE (Paintlia, Paintlia et al. 2008). Furthermore, this combination also appears to afford neuroprotection and repair in the animal model (Paintlia, Paintlia et al. 2009). However, Blom and colleagues found different effects in their animal model and published findings suggesting that that the knock-out mouse model used in Chabas’ work could have knocked out OPN-linked polymorphic genes that explain the observed decrease in EAE severity.
(Blom, Franzen et al. 2003). The authors do however note that identifying the OPN-linked polymorphic genes that exert a strong influence on encephalomyelitis remain an important task.

Tajouri and colleagues undertook studies to determine the qualitative and quantitative changes in gene expression patterns that characterise MS plaques, using RNA from chronic active and acute MS lesions (Tajouri, Mellick et al. 2003). They identified 139 genes that were differentially expressed in the MS brain compared to normal healthy matched controls. Of those 139 genes, 69 showed a common pattern of expression and 70 transcripts were differently expressed in either acute or chronic plaques. The genes of significance reported by these investigators included myelin basic protein (MBP), glutathione S-transferase mu-1 (GSTM1), nerve growth factors such as ninjurin 1 (NINJ1), X-ray and excision DNA repair factors such as X-ray repair complementing defective repair 9 and excision repair cross-complementing 5 (XRCC9 & ERCC5), and ribosomal protein S4 X-linked (RPS4X). Markers of inflammation such as granulin (GRN) were found up-regulated. Granulins are expressed by leukocytes and have been shown to regulate cell growth (Bateman, Belcourt et al. 1990). The importance of this finding lies in the fact that healthy human brain does not express this gene. It has been detected however in glial tumours in rats (Liau, Lallone et al. 2000). The significantly varied expression of complement component 1-q-subcomponent-B chain (C1QB), which is involved in complement and inflammation (Klegeris, Bissonnette et al. 2000), and has been previously
associated with amyotrophic lateral sclerosis (ALS) (Grewal, Morgan et al. 1999) is also reported. Furthermore, important anti-inflammatory mediators such as endothelial protein C receptor (PROCR) showed significant down expression in plaques.

Mufson and colleagues make the important observation that gene expression profiling of Alzheimer's disease, Parkinson's disease, Schizophrenia, Multiple sclerosis, and Creutzfeld-Jakob disease (CJD) have revealed that vulnerable brain regions in many of these neurological diseases share putative pathogenic alterations in common classes of genes (such as decrements in synaptic transcript levels and increments in immune response transcripts) (Mufson, Counts et al. 2006). Kaminski et al. have recently investigated the possibility of using expression profiling to determine which MS patients will respond to IFN-β therapy, noting that further efforts to understand gene expression patterns, and development of reliable biomarkers is required (Kaminski and Achiron 2005). In another study, Achiron et al. undertook studies in peripheral blood mononuclear cells (PBMCs; lymphocytes and monocytes) finding that gene expression patterns in PBMCs contain information about the MS disease process in the CNS that may be useful for diagnosis and therapy (Achiron, Gurevich et al. 2004). Bomprezzi et al. undertook studies in an attempt to identify molecular markers indicative of disease status rather than susceptibility genes for MS, and showed that gene expression profiling of PBMCs can distinguish MS patients from healthy controls (Bomprezzi, Ringner et al. 2003).
Gene expression studies have contributed significantly to the literature, advocating the importance of gene expression profiling in determining the genes involved in disease. The underlying premise of these investigations has been that expression profiles of diseased populations may reveal mechanistic clues to molecular pathogenesis. While expression profiling in human brains remains important, animal models have also contributed to our current understanding of the pathogenesis of MS.
3.6.3 Gene Expression in Animal Models

Some important gene expression experiments have been carried out in the mouse model of MS; EAE. In 1998 Jeong and colleagues undertook differential cDNA analysis of a murine encephalomyelitis model following immunisation with MBP activated T-cells (Jeong, Izikson et al. 1998). The authors found increases of several factors one of which was IL-3, which is known to be a trophic factor for central cholinergic neurons (Kamegai, Nijima et al. 1990). In a different study, Celestin et al have found that IL-3 can enhance the presentation of superantigens to T-cells (Celestin, Rotschke et al. 2001). In 2001, Ibrahim and colleagues conducted gene expression experiments in EAE mice in an effort to define the expression of genes related to time of onset and peak of the disease (Ibrahim, Mix et al. 2001). The authors found that of the approximately 11,000 genes studied, 213 were expressed differentially, and 100 showed consistent differential expression throughout the disease. Inflammation resulted in a profile of increased gene expression of immune-related molecules, extracellular matrix and cell adhesion molecules, and molecules involved in cell division and transcription, and differential expression of molecules involved in signal transduction, protein synthesis and metabolism. Another study by Whitney and colleagues encompassed microarray gene expression profiling using EAE mouse brain tissues (Whitney, Ludwin et al. 2001). The investigators in that study found that 5-lipoxygenase, a key enzyme in the biosynthesis of proinflammatory leukotrienes was increased. Expression microarrays have also been used by
other investigators to show that in the animal model, nervous responsive genes (such as glial fibrillary acid protein; Gfap) were upregulated whilst neurotrophic factors (such as glial cell derived neurotrophic factor; Gdnf) were downregulated (Carmody, Hilliard et al. 2002). Two main findings have emerged that are the same between the animal and human forms of MS and they are that the MHC appears to be involved with MS susceptibility and that females show more severe phenotypes than males (Steinman 2001).
3.7 Research Design

3.7.1 Approaches to Complex Disease

Medical genetics was revolutionised during the 1980s by the introduction of genetic mapping to locate the genes responsible for simple Mendelian disorders (Lander and Schork 1994). However, most diseases and traits do not follow simple inheritance patterns. Investigators have thus begun taking up the challenging task of dissecting complex traits. Several major approaches have been developed including linkage analysis, allele-sharing methods, association studies, and gene expression studies. The genetics of complex disease is now entering a new era with exponential advances in knowledge and technologies. The impact of these new discoveries in neuroscience is incalculable at this stage but potentially revolutionary (Sawcer 2008). The findings of genome-wide and screening approaches are leading the way into focused candidate gene and gene expression investigations, which will hopefully unravel the key events leading to the development of diseases such as MS.

3.7.2 Genetic Investigations in MS

Genetic investigations aim to discern the degree and extent of heritability of a disorder. This research is based on the underlying principle that particular genetic variations and gene expression patterns distinguish the molecular
pathology of MS. This research aims to investigate numerous candidate genes and their variants for association with MS (see Appendix A-Candidate Genes Investigated for a full list of candidate genes) and to test specific genes to determine if they show gene expression differences in MS affected tissue versus normal tissue. The aims are addressed using case-control genetic association studies to investigate genetic variants in MS cases versus healthy matched controls, and a gene expression approach, undertaking real-time PCR based gene expression studies in MS affected brain tissues versus healthy matched neuropathology free controls. Genomic DNA from MS cases and matched healthy matched controls is available for genetic variation studies. Additionally, an MS brain-bank containing tissue samples from MS affected brains and healthy controls exist within our laboratory. Tissue samples and clinical information continue to be collected.

The first undertaking involves the collection and audit of all samples and review of the associated clinical information. A database incorporating all known information, including histopathology reports, is necessary. Accurate background information is integral to later analyses. For this research blood-derived lymphocytes served as the source of DNA for the genotyping studies, and brain tissues served as the source of RNA for the gene expression studies (see Figure 3-4 below).
Genotyping is undertaken in order to investigate genetic variations using various methods. The methods used in this research include restriction fragment length polymorphism (RFLP) analysis, capillary gel electrophoresis (GeneScan analysis), high-resolution melt analysis (HRM), and TaqMan genotyping assay. Statistical methods associated with genotyping allow for the determination of associations between particular genotypes (allele frequencies) and the presence or absence of disease (MS). Differences in allele frequencies between cases (MS affected) and controls (healthy unaffected individuals) may suggest MS susceptibility.

Real-time PCR is undertaken to quantify gene expression of candidate genes (and their associated transcriptional variants). Fluorescent dye based chemistries such as SybrGreen I now facilitate efficient integration of dye into polymerase chain reactions (PCR) allowing for accurate laser detection of PCR amplification products (gene quantification). Selected genes from gene expression experiments can be investigated for genetic variations which may be contributing to the disease being investigated. Access to well documented disease tissues (white and grey matter) is an incredibly valuable resource. Differences between markers in case and control populations may implicate genes and/or their involvement in MS susceptibility.
Figure 3-4 shows the basic macromolecular structures of DNA and RNA highlighting the minor difference at the 2' position between the two (H; Hydrogen in DNA, OH; hydroxyl group in RNA). This minor disparity results in fundamental differences between the two molecules. DNA is the “genetic-blueprint”, while RNA exists in numerous forms. Much interest is currently directed towards understanding the various roles of the different RNAs in human biology. The message that DNA sends to ribosomes in the cell to code for proteins is in the form of messenger RNA (mRNA). Variations in the nature or amount of that message (mRNA transcripts) are of great interest in the pursuit of disease biology. For this research DNA was used for the genetic variation studies, whilst RNA (specifically mRNA) was used for the gene expression studies.
3.7.3 Determining Genetic Variation in MS

Two approaches have been traditionally used to investigate genetic variations and disease; linkage and association. Both of these methods depend on the underlying assumption that DNA variants are inherited and that associated variants occur more frequently in cases than controls. A combination of inherited alleles constitutes a haplotype, and those analyses have become increasingly popular in MS research, particularly since the completion of the human genome project (Dyment, Herrera et al. 2005; Vyshkina and Kalman 2005; Chao, Barnardo et al. 2007; Urcelay, Blanco-Kelly et al. 2007). One difficulty with haplotype analysis is the appropriate dissection of causative alleles and the disease (Etzensperger, McMahon et al. 2008). For linkage analysis to be successful, several affected individuals from the same family are required. For association analysis however, random, non-related individuals may be used.

Genetic case-control association studies can be used to determine whether particular genetic variations are associated with a disease, these associations are deemed positive if alleles of specific markers occur more often in those affected with the disease than those unaffected. Genomic analysis most commonly involves the examination and analysis of mutations and polymorphic gene variants. Variations are characterised and then tested for association with disease risk or incidence. An important feature of case-control associations (positive associations) is that a positive result can occur if the disease causing
allele is being investigated or if the allele tested is in linkage disequilibrium with the disease causing marker. Subsequently, it is also important to note that combinations of alleles with distant loci should therefore theoretically decrease with increased genetic distance owing to the increased probability of crossing-over between the two (Giordano, D'Alfonso et al. 2002). This complication implies that successful disease mapping depends on the extent of linkage disequilibrium (LD) across the gene of interest (Kruglyak 1999). Indeed, the most notable limitation of the association approach is the proper matching of controls to cases. In order to overcome this limitation, family-based analyses may be used. However, it is difficult to obtain large families with multiple affected members with MS. Smaller families can still be used for linkage and association studies though.

Family based methods can use linkage approaches testing for coinheritance of co-segregation of disease with a tested marker or transmission disequilibrium testing (TDT). The rationale behind TDT is that in the absence of both linkage and association, markers should be transmitted at random from parent to offspring. Given the low prevalence rate of MS in the overall population, this approach has not been practical. The most notable contributions to MS research to date from association studies have been the implication of the human MHC region (the HLA system) located 6p21.3 to susceptibility and the implications of that finding to the study of MS haplotype diversity (Oksenberg, Baranzini et al. 2001; Dyment, Herrera et al. 2005). While some limitations exist, case-control
association studies remain a popular method for investigating complex disorders such as MS (Rasmussen, Kelly et al. 2001; Oksenberg, Barcellos et al. 2004).
3.7.4 Determining Gene Expression in MS

Gene expression studies require RNA as the source of genetic material. Specifically, mRNA is selected. Messenger RNA provides an indication of the expression of a particular gene. Numerous transcriptional variants can exist for any RNA, and this must be taken into consideration when planning experiments. The transcripts of interest selected for investigation code for particular gene products (proteins). RNA is sensitive to environmental and enzymatic degradation and therefore RNA handling differs quite pronouncedly from DNA, all equipment to be used for RNA work is first cleaned with >70% ethanol (EtOH), washed with diethylpyrocarbonate (DEPC) treated water, and treated with RNase inhibitors to eliminate degrading RNAses from the environment. Furthermore, RNA work is carried out using RNA-only equipment in a designated RNA workstation and/or laboratory area. RNA must be stored colder and for shorter periods of time than its counterpart, DNA. RNA is never truly free of contaminating DNA. A contamination check is carried out using a subtractive reverse transcription method in the lab. The ultimate goal of careful RNA handling is to harvest intact, complete samples of mRNA which will subsequently be converted to a complementary DNA template (cDNA) which is then used for further quantification (see Figure 3-5 below). Complementary DNA (cDNA) is synthesised from messenger RNA (mRNA) using oligo dT primers and a reverse transcriptase enzyme. Numerous methods exist for the synthesis of cDNA including use of gene specific and random primers. Reverse transcriptase
enzymes also differ in their efficiencies (Stahlberg, Kubista et al. 2004). Proficient recovery of intact mRNA with subsequently efficient conversion to cDNA allows for downstream quantification of gene expression.

Real-time PCR analysis is used in MS research to investigate gene expression differences between MS affected, and healthy control brain samples. Real-time PCR uses fluorescent reporters to monitor the amplification of products during each cycle of PCR. This combines the nucleic acid amplification and detection steps into one homogeneous assay and obviates the need for gel electrophoresis to detect amplification products. The use of appropriate chemistries and data analyses eliminates the need for Southern-blotting or DNA sequencing for amplicon identification. Its simplicity, specificity and sensitivity, and reproducibility have made real-time PCR the benchmark technology for the detection and comparison of gene expression (Bustin, Benes et al. 2005).

Studies investigating inflammatory disease using microarrays have been published (Devauchelle and Chiocchia 2004; Gebicke-Haerter 2005). To date, microarray analysis in MS has mainly focused on gene expression in active and chronic plaques. Genes reported to have shown altered expression include cell receptor adhesion molecule, inflammatory, growth factor, and apoptosis genes (CD47, SULT, MC4R, LOC243461, RNASE3, IGF-1, G-CSF, FGF2, VJC, IL-1, IL-6, IL-8, TNF, MAPKK, CASP9, NEUROD, GJB2, MS4A2, HRH2, PTAFR, c-erbA, IL-17, MOX1, IGHD TPS, MMP-1, COL9A3, TGF, and TNC) (Jacobs, Baker et al. 1991; Gijbels, Brocke et al. 1995; Mycko, Kwinkowski et al. 1998;
Lock, Hermans et al. 2002; Mycko, Papoian et al. 2003). Jacobs et al undertook studies in the experimental allergic encephalomyelitis model (EAE) and found that interleukin-1 (IL-1) may initiate or promote inflammation within the central nervous system. In addition, they concluded that specifically blocking the biological activity of IL-1 in vivo by soluble receptors may prove beneficial for the treatment of autoimmune or inflammatory diseases. Mycko and colleagues reported that increased frequency of ICAM-1 exon 6 allele T in the intracellular adhesion molecule 1 (ICAM-1) correlates with MS susceptibility. Those investigators also undertook complementary DNA (cDNA) microarrays in 2003 and reported several new MS susceptibility genes (as above).

Aberrant expression of apoptosis and DNA damage-regulatory genes (NR4A2, RIPK2, SODD, TRAIL, BCL2, and DAXX) has also been published (Satoh, Nakanishi et al. 2005). In those studies Satoh and colleagues found that among the 1258 genes examined, 173 were in T-cell related and 50 in non-T-cell related genes and report that those candidates were expressed significantly differently between MS patients and healthy matched controls. The investigators noted that in their studies downregulated genes greatly outnumbered upregulated genes with more than 80% of the top 30 most significant genes being categorised into apoptosis signalling-related genes of both proapoptotic and antiapoptotic classes.
Technologies are now emerging that will potentially allow for accurate, affordable, high throughput microarray screening of human samples for the detection of viral pathogens (Kennedy, Grinfeld et al. 2005). Repeated attempts to conclusively implicate a viral pathogen to date have failed. One complication regarding investigations in the viral hypothesis includes the fact that some pathogens do not grow in culture (Anderson, Jones et al. 1983). The eventual development and application of a microarray, or real-time technique to detect pathogens in MS research may prove rewarding. However, designing a platform to assay those targets is paved with complications owing to the diverse array of possible targets. Once potential chromosome regions or pathways are identified, specifically designed arrays are likely to become powerful resources in clinical settings (Oertelt, Selmi et al. 2005). Determining the gene expression signatures that distinctly characterise MS may reveal new aspects of an old disease and assist in the advancement of this process.
Figure 3-5 shows the process used to synthesise cDNA template from mRNA for this research (gene expression studies). RNA cannot be used for PCR (owing to its chemical form). The original mRNA transcript is reverse-transcribed into a complementary DNA template that can then be used for further experiments. (Source: Purves, W.K., Sadava, D., Orians, G.H., Heller, C.H.: Life: The science of Biology, Figure 16.8, Sinauer Associates, Inc. and W. H. Freeman & Co.).
Chapter 4

General Methodology
4.1 Introduction to General Methodology

The project encompassed two specific aims: the quantification of candidate gene expression in MS, and the investigation of genomic variations potentially associated with MS. We undertook sensitive case-control gene expression studies of five genes implicated in biological processes involved in neuroinflammation. The genes studied were claudin 11 oligodendrocyte transmembrane protein (CLDN11), glial cell derived neurotrophic factor (GDNF), neuregulin 1 (NRG1), the protein tyrosine phosphatase, receptor type, C (PTPRC), and the toll-like receptor 3 (TLR3) and expression of these genes was undertaken in chronic secondary-progressive multiple sclerosis (SPMS) affected brains. These studies investigated expression differences between frontally and temporally obtained white-matter and grey matter tissues by relative quantitative polymerase chain reaction (qRT-PCR), followed by immunoblotting to determine if there are any significant differences between the profiles of multiple sclerosis brains compared to healthy matched disease free controls.

Genetic variation studies within the genes 5,10-methylenetetrahydrofolate reductase (MTHFR), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR), protein tyrosine phosphatase, receptor type C (PTPRC), and the toll-like receptor 3 (TLR3) were also undertaken using various genomic techniques including restriction fragment length polymorphism (RFLP) restriction enzyme digest followed by agarose gel electrophoresis (AGE), capillary gel
electrophoresis (GeneScan analysis), high-resolution melt analysis (HRM), and TaqMan genotyping assay analysis.

4.2 Genetic Variation Studies Methodology

The genetic variation studies involved RFLP analysis of MTHFR A1298C and MTRR A66G; HRM and RFLP analysis of PTRPC C77G and C772T, respectively, and capillary gel electrophoresis (GeneScan) analysis and TaqMan genotyping assay for the TLR3 [-/A]_8 insertion-deletion and C1236T variants, respectively. MS versus age, gender, ethnicity, matched control DNA was used for the experiments. The aim of the variation studies was to determine whether allele frequencies within the tested markers differ between MS affected individuals and unrelated, healthy, matched controls.

4.2.1 DNA Preparation

205 MS and 205 control (age, gender, and ethnicity; Caucasian, matched) DNA samples were used for the genetic variation studies to investigate the polymorphic alleles within the chosen candidate genes (MTHFR, MTRR, PTPRC, and TLR3). The MS population was obtained from the Multiple Sclerosis Clinic at the Royal Brisbane Hospital, Brisbane, Queensland, Australia; all samples were obtained from patients residing in South East Queensland, Australia. The
populations consisted of 75% females and 25% males, and were categorised based on clinical course: Relapsing-Remitting (RR), Secondary-Progressive (SP), and Primary-Progressive (PP). The matched control population (matched for age ±5 years, gender, and ethnicity) was obtained via the Genomics Research Centre Clinic, Southport, Queensland, Australia. Genomic DNA was extracted from peripheral blood using a standard salting-out procedure as follows:

Genomic DNA was extracted using a QIAmp Blood Kit (Qiagen) according to manufacturer’s instructions; Lymphocytes were isolated and pelleted in phosphate buffered saline (PBS) followed by lysis with protease and lysis buffer (AL Buffer, Qiagen). Pure ethanol (100%) was added to precipitate the nucleic acid before transfer to a QIAmp Maxi-Column, the column was then centrifuges at 3000 rpm for 3 min. AW1 Buffer (Qiagen) was then applied to the column facilitating DNA binding (to the column) before centrifugation at 5000 rpm for a further 1 min. Buffer AW2 (Qiagen) was added and the column was again centrifuged at 5000 rpm for 15 min to facilitate ethanol drainage. The column was then transferred to a new collection tube and AE Buffer (Qiagen) was added. Column were left to stand at room temperature for 5 min. Genomic DNA was eluted following a 5000 rpm 5 min centrifuge step. The resulting DNA was quantified using spectrophotometry at 260nm and working concentrations of DNA were prepared (20ng/μL).
4.2.2 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was first described in literature in 1985 (Saiki, Scharf et al. 1985). The method uses oligonucleotide primers (of about 20bp in length) that complement the 5’ and 3’ ends of a DNA sequence. Specific primers result in amplification of the sequence spanned by the primer pair (forward and reverse primers) in an exponential manner (see Figure 4-1 below). For the reaction to proceed, a thermal cycle is necessary. A denaturation step begins the cycling (usually around 95°C). Primers anneal at lower temperatures (around 45-65°C) and in the presence of a Taq polymerase and free nucleotides, extension of the complementary template occurs. The extension temperature is variable from assay to assay but is usually around 72°C. Once this extension is complete, another denaturing step restarts the process. Thermal cycling is automated using laboratory equipment. The production of copies of DNA using this process is exponential and the process is efficient for approximately 40-50 cycles. It is necessary to first amplify DNA by PCR to ensure template concentration is sufficient for further manipulation, such as restriction enzyme digest.
Figure 4-1 Exponential Nature of PCR

Figure 4-1 shows the exponential nature of PCR (first 3 cycles shown only). Parental strands of DNA are denatured by heating, the temperature is lowered to allow forward and reverse primers to anneal to the regions of intended amplification, the temperature is again increased and extension of the amplified region occurs with the aid of dinucleotide triphosphates (dNTPs). The process repeats for the total number of cycles resulting in many copies of the original template region of interest.
4.2.3 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphisms (RFLPs) are widespread throughout the genome. It is possible to distinguish different RFLP sequences using restriction enzymes. Restriction enzymes originate from bacteria, they serve to recognise and specifically cleave particular sequences of DNA. Specific cleavage produces DNA fragments of different sizes that then characterise the particular sequence of interest. Visualisation of the result of this process is possible through agarose gel electrophoresis (AGE) or alternately, if multiple products in the reaction are similar in size, through capillary gel electrophoresis (GeneScan analysis). Three restriction enzymes were used during this research to genotype MTHFR A1298C (MboII), MTRR A66G (NspI), and PTPRC C772T (RsaI) (see Figure 4-2 below).

Figure 4-2 shows the recognition sites of the restriction enzymes used to genotype (A) MTHFR A1298C (MboII), (B) MTRR A66G (NspI), and (C) PTPRC C772T (RsaI).

A  
3’...CTTCT(N)₆...5’  
5’...RCATGY...3’  

B  
3’...YTACR...5’  
5’...CATG...3’

C  
5’...GAAGA(N)₆...3’  

Figure 4-2 Recognition Sites for MboII, NspI, and RsaI Restriction Enzymes.
4.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis (AGE) is a widely used technique for the separation of macromolecules which can be either nucleic acids or proteins, on the basis of their sizes and electric charges. Many important biological molecules such as amino acids, peptides, proteins, nucleotides, and nucleic acids possess charged side groups and therefore, at any given pH, exist in solution as electrically charged species. Depending on the nature of the net charge, the charged particles will migrate either to the cathode or the anode in an electrophoresis tank. Electrophoresis is used to resolve products, to determine if the correct product was amplified during PCR, and to determine results for genotyping (see Figures 4-3 and 4-4 below). Agarose (a polysaccharide consisting of 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose) powder is mixed into buffer, generally 1XTAE (40mM Tris acetate + 1mM ethylenediaminetetraacetic acid; which contains Tris-base, Glacial acetic acid, and EDTA) or 1XTBE (45mM Tris-borate + 1mM ethylenediaminetetraacetic acid; which contains Tris-base, Boric acid, and EDTA), heated until dissolved, then pre-stained with a fluorescent agent (generally Ethidium Bromide; EtBr), and finally cast. Different gels, for example for RNA electrophoresis, are prepared differently (with the addition of formaldehyde to keep RNA denatured during electrophoresis).
Figure 4-3 Characteristic Banding Patterns in AGE

Figure 4-3 shows RFLP genotyping by agarose gel electrophoresis (AGE) after PCR and restriction enzyme digest. Patterns of banding correspond to different genotypes (C/C, C/T, and T/T). (M) is the MW marker used to estimate bp sizes of the test bands.

Figure 4-4 Real-Time PCR Amplicons in AGE

Figure 4-4 shows PCR amplicons being resolved post real-time PCR; (A) ≈200bp products lanes 1-6, (B) ≈400bp amplicons lanes 7-12, and (C) negative controls.
4.2.5 Capillary Gel Electrophoresis

Capillary gel electrophoresis (GeneScan analysis) uses fluorescently labelled markers (PCR primers) incorporated during PCR to produce amplified DNA segments (amplicons) that are fluorescently tagged. Minor preparation of the PCR product occurs (heating the DNA to denature the products), and a GeneScan machine is used to electrophorese the products. The GeneScan equipment excites the incorporated fluorescent markers using a laser and then determines the size of the amplicons that were detected during electrophoresis.

For GeneScan analysis starting DNA concentrations must be equal (adjusted to 10ng/μL for each sample for our experiments). Specific forward and reverse PCR primers are designed and the reverse primers are labelled with fluorescent reporter dye (FAM in our experiments). GeneScan requires a multi-stage process of standard PCR, clean-up, and then subsequent resolution by capillary gel electrophoresis. In this way the GeneScan component of the method can be viewed as analogous with the agarose gel electrophoresis used to resolve genotypes by standard AGE. Genomic DNA is used as the template to generate PCR products for subsequent genotyping by capillary gel electrophoresis. Assay conditions were empirically determined (as for standard PCR with AGE). In our experiments 40ng total DNA was amplified on a PC-960 Thermocycler (Corbett Life Science, Australia), PCR products were electrophoresed on 2% w/v agarose gels to confirm the presence of the predicted DNA fragments, and PCR product
was diluted to a working concentration for the next step. In the preliminary experiments the correct DNA dilutions were determined with regards to relative fluorescence from the GeneScan-350 Size Standard marker. As agarose gel electrophoresis lacks the sensitivity to resolve small differences in the order of several nucleotide base pairs (all PCR amplicons look the same upon AGE) the PCR products were genotyped using ABI PRISM® 310 Genetic Analyser. Equimolar concentrations of PCR product were combined with Hi-DiTM Formamide (Applied Biosystems) and ABI310 Genetic Analyser Performance Optimised Polymer-4 (POP-4TM) (Applied Biosystems) according to the manufacturer’s instructions, and then run at 60V for approximately 20 minutes per sample. The Genetic Analyser processes samples using electro-kinetic injection and detects products using a laser capture charge-coupled device (CCD) (see Figure 4-5 below). Data was analysed on a computer (see Figure 4-6 below), which serves as both the control unit for the machine and the analysis platform GeneScan analysis is ideal for both high-throughput applications (multiplexing several markers each with independent fluorescent markers) or as in our research, the discrimination of multiple similarly sized PCR products. For our experiments, a single capillary genetic analyser (operating on the principle depicted in Figure 4-5 below) was used for all experiments.
Figure 4-5 Principle of Electro-Kinetic Injection

Figure 4-5 shows the theory of electro-kinetic injection which the GeneScan machine employs. PCR samples (sample) are electrophoresed through a glass capillary (capillary) with the application of a current. The products (which were fluorescently labelled during previous PCR) travel through the capillary and past the laser charge-coupled device and detector (detection window/laser). The sample is then collected in a reservoir (GA Buffer). The process is controlled by a computer (not shown) that serves as both the control unit for the machine (not shown) and the collection and analysis of data (Source: Applied Biosystems, GeneScan Analysis Software 310 Compendium Version 3, p3, Applied Biosystems, USA).
Figure 4-6 Capillary Gel Electrophoresis Results

Figure 4-6 shows an example of capillary gel electrophoresis (GeneScan) results: (A) shows the background size standard (molecular weight marker) which is the reference point for all samples peaks, (B) this area lists the exact bp sizes of the peaks detected as the electrophoresis proceeds (shown along the x-axis), (C) shows different loci alleles (heterozygotes; 2 peaks each loci) and (D) shows their corresponding sizes. The point at (E) shows the difference between the heterozygotes (detected at C) and the appearance of a homozygote (with only 1 peak at E).
4.2.6 TaqMan Genotyping Assay

The TaqMan single-nucleotide polymorphism (SNP) genotyping assay incorporates probes labelled with FAM or VIC (fluorescent dyes) at the 5’ end, and a minor-groove binder (MGB) and non-fluorescent quencher at the 3’ end. During the first step of a TaqMan SNP genotyping assay experiment, AmpliTaq polymerase from the TaqMan Universal PCR Master Mix, and No AmpErase UNG amplify target DNA using sequence-specific primers. MGB probes from the SNP genotyping assay provide a fluorescence signal for the amplification of each allele (see Figure 4-7 below). The MGB increases the melt temperature ($T_m$) for a given probe length creating a discernable difference between matched and mismatched probes, which allows for allelic discrimination (Kutyavin, Lukhtanov et al. 1997; Kutyavin, Afonina et al. 2000). TaqMan genotyping assays are highly specific and enable genotyping of markers that would otherwise prove problematic due to confounding factors such as nearby variations and highly repetitive neighbouring sequences. Fluorescence data are collected on a real-time PCR machine, collecting cycling information on separate channels (designated green and yellow). Cycle-thresholds are applied and discrimination plots allow for clustering of individual genotypes (see Figure 4-8 below).
Figure 4-7 shows the chemistry involved with TaqMan MGB probe-based genotyping assays. Each TaqMan Assay contains sequence specific primers for amplifying the polymorphism of interest as well as MGB probes for detecting alleles of the specific polymorphism of interest. Each TaqMan MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites. When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye causes the reporter dye signal to be quenched. AmpliTaq Gold DNA polymerase extends the primers bound to the genomic DNA template. AmpliTaq Gold DNA polymerase (a 5´ nuclease) cleaves probes that are hybridized to the target sequence. When the hybridized probes are cleaved, the quencher dye is separated from the reporter dye, increasing the
fluorescence of the reporter dye. Therefore, the fluorescence signal generated by PCR amplification indicates which alleles are present in the sample. (Source: Applied Biosystems, TaqMan Drug Metabolism Genotyping Assay Reference Manual, section 1-10, Figure 1-2, Applied Biosystems, USA).
Figure 4-8 shows an example of the allelic discrimination plot obtained after PCR cycling analysis for a bi-allelic marker. Samples with identical genotypes cluster together owing to their alleles. In this example a substantial increase for the VIC dye (only) indicates homozygosity for allele 1, an increase in both VIC and FAM dyes (together) indicate heterozygosity for alleles 1 and 2, and a FAM dye increase (alone) indicates homozygosity for allele 2 alone (Source: Applied Biosystems, TaqMan Drug Metabolism Genotyping Assay Reference Manual, section 1-10, Figure 1-3, Applied Biosystems, USA).
4.2.7 High Resolution Melt Genotyping

High resolution melt analysis (HRM) is a method for genotyping that allows a high throughput and more efficient analysis over traditional methods, however not all markers can be genotyped using this method. In a recent study, Garritano and colleagues found that HRM may have limited sensitivity for detection of nucleotide insertion-deletion variants located immediately adjacent to mononucleotide runs because they may stutter during PCR (Garritano, Gemignani et al. 2009). For those markers that are in regions allowing for efficient primer design, with no closely neighbouring variations, HRM presents an excellent choice for efficient and reproducible genotyping. HRM is made possible by advances in specialised instrumentation and software, and the introduction of third generation fluorescent double stranded DNA (dsDNA) dyes such as SYTO-9 (Invitrogen Corp., Carlsbad, CA). SYTO-9 was used for all experiments in this project. SYTO-9 has low assay toxicity and can therefore be used at higher concentrations than traditional dyes. Using higher concentrations of dye allows for improved saturation of dsDNA leading to increased sensitivity even at low concentrations of starting template. DNA samples are amplified in a real-time PCR cycler followed by resolution of fluorescence in a final melt phase of the run (see Figure 4-9 below for an overview of melt kinetics). Individual products produce distinct melt patterns distinguishing the different genotypes of interest owing to the nature of their base sequences. Genotypes are determined by interpreting shifts in the amplification profiles and product melts of the samples.
using the Rotor-Gene software (Corbett Life Science) (refer to Figure 4-10 below for product melt examples).
Figure 4-9 Melt Kinetics of High-Resolution Melt Analysis

Figure 4-9 shows the fundamental theory of typical HRM analysis. The melt curve (green) plots the transition from high fluorescence of the initial pre-melt phase through the sharp fluorescence decrease of the melt phase to basal fluorescence at the post-melt phase. Fluorescence decreases as DNA intercalating dye is released from double-stranded DNA (dsDNA) as it dissociates (melts) into single strands (ssDNA). The midpoint of the melt phase, at which the rate of change in fluorescence is greatest, defines the temperature of melting ($T_M$) of the particular DNA fragment under analysis (Source: High Resolution Melt Assay Design and Analysis on the Rotor-Gene 6000 Real-Time Rotary Analyser, p3, Corbett Life Sciences, Australia).
Figure 4-10 SNP Genotyping by High-Resolution Melt Analysis

Figure 4-10 shows (A) homozygous wild type, mutation and heterozygote samples shown on an example normalised melt plot and (B) a difference plot normalised to mutant samples (Source: SNP Genotyping by High Resolution Melt (HRM), Discrimination of human ACTN3 (R577X) SNP genotypes (C to T substitution) using SYTO® 9 intercalation dye., Corbett Life Science, Australia).
Prior to performing HRM analysis, a target sequence is first amplified to high copy number. This is normally done using PCR in the presence of a dsDNA intercalating fluorescent dye (SYTO-9 used in all experiments). The dye does not interact with ssDNA but actively intercalates with dsDNA. The difference in fluorescence created can be used to measure the increase in DNA concentration during a pre-HRM amplification and then to directly measure thermally-induced DNA dissociation by HRM. Initially, fluorescence is high in a melt analysis because the sample starts as dsDNA, but fluorescence diminishes as the temperature is raised and DNA dissociates into ssDNA. DNA melt curves are distinct between different type of samples (based on sequence length, GC content and DNA sequence complementarity). HRM is a simpler and more cost effective way to characterise samples than probe-based genotyping assays and, unlike conventional methods, it is a closed assay system requiring no post-PCR processing. Results are comparable to more time consuming and expensive methods such as single-stranded conformational polymorphism (SSCP), DHPLC (denaturing high-pressure liquid chromatography), RFLP and DNA sequencing (White, Hall et al. 2007). HRM genotyping was used to analyse the PTPRC C77G marker in this research.
4.2.8 Statistical Analysis of Genotyping

Genotyping results are assessed by firstly performing descriptive statistical analyses. Analysis entailed the use of a combination of the statistics package for the social sciences (SPSS), Microsoft Excel, and the Quantitative Skills Tables package (Uitenbroek 1997). The G-Power statistical package was used to extrapolate study power \textit{a-priori} for the genetic variation studies (Faul, Erdfelder et al. 2007). Genotype frequencies are tested for Hardy-Weinberg Equilibrium (HWE) to compare expected and observed allele frequencies and to check for possible genotyping anomalies. Model free Chi-square ($\chi^2$) tests are used to determine whether significant differences exist between case and control populations. The Bonferroni correction can be applied to correct for comparisons between several repeated samples. Adjacent markers may be tested for linkage disequilibrium (LD) using the methodology of Zhao et al (Zhao 2004). LD analysis entails calculating pair-wise linkage disequilibrium statistics by estimating haplotype frequencies using a heuristic algorithm. Chi-square goodness-of-fit tests were carried out to determine if significant differences exist in allele frequencies between cases and controls ($\alpha=0.05$). Analysis of male cases versus male controls and female cases versus female controls was also carried out to test for significant differences by gender. Significant differences between cases and controls indicate association of the tested marker with the disease.
4.3 Gene Expression Studies Methodology

The gene expression studies entailed relative quantitative real-time PCR (qRT-PCR) of five genes implicated in neuroinflammation; claudin 11 oligodendrocyte transmembrane protein (CLDN11), glial cell derived neurotrophic factor (GDNF), neuregulin 1 (NRG1), the protein tyrosine phosphatase, receptor type, C (PTPRC), and the toll-like receptor 3 (TLR3) in chronic secondary-progressive multiple sclerosis (SPMS) brains in frontally and temporally obtained white-matter (NAWM) and grey matter (NAGM) tissues as compared to healthy matched controls, followed by immunoblotting for qualitative protein confirmation.

4.3.1 RNA Preparation

Gene expression studies require RNA as the source of genetic material. Specifically, total RNA is required. Messenger RNA provides an indication as to the expression of a particular gene. RNA handling differs quite pronouncedly from DNA. All equipment used for RNA work was first cleaned with >70% ethanol (EtOH), washed with diethylpyrocarbonate (DEPC) treated water, and treated with RNaseZAP (Sigma), to eliminate degrading RNAses from the environment. Furthermore, RNA work was carried out using RNA-only equipment in a designated RNA workstation (Gelaire). Total RNA extraction was performed using Trizol reagent (Invitrogen). A range of 0.1 to 1.5g of human brain tissue
was added to volumes of Trizol. The tissue was then disrupted using a series of syringes and needles (19G, 23G, and 25G) and then a Rotor-stator homogeniser (with samples on ice). The homogenate was left to stand at room temperature for few minutes followed by addition of chloroform and incubation for 3 min. Centrifugation at high speed (11,000 rpm) separated the phases of the lysate. The aqueous solution was transferred to a fresh tube and mixed with cold isopropanol. After 10 minutes of incubation the sample was centrifuged at high speed again. The RNA was pelleted and washed with DEPC EtOH twice. Resuspension in DEPC treated water followed. The total RNA was quantified and its purity assessed by the 260nm/280nm ratio provided by a spectrophotometer. Treatment with DNAse 1 (to remove genomic DNA contamination) was also carried out. A mix of DEPC water, RQ1 buffer (Qiagen), dithiothrietol (DTT), and DNAse 1 was added to the RNA. Incubation at 37°C for 15 min ensued and then RNA purification was necessary. Total RNA was purified using columns (Qiagen) to obtain a purified product. Total RNA was mixed with pure EtOH and poured into a column (Qiagen). Steps were carried out using RW1 buffer, RPE buffer, wash buffer, and finally RNA elution with DEPC-water. RNA quantification was performed again using a spectrophotometer. The integrity of the RNA was visualised using a formaldehyde agarose (FA) gel. The gel was prepared using agarose powder, FA-gel buffer (MOPS, sodium acetate, EDTA, and DEPC-water). RNA was mixed with an RNA loading dye (bromophenol blue, EDTA, formaldehyde), glycerol, formamide, and FA-gel buffer. The RNA mixtures were
incubated for 3 min at 65°C before electrophoresis. The gel was visualised under ultra-violet (UV) illumination and photographed with a digital camera (Kodak).

RNA is never truly free of DNA contamination. Quality extraction of RNA from post-mortem brain tissues can be problematic (Schramm, Falkai et al. 1999). A contamination check is possible via a reverse transcription PCR step (RT-PCR). RT-PCR was performed using dNTPs, (Promega), RT-PCR buffer (Tris-HCL, DTT, KCl, Triton X-100, EDTA, and MgCl$_2$), PCR primers, Taq polymerase (Perkin Elmer), and superscript III reverse transcriptase (Invitrogen). Thermal cycling was then performed. Triplicate controls for each reverse transcription (RT-PCR) were used. One control lacked reverse transcriptase, one lacked RNA, and one lacked both reverse transcriptase and RNA (but contained genomic DNA). PCR products were resolved using AGE, and visualisation was carried out under UV light. This process generated cDNA that was then used for real-time PCR (rt-PCR).

4.3.2 cDNA Synthesis

RNA cannot be used for PCR. RNA must first be converted to complementary DNA before molecular analyses are possible. The process of cDNA (complementary DNA; DNA that is complementary to an RNA sequence) synthesis is known as reverse transcription. Reverse transcription is achieved by reverse transcription polymerase chain-reaction (RT-PCR). The process of RT-
PCR was performed by adding 200 μM dNTPs (Promega), 5X rt-PCR buffer (300 mM Tris-HCL-pH 8.3, 2.5 mM DTT, 250 mM KCl, 0.5% Triton X-100 (Evergreen Scientific), 30 μM EDTA, 7.5 mM MgCl2), 0.4 μM each primer, 1 unit Taq polymerase (Perkin Elmer, ABI), and 1.2 units recombinant Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega). Thermal cycling was 30 minutes at 50°C (reverse transcription) followed by 5 minutes at 94°C, 5 cycles of 45 seconds at 94°C, 60 seconds at 57°C, 2 minutes at 72°C added to 30 cycles of 30 seconds at 94°C, 30 seconds at 57°C, 45 seconds at 72°C (with 3 seconds extension) and ended with a final incubation of 4 minutes at 72°C. Three Controls for each RT-PCR were carried out. These controls comprised one reaction without AMV, one without RNA, and one without AMV or RNA (but with genomic DNA) and Taq polymerase. This process ensured ability to check for PCR from RNA and not genomic DNA, and for presence of contaminants. PCR products were resolved using 2.0% agarose gel. Visualisation was possible under ultraviolet illumination after ethidium bromide staining. Multiple Sclerosis case, and matched control cDNAs were synthesised in this manner.
4.3.3 Quantitative Real Time Polymerase Chain Reaction

The use of precise sequence specific primers is of paramount importance for real-time (rt-PCR). Sequence specific polymerase chain reaction (PCR) primers facilitate the amplification of gene segments within regions of interest. Those primers can be designed to interrogate isolated targets, or alternately if they are directed at conserved regions, multiple transcripts of interest within a gene. To this end, we designed primer sets specific for the transcripts of interest for the genes being quantitated: claudin 11 oligodendrocyte transmembrane protein (CLDN11), glial cell derived neurotrophic factor (GDNF), neuregulin 1 (NRG1), the protein tyrosine phosphatase, receptor type, C (PTPRC), and the toll-like receptor 3 (TLR3). Primers were designed in conserved regions across numerous transcripts of interest to facilitate successful PCR quantification (see Appendix C for qRT-PCR Primers and Transcriptional Targets). Thorough optimisation is necessary for all individual targets and annealing temperature tests as well as MgCl₂ titrations were carried out. Quantitative PCR (Q-PCR) allows for investigations of gene expression (Klein 2002). Q-PCR encompasses a range of absolute, competitive, and relative PCR methodologies. For this research the Q-PCR undertaken may be termed relative quantitative real-time PCR (qRT-PCR) because relative differences in gene expression are being investigated between cases (MS affected samples) versus healthy matched controls. Conventional reverse transcriptase PCR (RT-PCR) is not conducive to quantification because the target of interest is amplified non-linearly
(exponentially) (Kainz 2000). Several methods aim to circumvent this phenomenon. Competitive approaches and real-time detection methods have been developed that allow quantification after applied analysis (Lekanne Deprez, Fijnvandraat et al. 2002). Real-time detection allows for the accurate determination of the exact PCR cycle at which a product is amplified (Ginzinger 2002). The point of linear exponential amplification (which is most informative) is termed the cycling-threshold value (CT) (see Figure 4-11 below). The CT value is determined by the investigator empirically and is set at the same value for each experiment to allow for reproducibility. Individual CT values can be easily determined using software allowing for collection of data that is then compared between samples (relative quantification). Those CT values are also corrected before use subtracting a baseline ubiquitous reference (incorporated into each experimental run) to give a true normalized CT value. Melt identities of the PCR products were also resolved (see Figure 4-12 below). RT-PCR is being increasingly implemented for the analysis of complex samples and is of particular interest in the neurosciences (Aldape, Ginzinger et al. 2002). These techniques have been used to assay the expression of genes accurately in both animal and human studies and continue to be the method of choice for these approaches to genetic investigation.
Figure 4-11 Real-Time PCR Cycling

Figure 4-11 shows real-time PCR fluorescent cycling for 3 distinct samples (A), (B), and (C). The PCR cycle at which amplification occurs is shown on the x-axis and relative fluorescence is shown on the y-axis. Point (D) shows the cycle-threshold (CT) that has been applied to derive the CT values for each sample (A, B, and C). In this example the CT values for A, B, and C would be approximately 14, 27, and 34, respectively. The CT value serves as the reference point for further analysis of gene expression. Targets with increased expression will amplify to the left on the x-axis with lower CT values (earlier in the PCR owing to increased abundance) and targets with decreased expression will amplify further to the right; with higher CT values (owing to their lower abundance).
Figure 4-12 shows melt phase analysis of 3 samples. Each sample has a distinct melting temperature (shown increasing left to right on the x-axis). All the samples of (A) type cluster together and are dissociating (melting) around 76°C. Samples of the type (B) are melting around 80.5°C, and samples of the (C) type are melting around 87°C. A threshold line can be seen at (D), this vertical line represents a reference that is applied at analysis (which can be shifted) to accurately determine the exact melting temperature of a product (peak). The exact melting temperature (represented by the uppermost pinnacle of the peak) uniquely identifies a sample. Note that it is favourable to design the assay so that each target being amplified has a distinct melt signature, otherwise distinguishing individual products can become difficult, or even impossible (depending on amplicon size and base composition).
Intron-spanning primers were designed where possible (for CLDN11, GDNF, and PTPRC) to control for possible genomic DNA contamination. Lone transcripts of interest were isolated for CLDN11 and TLR3. Primers spanning multiple transcripts of interest were designed for GDNF, NRG1, and PTPRC. Several normalisation genes were considered and tested for stability; the genes were glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (β-Actin), and 18S. Efficiency and stability parameters were assessed using the computer software package geNorm (Vandesompele, De Preter et al. 2002). Based on these results, the ubiquitous gene 18S was selected for use in all experiments. Real-time PCR using SYBR Green I (Bio-Rad) was optimised on a Corbett Life Science Rotorgene-6000 platform (Corbett Life Science, Australia). Cycling and melt data were resolved with follow-up normal 2% agarose gel electrophoresis to confirm amplicon specificity for each set of primers. Conditions were optimised for uniform amplification efficiency within genes, and final primer concentrations (250nM), and optimal MgCl$_2$ concentrations (2-4mM) for each gene. Each reaction began with 20ng of template with each run including cDNA, genomic DNA, and no template control (NTC). All reactions were performed in triplicate. Rotorgene-6000 software (Corbett Life Science, Australia) was used to derive cycling threshold (CT) values from the exponential phases of the optimised PCRs before statistical analysis.
4.3.4 Statistical Analysis of Gene Expression

For relative gene expression analysis the \( \Delta\Delta CT \) method \( (2^{-\Delta\Delta CT}) \) was used (Livak and Schmittgen 2001). Triplicate averages were corrected to the housekeeping gene (18S) for each sample ensuring that standard deviations (SDs) remained less than 0.20 in each set. Shapiro-Wilk and Kolmogorov-Smirnov tests were used to test data distribution. Levene’s test for homogeneity of variance followed by independent samples T-Test was used to determine mean differences between cases and controls. Fold regulation was analysed and compared between affected (case) and unaffected (control) samples.

4.3.5 Protein Preparation and Immunoblotting

Proteins were extracted from the same brain tissues as used for the gene expression studies in order to undertake immunoblotting to demonstrate the protein associated with the gene transcripts of interest. Protein extraction was with a Protein Extraction Reagent Kit (Pierce). The kit used allowed for isolation of hydrophilic and hydrophobic fractions of both grey matter and white matter samples for each patient sample. Protease inhibitor cocktail (at a concentration of 1:100) was added to the lysates. A protein precipitation was carried out next using chloroform and methanol, before quantification using a BCA Protein Assay Kit (Pierce, USA) and NanoDrop spectrophotometer (Shimadzu).
The precipitation was carried out by adding 400μL of methanol to 100 μL of samples, vortexing, adding 100 μL chloroform, vortexing, adding 300 μL water, vortexing, centrifuging for 1 minute at 14,000 g, removing the top aqueous layer, adding 400 μL methanol, vortexing, centrifuging for another 2 minutes at 14,000 g, removing the methanol without disturbing the pellet, speedvacuum drying the sample briefly, and then reconstituting the sample in polyacrylamide gel electrophoresis (PAGE) buffer. This method is known to precipitate soluble as well as hydrophobic proteins from dilute solutions and is not affected by the presence of contaminants such as detergents, lipids, salt, buffers, or beta-mercaptoethanol (Wessel and Flugge 1984).

Samples were quantified on a FLUOstar Optima plate reader as well as a NanoDrop Spectrophotometer reading at 562nm (Shimadzu) (see Figure 4-13 below). Quantification of protein standards was carried out in order to construct a standard curve for protein quantification. Six standard of known concentration (of 125, 250, 750, 1000, 1500, and 2000mg/mL) were included in the quantification (see Figure 4-14 below). A standard curve was generated by plotting the obtained absorbance readings at 562nm (AU) against the known concentrations of protein standard.
Figure 4-13 shows protein quantification by spectrophotometry using a BCA protein detection kit. Results include the measured absorbance at 562nm wavelength and the calculated concentration of protein contained in each tested sample measured in mg/mL.
Figure 4-14 shows the protein standard curve obtained using known protein standards (125, 250, 750, 1000, 1500, and 2000mg/mL concentrations). Protein concentration (mg/mL) is shown on the x-axis, while absorbance (AU) is shown on the y-axis. These protein standards were used to extrapolate the concentration of the unknown samples that were measured. A linear regression model was used to calculate those values.
Western blotting (with SDS-PAGE) and immunoblotting (without SDS-PAGE) were attempted for all protein samples. The western-blot procedure entailed SDS-PAGE, nitrocellulose transfer, antibody hybridisation steps, and development and chemiluminescent photography. The immunoblot procedure entailed quantification followed by nitrocellulose blotting, antibody hybridisation steps, and developing followed by chemiluminescent photography on a Bio-Rad VersaDock system (Bio-Rad, USA).

SDS-PAGE was carried out using running buffer (25mM Tris, 192mM Glycine, 0.1% w/v SDS pH 8.3), Laemmli sample loading buffer (Laemmli buffer: 2-mercaptoethanol 95:5). A 50:50 solution of sample and loading buffer was boiled for 5 minutes, put on ice and then briefly spun down. A gel cassette was prepared and a Western blot electrophoresis tank was filled with running buffer (as before). Samples were loaded into set gels and molecular weight marker was added to the first lane. The gel was electrophoresed for 1 hour at 150V, 400mA. Following electrophoresis the gels were removed, used for Coomasie staining and nitrocellulose transfer. For Coomasie staining, gels were stained with 40% methanol, 10% acetic acid, 50% water, and 0.1% w/v Coomasie 250 brilliant blue for 1 hour. Destaining followed, with 40% methanol, 10% acetic acid, and 50% water, changing the buffer frequently.
A selection of Coomasie gels were visualised and photographed with Bio-Rad photographic equipment (see Figure 4-15 below). An appropriate protein marker was used for comparison to the samples tested and the Bio-Rad photographic software suite estimated the sizes of the protein fragments in the tested samples in comparison to this (size scale in kilodaltons; kDa).

Nitrocellulose transfer followed using transfer buffer (containing Tris base, Glycine, water, methanol, and 10% SDS). The SDS-PAGE gels were rinsed, and blotting paper and nitrocellulose membrane was cut to size. Gels were wedged between the membrane and wet (with transfer buffer) blotting paper before electrophoresis at 100V, 400mM for 1-1.5 hours.

The following steps were carried out for Western blotting: Tris buffered saline(TBS) (125mM NaCl, 25mM Tris) and TBS with Tween-20 (TBS-T) were prepared (125mM, NaCl, 25mM Tris, 0.1% v/v, and 1% Tween-20; pH)adjusted to pH 7.5 with HCl acid). Blocking solution was prepared (TBS-T with 5% w/v skim milk powder). Non-specific binding was blocked with blocking solution for 30-60 minutes. Primary antibodies (rabbit anti-human) (Abcam) were prepared (0.2-1 ug/ml in blocking solution) before incubation of the membranes with primary antibody. β-Actin was used as the ubiquitous positive control. The incubation lasted 1 hour with mechanical agitation. Next, 5 wash steps were carried out with TBS-T for 5-10 minutes each. Secondary antibody was prepared (1:1000 concentration horseradish peroxidase (HRP) conjugated secondary
antibody: 1:50,000) (Sigma). Membranes were then incubated for 1 hour, again with agitation. Wash steps were repeated again with TBS-T before development with western-blot chemiluminescence substrate (1:1) luminol enhancer solution (containing peroxide) (Santa Cruz Biotechnology, USA). Membranes were briefly incubated with the developer solution before photography with a BioRad VersaDock imaging system (Bio-Rad, USA), with manual exposure set at between 150-300 seconds (see Figure 4-16 below).
Figure 4-15 shows a photograph of a Coomasie blue stained protein gel after electrophoresis. Lane (1) shows separation of proteins in a hydrophilic sample. Lane (2) shows separation of protein from a hydrophobic sample. Lane (3) shows the protein weight marker with corresponding mass marked in kilodaltons (kDa).
Figure 4-16 shows an example of detection of a protein of interest using a Western blot. Only the protein of interest is visible in this exposure because of the specificity of the antibody in use (secondary antibody with HRP conjugate attached to the primary antibody for the target of interest). Different exposures of the membrane are necessary to visualise all components of the blot (molecular weight marker, separated proteins, and protein of interest).
Chapter 5

Gene Expression Studies in the Normal Appearing White Matter of Multiple Sclerosis

Expression of neuregulin-1 and toll-like receptor-3 genes are increased in secondary progressive white matter tissue of multiple sclerosis.
Multiple Sclerosis (MS) is a chronic debilitating disease that attacks the central nervous system. Recent efforts to describe the disease have included both clinical revisions (Polman, Reingold et al. 2005; Roxburgh, Seaman et al. 2005) and molecular advances (Booth, Arthur et al. 2005; Hafler, Compston et al. 2007). Despite concerted efforts, the exact mechanisms underlying the molecular pathology of MS still remain undefined (Kantarci 2008; Lindsey and Patel 2008). MS is thought to be of autoimmune origin developing in genetically susceptible individuals, possibly triggered by environmental factors (Vanderlocht, Hellings et al. 2006). Immunological phenomena are fundamental to the pathogenesis of the disease warranting investigation of immune related genes (Bar-Or 2008; Bar-Or and Antel 2008). The changes seen in MS plaques have been a particular focus of past studies with efforts concentrated on characterizing the nature of lesion pathology. Genes that have been identified as differentially regulated within plaques include the family of receptor-type protein tyrosine phosphatases (RPTPs), insulin-like growth factor receptor (IGFR), and neuronal growth regulators such as microtubule associated proteins (MAPs) (Nakagawa, Koyama et al. 2000; Tajouri, Mellick et al. 2003). Damaging MS lesions termed plaques are known to form in brain normal appearing white matter (NAWM). Acute MS plaques are oedematous and are marked by myelin debris containing macrophages. Chronic active plaques are well delineated and show macrophage activity at the lesion borders. The benefit of transcriptional profiling, from several
sources in various models, continues to be topical in neurological research (Sharp, Xu et al. 2006; Comabella and Martin 2007). Quantification of mRNA using real-time RT-PCR remains the method of choice for accurate determination of gene expression (Nolan, Hands et al. 2006). Genes associated with central nervous system (CNS) myelin, neurotrophic processes, glial support, immune cell recognition, and viral response, remain ideal targets for gene expression profiling in the brain. Gene expression profiling is beginning to reveal underlying molecular mechanisms in neurological disease and may potentially aid in the identification of therapeutic targets (Mufson, Counts et al. 2006). We undertook studies in NAWM tissues (which are primarily comprised of neuroglia; oligodendrocytes and astrocytes) in order to better understand gene expression in these tissues.

The protein encoded by the claudin family of tight junction associated proteins is a major component of the central nervous myelin which is essential for normal central nervous system function; furthermore the myelin protein Claudin11 (encoded by the gene CLDN11) is central to oligodendrocyte differentiation and proper myelin membrane formation (Anitei, Ifrim et al. 2006). GDNF encodes a highly conserved neurotrophic factor. The mature protein product of this gene exists as a homodimer and has been shown to promote the survival and differentiation of dopaminergic and motor neurons, recently the balance between GDNF mediated neurite outgrowth and inhibition via multi-component receptor complex interaction has been demonstrated (Yoong, Wan et al. 2006; Yoong and
Too 2007). Neuregulins are a family of structurally related proteins of the epidermal growth factor (EGF) family. Several isoforms of neuregulin-1 (NRG1) exist as a result of alternative splicing: Type I (Heregulin/Neu Differentiation Factor/Acetylcholine Receptor Inducing Activity), Type II (Glial Growth Factor 2), Type III (Sensory-Motor Derived Factor), as well as types IV, V, and VI. Interaction of NRG1 with ERBB receptors induces the growth and differentiation of neuronal and glial cells (Edwards and Bottenstein 2006). Recently, Type III neuregulin-1 has been shown to promote oligodendrocyte myelination (Taveggia, Thaker et al. 2008). PTPRC codes for the leukocyte common antigen CD45 and is an essential regulator of T- and B-cell antigen receptor signalling. Direct interaction with antigen receptor complexes as well as activation of Src kinases has been demonstrated reiterating CD45s role in both direct and indirect immune response (Huntington and Tarlinton 2004). Toll-like receptors (TLRs) play a fundamental role in pathogen recognition by identification of pathogen-associated molecular patterns resulting in both innate and adaptive immune responses (Huang, Zhao et al. 2008). TLRs have been shown to recognise pathogen-derived ligands on dendritic cells and T-cells triggering nuclear factors such as NFκB and type-1 interferons leading to the production of proinflammatory cytokines (Wang, Miyahara et al. 2008).

We present a sensitive case-control gene expression study of five genes implicated in biological processes involved in neuroinflammation; claudin 11 oligodendrocyte transmembrane protein (CLDN11), glial cell derived neurotrophic
factor (GDNF), neuregulin 1 (NRG1), the protein tyrosine phosphatase receptor type C (PTPRC), and the toll-like receptor 3 (TLR3) in chronic secondary-progressive multiple sclerosis (SPMS) affected brains investigating expression differences between frontally and temporally obtained white-matter by relative quantitative polymerase chain reaction (qRT-PCR) to determine if there are any significant differences between the profiles of multiple sclerosis brains compared to healthy matched controls.

5.2 Materials & Methods

5.2.1 Tissue Samples

Institutional ethics approval was granted by the Griffith University Ethics Committee for research involving human specimens (HSC/09/03/HREC). Age, gender, ethnicity, and anatomically matched control samples were obtained from the National Health and Medical Research Brain Bank, University of Queensland, Australia, and MS case samples from the Human Brain and Spinal Fluid Resource Center, Los Angeles, USA, with neuropathology reports issued by an independent neuropathologist. A total of 7 SPMS normal appearing white matter samples adjacent to plaques (as shown in Appendix B) from patients with median age 63±11 years and disease duration 29±13 years were used (6 Female, 1 Male). The control group was comprised of 7 matched controls certified free of neurological disease. Tissues were originally sectioned into 8mm coronal
sections, labelled, imaged, bagged air tight, frozen between 2 pre-chilled aluminium plates in -150 degree ultra low temperature freezer, then stored at -80ºC. Post-mortem interval (autolysis) time (time between death and quick freezing) was kept to the minimum possible (15±6 hours). Computerised full coronal digital images were supplied by the neurospecimen brain bank. Plaque classification was carried out by an experienced neuropathologist (1 Acute, 1 Chronic Inactive, and 5 Chronic Active). Patient samples were carefully selected taking into account detailed medical history. Total RNA was extracted using a modified phenol guanidine isothiocyanate protocol optimised in our lab for brain tissue. For each sample 0.5-1.0g of tissue was homogenized using high-speed sonication in TrizolTM solution (Invitrogen). RNeasyTM (Qiagen) Mini Kits were used for purification followed by DNaseI treatment. RNA integrity was assessed by denaturing gel formaldehyde agarose electrophoresis, spectrophotometry, and capillary electrophoresis using the 6000 Nano Lab ChipTM (Agilent Technologies). One-step RT-PCR was performed using previously published methods (Tajouri, Mellick et al. 2003). Complementary DNA (cDNA) templates were generated from the anatomical localisations (as shown in Appendix B), and matched healthy controls.
Transcripts of interest were chosen based on their functional relevance using Entrez Gene (NCBI). Primers were designed based on mRNA sequence information and intended transcriptional target (as shown in Appendix C). Intron-spanning primers were designed where possible (CLDN11, GDNF, and PTPRC) as an added measure to control for possible genomic DNA contamination. Lone transcripts of interest were isolated for CLDN11 and TLR3. Primers spanning multiple transcripts were designed for GDNF, NRG1, and PTPRC. Several normalisation genes were considered and tested for stability in brain tissues; the targets included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (β-Actin), and 18S. Efficiency and stability parameters were assessed using geNorm (Vandesompele, De Preter et al. 2002). The ubiquitous normalisation gene 18S was selected for use in all experiments. Real-time PCR using SYBR Green I (Bio-Rad) was optimised on a Corbett Life Science Rotorgene-6000 platform.

Cycling and melt data were resolved with normal 2% agarose gel electrophoresis to confirm amplicon specificity for each separate primer set. Conditions were optimised for uniform amplification efficiency within genes (as shown in Appendix D), and final primer concentration (250nM), and optimal MgCl₂ concentrations (2-4mM) for each gene. Each reaction started with 20ng of template with each run including cDNA, genomic DNA (gDNA), and no template control (NTC) in
triplicate. Rotorgene-6000 software (Corbett Life Science) was used to resolve cycling threshold (CT) values in the exponential phase of the optimised PCRs before statistical analysis.

5.2.3 Protein Isolation and Immunoblot in NAWM

Proteins were extracted from tissues using a Protein Extraction Reagent Kit (Pierce) including protease inhibitor cocktail. A protein precipitation using Chloroform/Methanol was carried out before quantification using BCA Protein Assay Kit (Pierce). Samples were quantified on a FIUOstar Optima plate reader as well as a spectrophotometer (Shimadzu). Equal amounts of protein were prepared followed by blotting onto nitrocellulose membrane (Sigma, USA). Membranes were blocked in 5% low-fat milk TBS-T for 1 hour at room temperature and then stained overnight with rabbit anti-human primary antibody (Abcam) and β-Actin was used as ubiquitous control. Following three 10 minute washes with TBS-T the membrane was incubated with 1:1000 horseradish peroxidase-conjugated secondary antibody for 1 hour (Sigma). Luminol chemiluminescent reagent (Santa Cruz Biotechnology, USA) was used to develop the membranes before photography with a BioRad VersaDock system. Positivity of expression was confirmed by signal upon photography.
5.2.4 Statistical Analysis in NAWM

The ΔΔCT method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen 2001) was used for relative quantitative real-time analysis. Triplicate averages were used corrected to the housekeeping gene (18S) expression for each sample with standard deviation (SD) less than 0.20 in each set. Shapiro-Wilk and Kolmogorov-Smirnov tests were implemented for testing data distribution. Levene’s test for homogeneity followed by independent samples T-Test was used to determine mean difference between cases and controls. Fold regulation was analysed and compared between affected and unaffected samples.

5.3 Results in NAWM

The aim of the experiment was to determine gene expression profiles for 5 specifically tested genes (CLDN11, GDNF, NRG1, PTPRC, and TLR3) in normal appearing white matter tissues of secondary-progressive multiple sclerosis affected brains by relative quantitative real-time PCR, and to assess whether these expression patterns differ from matched healthy controls.
Detailed neuropathology reports were obtained and cDNA templates generated representative of frontal and temporal tissues as described in methods. Real-time PCR cycling and melt data were resolved and CT values were determined. PCR conditions were previously optimised to give consistent amplification efficiencies as evidenced by identical amplification slopes. PCR product melts were used to ensure amplification specificity.

Example real-time PCR results for the genes that were found to be significantly differentially expressed (NRG1 and TLR3 cases versus controls) are shown below: Figure 5-1 shows NRG1 Real-Time PCR Cycling in NAWM Controls. Figure 5-2 shows NRG1 Real-Time PCR Melt in NAWM Controls. Figure 5-3 shows NRG1 Real-Time PCR Cycling in NAWM Cases. Figure 5-4 shows NRG1 Real-Time PCR melt in NAWM Cases. Figure 5-6 shows TLR3 Real-Time PCR Cycling in NAWM Controls. Figure 5-7 shows TLR3 Real-Time PCR Melt in NAWM Controls. Figure 5-8 shows TLR3 Real-Time PCR Cycling in NAWM Cases. Figure 5-9 shows TLR3 Real-Time PCR Melt in NAWM Cases. A selection of representative samples was also checked for expected amplicon size and specificity by normal 2% agarose gel electrophoresis. Figure 5-5 shows Agarose Gel Electrophoresis of NRG1 cDNA Amplicons, whilst Figure 5-10 shows Agarose Gel Electrophoresis of TLR3 cDNA Amplicons. A summary of normalised CT values from Real-Time PCR for NRG1 and TLR3 can be seen in Table 5-1. Immunoblotting was undertaken purely as a qualitative procedure to
ascertain whether the proteins of interest could be detected, positivity of expression was confirmed by signal upon photography.

Statistical analysis by the $2^{\Delta\Delta CT}$ method involved the averaging of triplicate samples within runs to within 0.20 standard deviations within each set. Each case and control sample CT value was adjusted for its internal control (18S) giving a true representation of quantitative expression. Cases and controls were then compared taking into account regional localisation. Independent samples T-Test was carried out, first evaluating data distribution using Levene’s test for homogeneity. Table 5-2 shows Statistical Analysis Results for CLDN11 in NAWM. Table 5-3 shows Statistical Analysis Results for GDNF in NAWM. Table 5-4 shows Statistical Analysis Results for NRG1 in NAWM. Table 5-5 shows Statistical Analysis Results for PTPRC in NAWM. Table 5-6 shows Statistical Analysis Results for TLR3 in NAWM.
Figure 5-1 NRG1 Real-Time PCR Cycling in NAWM Controls

Figure 5-1 shows real-time PCR cycling results for the gene NRG1 in NAWM control samples.

Figure 5-2 NRG1 Real-Time PCR Melt in NAWM Controls

Figure 5-2 shows real-time PCR melt curve analysis for the gene NRG1 in NAWM control samples.
Figure 5-3 NRG1 Real-Time PCR Cycling in NAWM Cases

Figure 5-3 shows real-time PCR cycling results for the gene NRG1 in NAWM case samples.

Figure 5-4 NRG1 Real-Time PCR melt in NAWM Cases

Figure 5-4 shows real-time PCR melt curve analysis for the gene NRG1 in NAWM case samples.
Figure 5-5 shows agarose gel electrophoresis (AGE) of (A) NRG1 cDNA amplicons by 2% agarose gel stained with Ethidium Bromide and (B) no template controls (NTCs) in normal appearing white matter (NAWM) samples.
Figure 5-6 TLR3 Real-Time PCR Cycling in NAWM Controls

Figure 5-6 shows real-time PCR cycling results for the gene TLR3 in NAWM control samples.

Figure 5-7 TLR3 Real-Time PCR Melt in NAWM Controls

Figure 5-7 shows real-time PCR melt curve analysis for the gene TLR3 in NAWM control samples.
Figure 5-8 TLR3 Real-Time PCR Cycling in NAWM Cases

Figure 5-8 shows real-time PCR cycling results for the gene TLR3 in NAWM case samples.

Figure 5-9 TLR3 Real-Time PCR Melt in NAWM Cases

Figure 5-9 shows real-time PCR melt curve analysis for the gene TLR3 in NAWM case samples.
Figure 5-10 Agarose Gel Electrophoresis of TLR3 cDNA Amplicons in NAWM

Figure 5-10 shows agarose gel electrophoresis (AGE) of (A) TLR3 cDNA amplicons by 2% agarose gel stained with Ethidium Bromide and (B) no template controls (NTCs) in normal appearing white matter (NAWM) samples.
Table 5-1 NRG1 and TLR3 Average Normalised CT Values from qRT-PCR in NAWM

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue</th>
<th>Analysis</th>
<th>Cases Avg CT</th>
<th>Cases SD</th>
<th>Controls Avg CT</th>
<th>Controls SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRG1</td>
<td>NAWM</td>
<td>Overall</td>
<td>15.59</td>
<td>2.25</td>
<td>19.89</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NRG1</td>
<td>15.67</td>
<td>2.67</td>
<td>19.94</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frontal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>NAWM</td>
<td>Overall</td>
<td>15.57</td>
<td>3.68</td>
<td>24.66</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TLR3</td>
<td>16.08</td>
<td>4.28</td>
<td>23.93</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Frontal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-1 shows the average normalised (samples corrected for their internal ubiquitous control) CT values for the significant genes NRG1 and TLR3 in NAWM.
Table 5-2 shows statistical analysis results for the gene expression of CLDN11 in NAWM. All P-values exceeded 0.05 (t-test for Equality of Means Sig. (2-tailed) column) therefore no statistically significant results were observed for this gene in NAWM.
Table 5-3 shows statistical analysis results for the gene expression of GDNF in NAWM. All P-values exceeded 0.05 (t-test for Equality of Means Sig. (2-tailed) column) therefore no statistically significant results were observed for this gene in NAWM.
Table 5-4 shows statistical analysis results for the gene expression of NRG1 in NAWM. P-values (t-test for Equality of Means Sig. (2-tailed) column) for overall (0.0040) and frontal expression (0.0206) were statistically significant for this gene in NAWM.
Table 5-5 Statistical Analysis of PTPRC in NAWM

Table 5-5 shows statistical analysis results for the gene expression of PTPRC in NAWM. All P-values exceeded 0.05 (t-test for Equality of Means Sig. (2-tailed) column) therefore no statistically significant results were observed for this gene in NAWM.
Table 5-6 Statistical Analysis of TLR3 in NAWM

<table>
<thead>
<tr>
<th></th>
<th>t</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
<th>Mean Difference</th>
<th>Std. Error Difference</th>
<th>95% Confidence Interval of the Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>frontal</td>
<td>3.6159</td>
<td>6</td>
<td>.0112</td>
<td>7.8475</td>
<td>2.1703</td>
<td>2.5370 - 13.1580</td>
</tr>
<tr>
<td>temporal</td>
<td></td>
<td></td>
<td></td>
<td>13.0250</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-6 shows statistical analysis results for the gene expression of TLR3 in NAWM. P-values (t-test for Equality of Means Sig. (2-tailed) column) for overall (0.0007) and frontal expression (0.0112) were statistically significant for this gene in NAWM.
Expression profiles are summarised in Table 5-7 below while individual cycling threshold (CT) values and their standard deviations are shown in Figures 5-11 (NRG1) and 5-12 (TLR3). Statistically significant gene expression differences (where P<0.05) are shown in Figure 5-13 and non-statistically significant results (where P>0.05) in Figure 5-14.

Statistically significant increased expression was observed for NRG1 (↑19.72-fold, P=0.004) and in frontally obtained samples (↑19.94-fold, P=0.021), and for TLR3 (↑543-fold, P=0.0007) as well as for frontally obtained samples (↑230-fold, P=0.0112) compared to control samples as shown in Figure 5-13. Non-significant gene expression differences (where P>0.05) were observed for CLDN11 (↑1.75-fold in frontal, ↑31.12-fold in temporal), GDNF (↑2.46-fold in frontal, ↑2.38-fold in temporal), PTPRC (↑3.49-fold in frontal, ↑2.64 in temporal), and NRG1 (↑19.29-fold in temporal) samples as shown in Figure 5-14. TLR3 expression in temporal tissues was not assessed due to small sample size (n=2). Notable variations may be a result of tissue autolysis times, or perhaps to duration of the disease (refer to Appendix B for autolysis times).
Table 5-7 shows a summary of the relative gene expression differences between cases and controls in normal appearing white matter (NAWM) samples. Statistically significant findings include expression of NRG1 in overall (0.004) and frontal (0.021) samples, and TLR3 in overall (0.0007) and frontal (0.0112) samples. The other tested genes were not found to be significantly different (all P-values >0.05).

Table 5-7 Relative Expression Differences between Cases & Controls in NAWM

<table>
<thead>
<tr>
<th>Gene</th>
<th>Analysis</th>
<th>Fold-Change</th>
<th>p-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLDN11</td>
<td>Overall</td>
<td>↑ 4.56</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Frontal</td>
<td>↑ 1.75</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>↑ 31.12</td>
<td>0.20</td>
</tr>
<tr>
<td>GDNF</td>
<td>Overall</td>
<td>↓ 1.52</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>Frontal</td>
<td>↓ 2.46</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>↑ 2.38</td>
<td>0.69</td>
</tr>
<tr>
<td>NRG1³</td>
<td>Overall³</td>
<td>↑ 19.72</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Frontal³</td>
<td>↑ 19.94</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>Temporal³</td>
<td>↑ 19.29</td>
<td>0.31</td>
</tr>
<tr>
<td>PTPRC</td>
<td>Overall⁴</td>
<td>↓ 3.18</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Frontal⁴</td>
<td>↓ 3.49</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Temporal⁴</td>
<td>↓ 2.64</td>
<td>0.77</td>
</tr>
<tr>
<td>TLR3³</td>
<td>Overall³</td>
<td>↑ 543</td>
<td>0.0007</td>
</tr>
<tr>
<td></td>
<td>Frontal³</td>
<td>↑ 230</td>
<td>0.0112</td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>n/a⁵</td>
<td></td>
</tr>
</tbody>
</table>

¹Normal Appearing White Matter
²Independent Samples T-Test
³Statistically Significant Result
⁴Homogeneity of Variance Violated
⁵Sample Size Inadequate
Figure 5-11 NRG1 Expression Differences between Cases & Controls in NAWM

Figure 5-11 shows significant relative gene expression differences expressed as average CT values between cases and controls for the gene neuregulin-1 (NRG1) in the overall and frontal analyses in normal appearing white matter (NAWM). NRG1 average overall CTs were 15.59 (cases) versus 19.89 (controls) and NRG1 average frontal CTs were 15.67 (cases) versus 19.94 (controls).
Figure 5-12 TLR3 Expression Differences between Cases & Controls in NAWM

Figure 5-12 shows significant relative gene expression differences expressed as average CT values between cases and controls for the gene toll-like receptor 3 (TLR3) in the overall and frontal analyses in normal appearing white matter (NAWM). TLR3 average overall CTs were 15.57 (cases) versus 24.66 (controls) and TLR3 average frontal CTs were 16.08 (cases) versus 23.93 (controls).
Figure 5-13 Statistically Significant Differences between Cases & Controls in NAWM

Figure 5-13 shows statistically significant (P<0.05) relative gene expression differences between cases and controls in normal appearing white matter (NAWM). NRG1 gene expression was increased 19.72-fold (overall) and 19.94-fold (frontal), while TLR3 gene expression was increased 543-fold (overall) and 230-fold (frontal) in MS cases compared to healthy matched controls.
Figure 5-14 shows non-statistically significant relative gene expression differences between cases and controls in normal appearing white matter (NAWM). Although gene expression differences were seen between cases and controls in these samples those results did not reach statistical significance (all P>0.05).
5.4 Discussion of NAWM Gene Expression

A broad spectrum of changes appears to accompany the underlying mechanisms responsible for the complex neuropathology of multiple sclerosis (Lassmann, Bruck et al. 2007). Histopathological studies have reported widespread heterogeneity at the cellular and molecular levels with notable homogeneity within patients (Lucchinetti, Bruck et al. 2004; Morales, Parisi et al. 2006). Molecular profiling is beginning to provide insight into the biology of neurodegenerative diseases (Mufson, Counts et al. 2006). Crude methods of detection are giving way to sensitive techniques for the quantification of mRNA levels of specific transcripts within distinct tissues (Pantelidou, Zographos et al. 2007). We set out to determine gene expression profiles of CLDN11, GDNF, NRG1, PTPRC, and TLR3 in the normal appearing white matter of secondary-progressive multiple sclerosis brains with mainly chronic active plaque pathology (n=5 of 7 total samples), compared to 7 healthy matched controls.

NRG1 expression in this study was found to be significantly increased in frontal samples (↑19.94-fold, P=0.021) and also in temporal lobe tissues (↑19.29-fold) although the latter did not reach statistical significance (P=0.31) due partly to a violation within the homogeneity of variance within those samples. A combined analysis of the two regions together gave a statistically significant overall average increase of 19.72-fold (P=0.004). Increased expression of NRG1 in chronic affected multiple sclerosis brains may signify glial induction or remyelination as
previously discussed in literature (Bieber 2008; Lucchinetti 2008). Changes in the expression of NRG1 are likely to affect the regulation of transmembrane proteolipid proteins (predominantly myelin proteins). PLP1 is known to play a role in compaction, stabilization, and maintenance of myelin sheaths and mutations leading to a primary-progressive MS phenotype have been described (Warshawsky, Rudick et al. 2005). TLR3 expression was found to be increased 230-fold (P=0.011) in frontal samples, but meaningful quantitative results were not available for isolated temporal tissues. Overall expression analysis yielded a 543-fold increase in TLR3 (P=0.0007) with frontal and temporal samples combined, but this result does not allow for the accurate extrapolation of temporal expression due to a restricted sample size for those experiments (n=2).

Increased expression of TLR3 may indicate a T-cell subset imbalance or reflect production of proinflammatory cytokines. Toll-like receptors (in particular TLR3) regulate interferon-regulatory factors such as interferon regulatory factor 1 (IRF1) and toll-interleukin 1 receptor domain containing adaptor proteins (TIRAP). Toll-like interleukin 1 receptor (TIR) is responsible for signal transduction and activation of the TLR4 immune pathway which leads to transcriptional activation of proinflammatory genes (Henneke and Golenbock 2001). Tight junction associated proteins are key components of normal central nervous system function, oligodendrocyte differentiation, and myelin formation. Increased expression of CLDN11 was found in both frontal (↑1.75-fold,) and temporal (↑31.12-fold) samples compared to controls, however did not reach statistical
significance (P=0.14 and P=0.20). Given the role of CLDN11 in proper myelin function, and proposed mechanisms of targeted myelin destruction (Breij, Brink et al. 2008) it is interesting to note the relative difference between expression levels in frontal versus temporal regions, where a 29-fold difference was demonstrated between the two regions (see Table 5-7).

In this study GDNF expression (a neurotrophic element) was decreased in frontal (↓2.46-fold) but increased in temporal lobe (↑2.38-fold) regions, although not reaching statistical significance (P=0.13 and P=0.69). Previous studies have investigated the expression of GDNF in the spinal cord of amyotrophic lateral sclerosis (ALS) patients and the Parkinsonian brain (Mitsuma, Yamamoto et al. 1999; Gill, Patel et al. 2003). Those studies report glial derived neurotrophic factors to be potential therapeutic targets for neurodegenerative diseases. The involvement of T-cells and the T-cell receptor in general is well established in multiple sclerosis (Weiner 2004; McDole, Johnson et al. 2006). T-cell regulation and balance involving T-regulatory (T-reg) cells is now thought to be a key aspect of autoimmune-mediated inflammatory disease (Dittel 2008; O' Connor and Anderton 2008). PTPRC expression was found to be decreased in both frontal (↓3.49-fold) and temporal (↓2.64-fold) tissues. Overall, expression was decreased on average 3.18-fold, but failed to reach significance (P=0.32). The link between immunological transcripts and T-cell dysregulated phenotypes has been previously proposed (Learn, Fecci et al. 2006). Differential expression of PTPRC in multiple sclerosis lends credence to an autoimmunological hypothesis.
which has been a fundamental topic of discussion on previous occasions (Barnett and Prineas 2004).

Overall, expression of NRG1 (↑19.94-fold, P=0.021) and TLR3 (↑230-fold, P=0.011) in frontal white matter was determined to be significantly different between multiple sclerosis samples compared to healthy matched controls. NRG1 protein has been previously demonstrated in the prefrontal cortex, within neurons, somatodendritically, and at axons (Law, Shannon Weickert et al. 2004). Persistent expression of neuregulins in the adult brain supports a continuing role for these factors in normal brain function.

TLR3 has been studied by immunohistochemical methods in adult brain and has been shown to be both highly expressed and regulated in late-active as well as early-active lesions but not healthy controls (Bsibsi, Persoon-Deen et al. 2006). Expression differences were observed for CLDN11 in frontal (↑1.75-fold) and temporal (↑31.12-fold), GDNF frontal (↓2.46-fold) and temporal (↑2.38-fold), NRG1 temporal (↑19.29-fold), PTPRC frontal (↓3.49-fold) and temporal (↓2.46-fold) samples, but those analyses did not reach statistical significance (P>0.05). The latter results may be attributable to disparate expression patterns observed within groups.

Determining tissue specific gene expression patterns by sensitive methods should provide us with valuable information regarding the overall involvement of
genes in complex diseases such as multiple sclerosis. Profiling larger groups of well-classified samples may provide explanations into the reasons behind the heterogeneity observed in complex disorders. Changes in the expression of genes in normal appearing white matter in MS continue to be of interest and may provide new insights into the disease that studies focused solely on plaque pathology may have overlooked. Further investigations to map the expression of genes in the MS brain are warranted and may provide exciting new findings linking the pathogenesis of the disease to the molecular mechanisms underlying its aetiology.
Chapter 6

Gene Expression Studies in the Normal Appearing Grey Matter of Multiple Sclerosis

Expression of glial cell derived neurotrophic factor, protein tyrosine phosphatase receptor type C, neuregulin 1, and toll-like receptor 3 genes are increased in the normal appearing grey matter of secondary-progressive multiple sclerosis brains.
6.1 Introduction to Studies in NAGM

Multiple sclerosis (MS) is a neuroinflammatory disorder of the brain and spinal cord in which lymphocyte infiltration leads to the damage of myelin and axons. The cause of the disease involves both environmental exposure and genetic susceptibility (Compston and Coles 2008). MS remains the most common idiopathic inflammatory disease of the central nervous system, predominantly affecting Caucasians between the ages of 18 and 40 years with females accounting for approximately 60-70% of cases (Weinshenker 1994). Previous investigations demonstrate leukocyte response as evidenced by IgG oligoclonal bands in the cerebrospinal fluid (CSF) in two-thirds of patients, additionally myelin basic protein (MBP), phospholipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) antibodies have been reported (Sellebjerg, Jensen et al. 2000). The precise cause of the immune response in MS is unknown although there is evidence to suggest a temporal relationship between infectious triggers, such as increased Epstein-Barr virus (EBV) titres, and the onset of neurological symptoms (Levin, Munger et al. 2005).

Post-mortem studies have shown that demyelinating grey matter plaques are frequently observed in the brain and spinal cord in MS (Bo, Vedeler et al. 2003; Gilmore, Bo et al. 2006). Because the clinical significance of these plaques is not completely understood, the sequence of events underlying the development of inflammatory plaques remains a central focus of MS research efforts (Frohman,
Acute MS plaques are oedematous and are marked by myelin debris containing macrophages. Chronic active plaques are well delineated and show macrophage activity at the lesion borders. Molecular events triggering and accompanying onset and progression of most diseases can be viewed as originating from perturbations of gene transcriptions eventually compounding protein functions (Gebicke-Haerter 2005). Magnetic resonance studies of normal appearing brain tissues flanking plaques have found that damage in these tissues can be clearly linked to cognitive impairment in MS (Filippi, Tortorella et al. 2000). Grey matter abnormalities have been investigated by advancing imaging techniques and the observed changes in the MS brain have been recently attributed to neuronal damage and reactive glial proliferation further emphasizing the need for investigating grey matter tissue in MS (Ceccarelli, Rocca et al. 2007). Relative quantitative real-time polymerase chain reaction (qRT-PCR) remains the leading choice for precise quantification of gene expression (Nolan, Hands et al. 2006). Genes implicated in central nervous system (CNS) disorders represent ideal targets for gene expression profiling. We undertook studies in NAGM tissues (which are primarily comprised of neurons) in order to better understand gene expression in these tissues.
In this study we set out to investigate the gene expression of five genes implicated in the biological processes of neuroinflammation, immune response, and myelination; claudin 11 oligodendrocyte transmembrane protein (CLDN11), glial cell derived neurotrophic factor (GDNF), neuregulin 1 (NRG1), the protein tyrosine phosphatase receptor type C (PTPRC), and the toll-like receptor 3 (TLR3) in chronic secondary-progressive multiple sclerosis (SPMS) affected brains. GDNF encodes a highly conserved neurotrophic factor and the mature protein product of this gene has been shown to promote the survival and differentiation of dopaminergic and motor neurons (Yoong and Too 2007). PTPRC codes for the leukocyte common antigen CD45 and is an essential regulator of both T- and B-cell antigen receptor signalling. CD45 plays a key role in both direct and indirect immune responses (Huntington and Tarlinton 2004). Neuregulins comprise a family of structurally related proteins of the epidermal growth factor (EGF) lineage. Various isoforms of neuregulin-1 (NRG1) exist as a result of alternative splicing: Type I (Heregulin/Neu Differentiation Factor/Acetylcholine Receptor Inducing Activity), Type II (Glial Growth Factor 2), Type III (Sensory-Motor Derived Factor), as well as types IV, V, and VI. ERBB receptors have been shown to interact with NRG1 to induce the differentiation of glial cells (Edwards and Bottenstein 2006). In a recent study, Type III neuregulin-1 was show to be capable of promoting oligodendrocyte myelination (Taveggia, Thaker et al. 2008). Toll-like receptors (TLRs) play an important role in pathogen recognition through the identification of pathogen-associated molecular patterns (Huang, Zhao et al. 2008). Additionally, TLRs are capable of inducing the
production of proinflammatory cytokines through interactions with nuclear factors in response to recognition of pathogen-derived ligands on dendritic and T-cells (Wang, Miyahara et al. 2008).

This study focused on quantification of the expression of secondary-progressive grey-matter by relative quantitative real-time polymerase chain reaction (qRT-PCR) and follow-up immunoblot to determine whether there are any significant differences in a number of gene profiles in multiple sclerosis brains compared to healthy matched controls.

6.2 Materials & Methods

6.2.1 Tissue Samples

Institutional ethics approval (HSC/09/03/HREC) was obtained through the Griffith University Ethics Committee for research involving humans. Age, gender, ethnicity, and anatomically matched control samples were obtained from the National Health and Medical Research Brain Bank, University of Queensland, Australia, and MS case samples from the Human Brain and Spinal Fluid Resource Center, Los Angeles, USA. Neuropathology reports were obtained through an independent neuropathologist, and the matching controls were certified neuropathology free. A total of 7 SPMS normal appearing grey matter samples adjacent to plaques (as shown in Appendix B) from patients with median
age 63±11 years and disease duration 29±13 years were used (6 Female, 1 Male). Healthy controls were matched for age (±5 years), gender, and ethnicity (Caucasian), and anatomical localisation and were certified free of neurological disease. Tissues were originally sectioned into 8mm coronal sections, labelled, imaged, bagged air tight, frozen between 2 pre-chilled aluminium plates in -150 degree ultra low temperature freezer, and then stored at -80ºC. Post-mortem interval (autolysis) time (time between death and quick freezing) was kept as short as possible (15±6 hours). Computerised full coronal digital images were supplied by the neurospecimen brain bank. Plaque classification was carried out by an experienced neuropathologist (1 Acute, 1 Chronic Inactive, and 5 Chronic Active). Patient samples were carefully selected taking into account detailed medical history. Total RNA was extracted using a modified phenol guanidine isothiocyanate protocol optimised in our lab for brain tissue. For each sample 0.5-1.0g of tissue was homogenized using high-speed sonication in Trizol solution (Invitrogen) with cooling on ice. RNeasy (Qiagen) Mini Kits were used for purification followed by DNasel treatment. RNA integrity was assessed by denaturing gel formaldehyde agarose electrophoresis, spectrophotometry, and capillary electrophoresis using the 6000 Nano Lab ChipTM (Agilent Technologies). One-step RT-PCR was performed using previously published methods (Tajouri, Mellick et al. 2003). Complementary DNA (cDNA) templates were generated from the anatomical localisations shown in Appendix B from MS affected as well as matched healthy controls.
6.2.2 qRT-PCR Gene Expression Assay of NAGM

Transcripts of interest were identified based on their functional relevance using Entrez Gene (NCBI). PCR Primers were designed based on mRNA sequence information and transcriptional target as shown in Appendix C. Intron-spanning primers were designed where possible (CLDN11, GDNF, and PTPRC) as an added measure to control for possible genomic DNA contamination. Lone transcripts of interest were isolated for CLDN11 and TLR3. Primers spanning multiple transcripts were designed for GDNF, NRG1, and PTPRC. Several normalisation genes were considered and tested for stability in brain tissues; the targets included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (β-Actin), and 18S. Efficiency and stability parameters were assessed using geNorm (Vandesompele, De Preter et al. 2002). The ubiquitous normalisation gene 18S was selected for use in all experiments. Real-time PCR using SYBR Green I (Bio-Rad) was optimised on a Corbett Life Science Rotorgene-6000 platform. Cycling and melt data were resolved with follow-up normal 2% agarose gel electrophoresis to confirm amplicon specificity for each separate primer set. Conditions were optimised for uniform amplification efficiency within genes (as shown in Appendix D), and final primer concentration (250nM), and optimal MgCl2 concentrations (2-4mM) for each gene. Each reaction started with 20ng of template with each run including cDNA, genomic DNA (gDNA), and no template control (NTC) in triplicate. Rotorgene-6000 software (Corbett Life Science) was
used to resolve cycling threshold (CT) values in the exponential phase of the optimised PCRs before statistical analysis.

### 6.2.3 Protein Isolation and Immunoblot in NAGM

Proteins were extracted from brain tissues using a Protein Extraction Reagent Kit (Pierce) including protease inhibitor cocktail (1:100). A protein precipitation using Chloroform/Methanol was carried out before quantification using BCA Protein Assay Kit (Pierce). Samples were quantified on a FLUOstar Optima plate reader as well as a NanoDrop 1000 (Shimadzu) Spectrophotometer. Equal amounts of protein were prepared followed by blotting onto standard nitrocellulose membrane (Sigma, USA). Membranes were blocked in 5% low-fat milk TBS-T for 1 hour at room temperature and then stained overnight with rabbit anti-human primary antibody (Abcam) and B-Actin was used as ubiquitous control. Following three 10 minute washes with TBS-T the membranes were incubated with 1:1000 horseradish peroxidase conjugated secondary antibody (1:50,000) for 1 hour (Sigma). Luminol chemiluminescent reagent (50:50) (Santa Cruz Biotechnology, USA) was used to develop the membranes before photography with a BioRad VersaDock system. Positivity of expression was confirmed by presence of a signal upon photography.
6.2.4 Statistical Analysis in NAGM

The ΔΔCT method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen 2001) was used for relative quantitative real-time analysis. Triplicate averages were used corrected to the housekeeping gene (18S) expression for each sample with standard deviation (SD) less than 0.20 in each set. Shapiro-Wilk and Kolmogorov-Smirnov tests were implemented for testing data distribution. Levene’s test for homogeneity of variance followed by independent samples T-Test was used to determine mean difference between cases and controls. Fold regulation was analysed and compared between affected (case) and unaffected (control) samples.

6.3 Results in NAGM

The aim of the study was to determine gene expression profiles for the 5 tested genes in normal appearing grey matter of secondary-progressive multiple sclerosis affected brains by relative quantitative real-time PCR followed by immunoblotting, and to assess whether these expression patterns differ from that of matched healthy controls.

Detailed neuropathology reports were obtained and cDNA templates representative of frontal and temporal tissues were generated as described in methods. Real-time PCR cycling and melt data were collected and CT values
determined. PCR conditions were optimised to give consistent amplification efficiencies as identified by identical amplification slopes. PCR product melts were checked to ensure amplification specificity.

Example real-time PCR results for the genes that were found to be significantly differentially expressed (GDNF, NRG1, PTPRC, and TLR3 cases versus controls) are shown below in: Figure 6-1 shows real-time PCR cycling results for the gene GDNF in NAGM control samples. Figure 6-2 shows real-time PCR melt curve analysis for the gene GDNF in NAGM control samples. Figure 6-3 shows real-time PCR cycling results for the gene GDNF in NAGM case samples. Figure 6-4 shows real-time PCR melt curve analysis for the gene GDNF in NAGM case samples. Figure 6-6 shows real-time PCR cycling results for the gene NRG1 in NAGM control samples. Figure 6-7 shows real-time PCR melt curve analysis for the gene NRG1 in NAGM control samples. Figure 6-8 shows real-time PCR cycling results for the gene NRG1 in NAGM case samples. Figure 6-9 shows real-time PCR melt curve analysis for the gene NRG1 in NAGM case samples. Figure 6-11 shows real-time PCR cycling results for the gene PTPRC in NAGM control samples. Figure 6-12 shows real-time PCR melt curve analysis for the gene PTPRC in NAGM control samples. Figure 6-13 shows real-time PCR cycling results for the gene PTPRC in NAGM case samples. Figure 6-14 shows real-time PCR melt curve analysis for the gene PTPRC in NAGM case samples. Figure 6-16 shows real-time PCR cycling results for the gene TLR3 in NAGM control samples. Figure 6-17 shows real-time PCR melt curve analysis for the
gene TLR3 in NAGM control samples. Figure 6-18 shows real-time PCR cycling results for the gene TLR3 in NAGM case samples. Figure 6-19 shows real-time PCR melt curve analysis for the gene TLR3 in NAGM case samples.

A selection of representative samples was also checked for expected amplicon size and specificity by normal 2% agarose gel electrophoresis: Figure 6-5 shows Agarose Gel Electrophoresis of GDNF cDNA Amplicons in NAGM, Figure 6-10 shows Agarose Gel Electrophoresis of NRG1 cDNA Amplicons in NAGM, Figure 6-15 shows Agarose Gel Electrophoresis of PTPRC cDNA Amplicons in NAGM, and Figure 6-20 shows Agarose Gel Electrophoresis of TLR3 cDNA Amplicons in NAGM. A summary of Normalised CT Values from qRT-PCR for GDNF, NRG1, PTPRC, and TLR3 in NAGM is shown in Table 6-1. Immunoblotting was undertaken purely as a qualitative procedure to ascertain whether the proteins of interest could be detected, positivity of expression was confirmed by signal upon photography.

Statistical analysis involved using the $2^{-\Delta\Delta CT}$ method averaging triplicate samples within runs to within 0.20 standard deviations for each gene set. Each case and control sample CT value was normalised to its internal control (18S) giving an accurate representation of quantitative expression. Cases and controls were then analysed taking into account regional localisation. Independent samples T-Test was carried out, first evaluating data distribution using Levene’s test for homogeneity. Table 6-2 shows Statistical Analysis Results for CLDN11 in NAGM.
Table 6-3 shows Statistical Analysis Results for GDNF in NAGM. Table 6-4 shows Statistical Analysis Results for NRG1 in NAGM. Table 6-5 shows Statistical Analysis Results for PTPRC in NAGM. Table 6-6 shows Statistical Analysis Results for TLR3 in NAGM.
Figure 6-1 GDNF Real-Time PCR Cycling in NAGM Controls

Figure 6-1 shows real-time PCR cycling results for the gene GDNF in NAGM control samples.

Figure 6-2 GDNF Real-Time PCR Melt in NAGM Controls

Figure 6-2 shows real-time PCR melt curve analysis for the gene GDNF in NAGM control samples.
Figure 6-3 GDNF Real-Time PCR Cycling in NAGM Cases

Figure 6-3 shows real-time PCR cycling results for the gene GDNF in NAGM case samples.

Figure 6-4 GDNF Real-Time PCR Melt in NAGM Cases

Figure 6-4 shows real-time PCR melt curve analysis for the gene GDNF in NAGM case samples.
Figure 6-5 shows agarose gel electrophoresis (AGE) of (A) GDNF cDNA amplicons by 2% agarose gel stained with Ethidium Bromide and (B) no template controls (NTCs) in normal appearing grey matter (NAGM) samples.
Figure 6-6 NRG1 Real-Time PCR Cycling in NAGM Controls

Figure 6-6 shows real-time PCR cycling results for the gene NRG1 in NAGM control samples.

Figure 6-7 NRG1 Real-Time PCR Melt in NAGM Controls

Figure 6-7 shows real-time PCR melt curve analysis for the gene NRG1 in NAGM control samples.
Figure 6-8 NRG1 Real-Time PCR Cycling in NAGM Cases

Figure 6-8 shows real-time PCR cycling results for the gene NRG1 in NAGM case samples.

Figure 6-9 NRG1 Real-Time PCR Melt in NAGM Cases

Figure 6-9 shows real-time PCR melt curve analysis for the gene NRG1 in NAGM case samples.
Figure 6-10 shows agarose gel electrophoresis (AGE) of (A) NRG1 cDNA amplicons by 2% agarose gel stained with Ethidium Bromide and (B) no template controls (NTCs) in normal appearing grey matter (NAGM) samples.
Figure 6-11 PTPRC Real-Time PCR Cycling in NAGM Controls

Figure 6-11 shows real-time PCR cycling results for the gene PTPRC in NAGM control samples.

Figure 6-12 PTPRC Real-Time PCR Melt in NAGM Controls

Figure 6-12 shows real-time PCR melt curve analysis for the gene PTPRC in NAGM control samples.
Figure 6-13 PTPRC Real-Time PCR Cycling in NAGM Cases

Figure 6-13 shows real-time PCR cycling results for the gene PTPRC in NAGM case samples.

Figure 6-14 PTPRC Real-Time PCR Melt in NAGM Cases

Figure 6-14 shows real-time PCR melt curve analysis for the gene PTPRC in NAGM case samples.
Figure 6-15 shows agarose gel electrophoresis (AGE) of (A) PTPRC cDNA amplicons by 2% agarose gel stained with Ethidium Bromide and (B) no template controls (NTCs) in normal appearing grey matter (NAGM) samples.
Figure 6-16 TLR3 Real-Time PCR Cycling in NAGM Controls

Figure 6-16 shows real-time PCR cycling results for the gene TLR3 in NAGM control samples.

Figure 6-17 TLR3 Real-Time PCR Melt in NAGM Controls

Figure 6-17 shows real-time PCR melt curve analysis for the gene TLR3 in NAGM control samples.
Figure 6-18 TLR3 Real-Time PCR Cycling in NAGM Cases

Figure 6-18 shows real-time PCR cycling results for the gene TLR3 in NAGM case samples.

Figure 6-19 TLR3 Real-Time PCR Melt in NAGM Cases

Figure 6-19 shows real-time PCR melt curve analysis for the gene TLR3 in NAGM case samples.
Figure 6-20 shows agarose gel electrophoresis (AGE) of (A) TLR3 cDNA amplicons by 2% agarose gel stained with Ethidium Bromide and (B) no template controls (NTCs) in normal appearing grey matter (NAGM) samples.
Table 6-1 shows the average normalised (samples corrected for their internal ubiquitous control) CT values for the significant genes GDNF, NRG1, PTPRC and TLR3 in NAGM.
Table 6-2 Statistical Analysis of CLDN11 in NAGM

<table>
<thead>
<tr>
<th></th>
<th>t</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
<th>Mean Difference</th>
<th>Std. Error Difference</th>
<th>95% Confidence Interval of the Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>overall</td>
<td>-.0013</td>
<td>11</td>
<td>.9990</td>
<td>-.0019</td>
<td>1.4376</td>
<td>-.31659 to 3.1621</td>
</tr>
<tr>
<td>frontal</td>
<td>-.3323</td>
<td>7</td>
<td>.7494</td>
<td>-.5240</td>
<td>1.5768</td>
<td>-4.2524 to 3.2044</td>
</tr>
<tr>
<td>temporal</td>
<td>.2578</td>
<td>2</td>
<td>.8221</td>
<td>.8250</td>
<td>3.2001</td>
<td>-13.9130 to 15.5630</td>
</tr>
</tbody>
</table>

Table 6-2 shows statistical analysis results for the gene expression of CLDN11 in NAGM. All P-values exceeded 0.05 (t-test for Equality of Means Sig. (2-tailed) column) therefore no statistically significant results were observed for this gene in NAGM.
Table 6-3 shows statistical analysis results for the gene expression of GDNF in NAGM. P-values (t-test for Equality of Means Sig. (2-tailed) column) for overall (0.0002) and frontal expression (0.0009) were statistically significant for this gene in NAGM.
Table 6-4 shows statistical analysis results for the gene expression of NRG1 in NAGM. P-values (t-test for Equality of Means Sig. (2-tailed) column) for overall expression (0.0215) were statistically significant for this gene in NAGM.
Table 6-5 Statistical Analysis of PTPRC in NAGM

### Independent Samples Test

<table>
<thead>
<tr>
<th></th>
<th>t</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
<th>Mean Difference</th>
<th>Std. Error Difference</th>
<th>95% Confidence Interval of the Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>overall</td>
<td>4.3760</td>
<td>11</td>
<td>.0011</td>
<td>4.9612</td>
<td>1.1337</td>
<td>2.4659 - 7.4565</td>
</tr>
<tr>
<td>frontal</td>
<td>8.6951</td>
<td>7</td>
<td>5.34E-05</td>
<td>4.4980</td>
<td>.5173</td>
<td>3.2748 - 5.7212</td>
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<tr>
<td>temporal</td>
<td>1.7086</td>
<td>1</td>
<td>.3335</td>
<td>6.3100</td>
<td>3.6931</td>
<td>-38.5233 - 51.1433</td>
</tr>
</tbody>
</table>

Table 6-5 shows statistical analysis results for the gene expression of PTPRC in NAGM. P-values (t-test for Equality of Means Sig. (2-tailed) column) for overall (0.0011) and frontal expression (5.34E10^-5) were statistically significant for this gene in NAGM.
Table 6-6 shows statistical analysis results for the gene expression of TLR3 in NAGM. P-values (t-test for Equality of Means Sig. (2-tailed) column) for the overall analysis (0.0004) were statistically significant for this gene in NAGM.
Relative gene expression differences expressed in terms of fold-change are summarised in Table 6-7 while individual average cycling threshold (CT) values and their standard deviations are shown in Figures 6-21 (GDNF), Figure 6-22 (NRG1), Figure 6-23 (PTPRC), and Figure 6-24 (TLR3). Fold-changes for the statistically significant results (where P<0.05) are shown in Figure 6-25. Figure 6-26 shows non-statistically significant (P>0.05) relative gene expression differences. Independent Samples T-test analysis results are shown in Table 6-7.

Statistically significant increased expression was observed for GDNF in samples from overall (↑55.82-fold, P=0.0002) and frontal (↑54.55, P=0.0009), NRG1 overall (↑142.19, P=0.022), PTPRC overall (↑31.13, P=0.0011) and frontal (↑22.60, P=5.34X10^-5) and TLR3 overall (↑101.29, P=0.0004) tissues compared to control samples. We detected no statistically significant gene expression differences for CLDN11 overall (P=0.999), frontal (P=0.749), or temporal (P=0.822), NRG1 frontal (P=0.105), PTPRC temporal (P=0.334), or TLR3 frontal (P=0.051) tissues as also summarised in Table 6-7 (all P>0.05).
Table 6-7 Relative Gene Expression Differences between Cases & Controls in NAGM

<table>
<thead>
<tr>
<th>Gene</th>
<th>Analysis</th>
<th>Fold-Change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLDN11</td>
<td>Overall</td>
<td>↓1.00</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>Frontal</td>
<td>↓1.44</td>
<td>0.749</td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>↑1.77</td>
<td>0.822</td>
</tr>
<tr>
<td>GDNF</td>
<td>Overall</td>
<td>↑155.82</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>Frontal</td>
<td>↑54.55</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>NRG1</td>
<td>Overall</td>
<td>↑142.19</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>Frontal</td>
<td>↑19.75</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>PTPRC</td>
<td>Overall</td>
<td>↑31.15</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>Frontal</td>
<td>↑22.60</td>
<td>5.34X10^-5</td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>↑79.34</td>
<td>0.334</td>
</tr>
<tr>
<td>TLR3</td>
<td>Overall</td>
<td>↑101.29</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>Frontal</td>
<td>↑26.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temporal</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

1 Normal Appearing Grey Matter
2 Independent Samples T-Test
3 Statistically Significant Result
4 Sample Size Inadequate

Table 6-7 shows a summary of the relative gene expression differences between cases and controls in normal appearing grey matter (NAGM) samples. Statistically significant findings include expression of GDNF in overall (0.0002) and frontal (0.0009) samples, NRG1 overall (0.022) samples, PTPRC overall (0.0011) and frontal (5.34X10^-5) samples, and TLR3 in overall (0.0004) samples. The other tested genes were not found to be significantly different (all P-values >0.05).
Figure 6-21 GDNF Expression Differences between Cases & Controls in NAGM

Figure 6-21 shows significant relative gene expression differences expressed as average CT values between cases and controls for the gene glial cell derived neurotrophic factor (GDNF) in normal appearing grey matter (NAGM). GDNF average overall CTs were 11.33 (cases) versus 18.62 (controls) and GDNF average frontal CTs were 12.62 (cases) versus 18.39 (controls).
Figure 6-22 NRG1 Expression Differences between Cases & Controls in NAGM

Figure 6-22 shows significant relative gene expression differences expressed as average CT values between cases and controls for the gene neuregulin-1 (NRG1) in normal appearing grey matter (NAGM). NRG1 average overall CTs were 12.74 (cases) versus 19.89 (controls).
Figure 6-23 shows significant relative gene expression differences expressed as average CT values between cases and controls for the gene protein tyrosine phosphatase receptor type C (PTPRC) in normal appearing grey matter (NAGM). PTPRC average overall CTs were 18.37 (cases) versus 23.33 (controls) and PTPRC average frontal CTs were 19.21 (cases) versus 23.71 (controls).
Figure 6-24 shows significant relative gene expression differences expressed as average CT values between cases and controls for the gene toll-like receptor 3 (TLR3) in normal appearing grey matter (NAGM). TLR3 average overall CTs were 18.00 (cases) versus 24.66 (controls).
Figure 6-25 Statistically Significant Differences between Cases & Controls in NAGM

Figure 6-25 shows statistically significant (P<0.05) relative gene expression differences between cases and controls in normal appearing grey matter (NAGM). GDNF and PTPRC gene expression was increased in both the overall and frontal analyses, while NRG1 and TLR3 were increased in the frontal analyses only.
Figure 6-26 Non-Significant Differences between Cases & Controls in NAGM

Figure 6-26 shows non-statistically significant (P>0.05) relative gene expression differences between cases and controls in normal appearing grey matter (NAGM). Although gene expression differences were seen between cases and controls in these samples those results did not reach statistical significance (all P>0.05).
6.4 Discussion of NAGM Gene Expression

MS is a prominent member of a spectrum of CNS idiopathic inflammatory demyelinating disorders that share the basic pathological hallmark of CNS inflammatory demyelination (Lucchinetti 2008). Pathological observations from large series of MS cases report heterogeneity in relation to lesion formation (Lucchinetti, Bruck et al. 2004). Experimental neuroimmunology has previously reported the importance of genetic susceptibility to induction of inflammatory demyelinating lesions (Linker, Maurer et al. 2002). Gene expression profiling of diseased neuronal populations may reveal mechanistic clues as to the molecular pathogenesis underlying neurological diseases (Mufson, Counts et al. 2006). Previous gene expression studies have investigated changes in plaque tissues mainly by array-based technologies (Carmody, Hilliard et al. 2002; Tajouri, Mellick et al. 2003). We set out to determine whether gene expression patterns of the genes CLDN11, GDNF, NRG1, PTPRC, and TLR3, which are candidate genes involved in neuroinflammation, immune response, and myelination, are any different in the normal appearing grey matter of secondary-progressive multiple sclerosis brains (n=7) compared to healthy matched neuropathology free controls.
The significant findings of this study include increased expression of GDNF in samples from overall (↑55.82-fold, \(P=0.0002\)) and frontal (↑54.55, \(P=0.0009\)), NRG1 overall (↑142.19, \(P=0.022\)), PTPRC overall (↑31.13, \(P=0.0011\)) and frontal (↑22.60, \(P=5.34\times10^{-5}\)), and TLR3 overall (↑101.29, \(P=0.0004\)) tissues compared to control samples. Studies report glial derived neurotrophic factors to be potential therapeutic targets for neurodegenerative diseases (Mitsuma, Yamamoto et al. 1999). Increased expression of NRG1 in chronic affected multiple sclerosis brains may represent attempted remyelination (Bieber 2008). Increased PTPRC may be associated with regulatory defects that facilitate pathological cascades allowing autoreactive lymphocytes to initiate immune responses in the brain (Compston and Coles 2008). Regulatory lymphocytes within MS patients have previously been shown to fail to suppress effector cells (Viglietta, Baecher-Allan et al. 2004).

T-cell regulation and T-regulatory (T-reg) cell balance is now thought to be a key aspect of autoimmune-mediated inflammatory disease (Dittel 2008; O'Connor and Anderton 2008). TLR3 has been studied immunohistochemically in the adult brain and has been shown to be highly expressed and regulated in both early and late-active lesions but not healthy controls (Bsibsi, Ravid et al. 2002; Bsibsi, Persoon-Deen et al. 2006). PTPRC (temporal=↑79.34, \(P=0.334\)), as well as NRG1 (frontal=↑19.75, \(P=0.105\)) and TLR3 (frontal=↑26.26, \(P=0.051\)), and CLDN11 (overall=↓1.00, \(P=0.999\), frontal=↓1.44, \(P=0.749\), temporal=↑1.77,
P=0.822) were different from the tested controls but were not statistically significant (all P>0.05).

Tissue specific gene expression patterns quantified by sensitive methods could provide us with valuable insights into the involvement of genes in complex neuropathologies such as multiple sclerosis. Profiling larger groups of well-classified samples in the future may provide clues that could aid in explaining the heterogeneity observed in neurological disorders. Changes in the expression of genes in normal appearing grey matter in MS continue to be of interest and may represent systemic changes associated with the disorder and also provide new insights into the processes underpinning development of the disease. Furthermore, investigations to map the expression of genes thought to be involved in pathological mechanisms in the MS brain versus healthy controls, may provide novel findings that could help explain some of the molecular mechanisms behind its aetiology.
Chapter 7

Genetic Variation Studies in MTHFR & MTRR

Genomic investigation for association between MTHFR A1298C and MTRR A66G polymorphisms, and multiple sclerosis in an Australian cohort.
Multiple Sclerosis (MS) is a complex neurological disease that affects the central nervous system (CNS) resulting in debilitating neuropathology. Pathogenesis is primarily defined by CNS inflammation and demyelination of nerve axons. The precise aetiology of MS remains elusive with a complex interplay between environmental factors, genetic susceptibility, and age-dependant exposure to viral infection postulated (Levin, Munger et al. 2005). A significant genetic component has been demonstrated by studies examining familial concordance rates with risk being up to 300-fold increased in monozygotic twins and 20-40-fold increased for first-degree relatives as compared to a general population prevalence of 0.1% (Ebers, Sadovnick et al. 1995).

Methionine synthase reductase (MTRR) is an enzyme that catalyzes the remethylation of homocysteine (Hcy) to methionine via a cobalamin and folate dependant reaction. Hcy is an intermediate in the folate, vitamin B12, and B6 dependent pathways of one-carbon and sulphur amino acid metabolism (Feix, Winkelmayer et al. 2004). Cobalamin acts as an intermediate methyl carrier between methylenetetrahydrofolate reductase (MTHFR) and Hcy (Gaughan, Kluijtmans et al. 2001). The cobalamin cofactor cycles between cob(I)alamin and methylcob(III)alamin. Cob(I)alamin is a strong reductant and over time becomes oxidized to produce an inactive cob(II)alamin form of methionine synthase (Leclerc, Wilson et al. 1998). MTRR plays a critical role in maintaining cobalamin
in an active form and is consequently an important determinant of total plasma Hcy (pHcy) concentration. Elevated intracellular pHcy has been implicated in CNS dysfunction, neurodegenerative, and cerebrovascular disease. Dysfunction is thought to result from vasotoxic and neurotoxic effects of elevated Hcy, inhibition of the methylation of myelin basic protein (MBP) and membrane phospholipids, and disruption of biogenic amine metabolism (Troen 2005).

The A1298C polymorphism in the MTHFR gene has been associated with hyperhomocysteinemia (Rozen 1997). The A1298C allele is characterised by a point mutation at position 1298 of the MTHFR gene (located 1p36.3) causing the replacement of glutamine by alanine in the corresponding enzyme (van der Put, Gabreels et al. 1998). The A66G polymorphism in the gene MTRR (located 5p15.3) alters an isoleucine into a methionine residue. The 66G variant may constitute a functional mutation associated with a reduced reductive repair of methionine synthase due to the crucial localisation of methionine synthase reductase in the enzyme (Olteanu, Munson et al. 2002). We present an investigation of the possible association of the MTHFR A1298C and MTRR A66G polymorphisms with MS in a case-control study of an Australian cohort.
7.2 Materials and Methods

7.2.1 Study Population

Griffith University’s Ethics Committee approved research involving human experimentation (Approval HSC/09/03/HREC). Informed consent was obtained from all participants. The case-control populations consisted of 140 MS patients and 140 healthy controls, which had been matched for age, gender, and ethnicity (Caucasian). The MS population was initially obtained from the Multiple Sclerosis Clinic at the Royal Brisbane Hospital, Brisbane, Queensland, Australia; all samples were procured from patients residing in the South East Queensland region. The populations consisted of 75% females and 25% males, and were subcategorised based on clinical course: Relapsing-Remitting (RR), Secondary-Progressive (SP), and Primary-Progressive (PP). Frequencies were: RR=40%, SP=36% and PP=24%. The matched control (age ±5 years, gender, and ethnicity) population samples were obtained via the Genomics Research Centre Clinic, Southport, Gold Coast, Queensland, Australia. Genomic DNA was extracted from peripheral blood using a standard salting-out procedure.
7.2.2 Genotyping MTHFR A1298C and MTRR A66G

Genomic DNA was used as a template to generate polymerase chain reaction (PCR) products for subsequent restriction enzyme digest and genotyping. Kara et al and Feix et al have previously described PCR primers for MTHFR and MTRR, respectively and adaptations of their genotyping methods were used for this study (Kara, Sazci et al. 2003; Feix, Winkelmayer et al. 2004). Primers for MTHFR were: forward 5'- CTT TGG GGA GCT GAA GGA CTA CTA C-3', reverse 5'-CAC TTT GTG ACC ATT CCG GTT TG-3'. Primers for MTRR were: forward 5'- GCA AAG GCC ATC GCA GAA GAC AT-3', reverse 5'- AAA CGG TAA TTA AAT CCA CTG TAA CGC C-3'. PCR primers were obtained from Proligo Primers and Probes (Sigma-Aldrich). Assay conditions were optimised with thermal cycling conditions for MTHFR consisting of 95°C for 3 minutes, then 40 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, with a final extension of 72°C for 7 minutes, and MTRR consisting of 95°C for 2 minutes, then 40 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, with a final extension of 72°C for 2 minutes. MTHFR and MTRR PCR products were resolved on a 2%, and 5% high range agarose gel (Progen Biosciences) yielding 163bp and 118bp amplicons, respectively. Restriction enzyme digest of the MTHFR product was with MboII (Fermentas Life Science), and the MTRR product with NspI (New England Biolabs).
MTHFR A1298A produced five fragments (56, 31, 30, 28, and 18bp), and A1298C produced six fragments (84, 56, 31, 30, 28, and 18bp), and digest products were resolved using an 8% resolution plus agarose gel (Progen Biosciences). The MTRR sense-primer introduced a mismatch at the 3rd nucleotide (from the 3’ end) creating a restriction site for the Nspl enzyme whenever the G allele was present. In the presence of the A allele the 118bp product remained uncleaved, in the presence of the G allele two fragments were produced: 24 and 94bp. MTRR digest products were resolved using a 5% resolution plus agarose gel (Progen Biosciences).

7.2.3 Statistical Analysis of MTHFR & MTRR Variations

Descriptive statistics were explored using the SPSS Standard Package (Version 11.0 for Mac OS X). Genotype and allele frequencies were investigated using standard Chi-square ($X^2$) analysis using Microsoft Excel. Genotype distributions were checked for Hardy-Weinberg equilibrium (HWE).

7.2.4 Exclusion Criteria for Statistical Analysis

Of the cohort of 140 cases/controls, 10 cases and 9 matched controls (MTHFR) and 17 cases and 16 matched controls (MTRR) were excluded due to genotyping
difficulties, leaving 130 cases and 131 matched controls (MTHFR) and 123 cases and 124 matched controls (MTRR) for final analysis.

7.3 Results for MTHFR & MTRR Variations

Genotypes were determined after PCR amplification and restriction enzyme cutting prior to standard agarose gel electrophoresis (see Figures 7-1 and 7-2 below). Results for genotype and allele frequency are shown in Tables 7-1 and 7-2 below. Genotype frequencies for both the case and control groups, for both markers conformed to Hardy-Weinberg equilibrium expectations: (MTHFR A1298C Cases HWE P=0.34, Controls HWE P=0.07; MTRR A66G Cases HWE P=0.86, Controls HWE P=0.20). The observed control frequencies for this study also conformed well to previously published frequencies (Kara, Sazci et al. 2003; Feix, Winkelmayer et al. 2004). There was no statistically significant difference between case and control allele frequencies for either the MTHFR ($X^2=1.15$, $P=0.28$, $\alpha=0.05$); or MTRR ($X^2=0.005$, $P=0.95$, $\alpha=0.05$); tested variants. For MTHFR, the A allele was more frequently observed than the C allele with very similar count in the cases and controls (A=0.67 and 0.62). Similarly, for the MTRR variant, the A allele was more frequent than the G allele (A=0.64 and 0.63) but similar in both cases and controls.
Figure 7-1 MTHFR A1298C MboII Restriction Enzyme Digest Products by AGE

Figure 7-1 shows MTHFR A1298C MboII restriction enzyme digest products. (A) Undigested PCR product (163bp), (B) A1298C (84, 56, 31bp), (C) A1298A (56, 31bp), and (D) no template control (NTC) resolved by 8% resolution plus agarose gel.

<table>
<thead>
<tr>
<th>Genotype Frequencies</th>
<th>Control</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>63(48%)</td>
<td>53(41%)</td>
</tr>
<tr>
<td>AC</td>
<td>49(37%)</td>
<td>56(43%)</td>
</tr>
<tr>
<td>CC</td>
<td>19(15%)</td>
<td>21(16%)</td>
</tr>
<tr>
<td>N</td>
<td>131</td>
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<table>
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<tr>
<td>C</td>
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<table>
<thead>
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<th>Genotype Frequencies</th>
<th>Control</th>
<th>MS</th>
</tr>
</thead>
<tbody>
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<td>AC</td>
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<td>CC</td>
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<td>21(16%)</td>
</tr>
<tr>
<td>N</td>
<td>131</td>
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<table>
<thead>
<tr>
<th>Allele Frequencies</th>
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</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>162(62%)</td>
</tr>
<tr>
<td>C</td>
<td>98(38%)</td>
</tr>
<tr>
<td>N</td>
<td>260</td>
</tr>
</tbody>
</table>

\[X^2=1.42, \ P=0.49\] \[X^2=1.15, \ P=0.28\]

Table 7-1 shows allele and genotype frequencies for MTHFR A1298C genotyping. No significant differences were detected between cases and controls (all P>0.05).
Figure 7-2 MTRR A66G NspI Restriction Enzyme Digest Products by AGE

Figure 7-2 shows MTRR A66G NspI restriction enzyme digest products. (A) Represents A66A (118bp), (B) A66G (118, 94bp), (C) G66G (94bp fragment), and (D) not template control (NTC) resolved by 5% resolution plus agarose gel.

<table>
<thead>
<tr>
<th></th>
<th>Genotype Frequencies</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
</tr>
<tr>
<td>Control</td>
<td>47(38%)</td>
<td>64(52%)</td>
</tr>
<tr>
<td>MS</td>
<td>49(40%)</td>
<td>58(47%)</td>
</tr>
</tbody>
</table>

$\chi^2=0.64$, $P=0.73$  
$\chi^2=0.005$, $P=0.95$

Table 7-2 shows allele and genotype frequencies for MTRR A66G genotyping. No significant differences were detected between cases and controls (all $P>0.05$).
7.4 Discussion of MTHFR & MTRR Genetic Variation Studies

MS remains a paradigm of complex disease demonstrating variable clinical expression with an unpredictable course. Disease susceptibility is partly genetically determined; however the underlying molecular mechanisms of MS pathogenesis remain unidentified (Keegan and Noseworthy 2002). In vitro studies have demonstrated the ability of Hcy to induce neurotoxicity through over-stimulation of N-methyl-D-aspartate (NMDA) receptors resulting in neuronal damage due to excessive Ca$^{2+}$ influx and induction of reactive oxygen species (Lipton, Kim et al. 1997). MTHFR deficiency is the most common genetic cause of hyperhomocysteinemia (Chen, Ge et al. 2002). Homocysteine may also sensitize neurons to oxidative stress via oxidation of sulfhydryl groups subsequently resulting in generation of reactive oxygen species such as superoxide and hydrogen peroxide (Kruman, Culmsee et al. 2000). The A1298C mutation in MTHFR has previously been associated with hyperhomocysteinemia (Rozen 1997). MTRR plays a crucial role in homocysteine metabolism and is necessary to maintain the function of methionine synthase (Fodinger, Buchmayer et al. 1999). It has previously been hypothesised that the MTRR A66G variant may constitute a functional mutation associated with a reduced reductive repair of methionine synthase due to the crucial localisation of methionine synthase reductase in the enzyme (Olteanu, Munson et al. 2002).
We investigated the MTHFR A1298C and the MTRR A66G variants for association with MS in an Australian case-control cohort. The findings of this study suggest no association with the two tested markers and MS in our population. No statistically significant difference was observed for genotype or allele frequencies for either marker between our cases and controls. Despite these findings, it is possible that other variants in MTHFR and MTRR may still be associated with MS or in fact other neurodegenerative disorders.

The MTHFR variant C66T has previously been reported to be positively associated with schizophrenia in some populations (Muntjewerff, Kahn et al. 2006; Muntjewerff, Gellekink et al. 2008). An American study further supports the notion that a folate-sensitive defect in homocysteine metabolism contributes to cases of that disease (Susser, Brown et al. 1998). An Irish study of 601 participants has reported a significant association between elevated pHcy levels and another variant of MTRR, G66G (Gaughan, Kluijtmans et al. 2001). In another study, Lindenbaum and colleagues have suggested that measurement of pHcy levels before and after treatment can be valuable for confirmation of a differential diagnosis of neuropsychiatric disorder in patients presenting with paresthesia, sensory loss, ataxia, and dementia (Lindenbaum, Healton et al. 1988; Lindenbaum, Healton et al. 1995). A recent study by Ramsaransing et al. report finding elevated pHcy levels in MS patients and concluded that although the finding is unlikely to be related to immune activation, oxidative stress, or deficiencies in vitamin B6, B12, or folate, it could be of some as yet uncertain
importance (Ramsaransing, Fokkema et al. 2006). Other investigations by Baig, Vrethem, and Besler and colleagues also support the finding of elevated homocysteine levels in MS patients compared with healthy controls (Baig and ALI Qureshi 1995; Besler and Comoglu 2003; Vrethem, Mattsson et al. 2003).

It is hypothesised that the nervous system may be particularly sensitive to pHcy as it promotes excitotoxicity by stimulation of NMDA receptors resulting in damage to neuronal DNA, hence triggering apoptosis in neuronal cells (Kruman, Culmsee et al. 2000; Ho, Ortiz et al. 2002). Ergo, although our findings suggest no association between MTHFR A1298C and MTRR A66G and MS in the tested population, other variants associated with the homocysteine metabolism cascade may still be contributing to MS susceptibility.
Chapter 8

Genetic Variation Studies in PTPRC

An investigation of the C77G and C772T variations within the human protein tyrosine phosphatase receptor type C gene for association with multiple sclerosis in an Australian population.
8.1 Introduction to Variation Studies in PTPRC

Multiple sclerosis (MS) is an inflammatory T-cell mediated autoimmune disease that results in numerous neurological deficits (Weiner 2004). It is an inflammatory demyelinating disorder that demonstrates notable clinical heterogeneity, proceeding in relapsing-remitting or progressive fashion (Hauser 2005). Structural studies have revealed cellular mechanisms such as antibody mediated oligodendroglial apoptosis and myelin targeted necrosis in affected tissues (Genain, Cannella et al. 1999). A widely accepted notion in MS is that a dysregulated immune response against brain resident antigens is central to its complex aetiology (Jacobsen, Schweer et al. 2000).

The protein tyrosine phosphatase receptor type C (PTPRC) is an important member of the protein tyrosine phosphatase (PTP) family known to regulate cellular signalling as well as growth, differentiation, and transformation of cells. The PTPRC protein consists of a transmembrane domain with two tandem intracytoplasmic catalytic domains, which ultimately facilitate cell signal transduction. The gene encoding PTPRC (located 1q31-32) is expressed as an essential regulator of T- and B-cell antigen receptor signalling (Trowbridge and Thomas 1994). Furthermore, the PTPRC receptor (CD45; also known as the leukocyte common antigen; LCA) is known to regulate integrin-mediated adhesion and migration of cells of the immune system (Shenoi, Seavitt et al. 1999). CD45 plays a key role in cytokine receptor signalling through suppression of Src family
kinases, and variations within the PTPRC gene have generated much interest in recent times (Jacobsen, Schweer et al. 2000; Barcellos, Caillier et al. 2001). The PTPRC C77G variant has been previously reported to be involved in increasing susceptibility to MS in German patients. The nucleotide transition in exon 4 of the gene locus interferes with mRNA splicing and results in altered expression of CD45 isoforms on immune cells (Jacobsen, Schweer et al. 2000). Susceptibility to HIV infection in patients with this variant has been reported in the UK. In humans, naive T-cells express high molecular weight CD45 isoforms that are recognised by CD45RA monoclonal antibodies, activation of those cells results in expression of low molecular weight isoforms. In affected individuals, activated or memory lymphocytes continue to express both high and low molecular weight isoforms in contrast to the normal pattern of low molecular weight isoform expression (Tchilian, Wallace et al. 2001). Furthermore, a homozygous 6-bp deletion in PTPRC, which results in a loss of glutamic acid 339 and tyrosine 340 in the first fibronectin type III module of the extracellular domain of CD45, has been shown to be associated with failure of surface expression of CD45 resulting in severe combined immunodeficiency (SCID) (Tchilian, Wallace et al. 2001). The C772T variant could also be involved in disease susceptibility but has not been previously tested in MS or any other related neurological disease and thus constitutes a novel candidate gene.
The demyelination and failed remyelination of axons receives much attention in MS research and continues to be the focus of numerous investigations (Salzer 2008; Taveggia, Thaker et al. 2008). Recent findings confirm the importance of CD45 as a Fyn tyrosine kinase regulator of oligodendrocyte precursor cell (OPC) maturation from neural stem cells, which in turn induces myelin generation by mature oligodendrocytes (Nakahara, Seiwa et al. 2005). To date, four distinct isoforms of the PTPRC gene are known to be expressed in humans, and variations within the gene have previously been reported to be associated with altered immunity (Jacobsen, Hoffmann et al. 2002; Boxall, Stanton et al. 2004). CD45 remains a valid and relevant candidate in MS studies with investigations of variations within the gene in different populations (Vorechovsky, Kralovicova et al. 2001; Vyshkina, Leist et al. 2004).

In this study we set out to investigate the C77G single-nucleotide polymorphism (rs17612648) of the PTPRC gene by high-resolution melt analysis and a restriction length polymorphism (RFLP) C772T (rs7540378) of the same gene, to determine if allele frequencies differ between MS cases and matched healthy controls in an Australian Caucasian population.
8.2 Experimental Procedure

8.2.1 Subjects

Griffith University’s Ethics Committee approved research involving human experimentation (Approval HSC/09/03/HREC). Informed consent was obtained from all participants. The case-control populations consisted of 205 cases and 205 matched healthy controls however, the total number of successfully genotyped cases and controls were 155 cases and 171 matched healthy controls for the C77G marker and 181 cases and 180 healthy matched controls for the C772T marker. The remaining samples proved difficult to amplify efficiently or were not able to be definitively categorised during analysis. Healthy controls were matched for age (±5 years), gender, and ethnicity (Caucasian). The MS population was recruited from the Multiple Sclerosis Clinic at the Royal Brisbane & Women’s Hospital, Brisbane, Queensland, Australia; all samples were procured from patients residing in the South East Queensland region. The population consisted of approximately 75% females and 25% males, and was subcategorised based on clinical course: Relapsing-Remitting (RR), Secondary-Progressive (SP), and Primary-Progressive (PP). Frequencies were: RR=40%, SP=35% and PP=25%. The matched control samples were obtained via the Genomics Research Centre Clinic, Southport, Gold Coast, Australia. The control group was comprised of matched controls certified free of all neurological and immunological disease history. All participants completed a questionnaire which
included questions regarding place of residence up until 15 years of age, details of grandparents, sibling information, and other clinically relevant information. Genomic DNA was extracted from peripheral blood using a standard salting-out procedure. Genomic DNA was used as a template to generate polymerase chain reaction (PCR) products for both high-resolution melt (HRM) genotyping (C77G marker) and restriction enzyme digestion (C772T marker) of the PTPRC gene.

**8.2.2 PTPRC C77G Genotyping by HRM**

HRM PCR primers were designed ensuring specificity for PTPRC C77G: forward 5'-GAT GCC CAG TGT TCC ACT TT -3', and reverse: 5'- GGG GAT ACT TGG GTG GAA GT -3' (Proligo, Australia), isolating a 144bp region with no intervening variations. 10ng of starting template DNA was amplified in duplicate reactions using 1.5mM MgCl$_2$, 0.2mM dNTPs, 300nM PCR primers, 1.5μM SYTO-9, and 1.25U Platinum Taq DNA Polymerase (Invitrogen, Australia) per reaction, as well as no template controls (NTCs). Thermal cycling consisted of: 95°C for 3 minutes, then 95°C for 5 seconds and 57°C for 10 seconds; repeated 45 cycles, with a final melt phase of 80-90°C rising 0.1°C each step. Data were resolved and analysed on a Rotor-Gene 6000 Platform (Corbett Life Science, Australia). Genotypes were determined by interpreting shifts in the amplification profiles and product melts of the samples using the Rotor-Gene software.
8.2.3 PTPRC C772T Genotyping by RFLP

Genomic PCR primers were designed to amplify the C772T variant region producing a 1,037bp amplicon containing the single-nucleotide polymorphism of interest: forward 5’- AAA GCC TCT CTG TCC CCC TA -3’, and reverse 5’- CTC CAG GCA ACC ACT GAT CT -3’ (Proligo, Australia). Thermal conditions were empirically determined: 95ºC for 3 minutes, then 95ºC for 30 seconds, 57ºC for 30 seconds, 72ºC for 1 minute; repeated 40 cycles, with a final extension of 72ºC for 3 minutes. A specific restriction endonuclease was selected with the aid of NEBcutter V2.0 (Vincze, Posfai et al. 2003). Restriction enzyme digest conditions were optimised for each reaction: 0.5U RsaI, 1XNEBuffer, Incubation at 37ºC for 2-4 hours. Amplified PCR products were resolved for both C77G (144bp) and C772T (1,037bp) post-amplification using 2% w/v standard agarose gel electrophoresis (Progen Biosciences). Restriction enzyme digest products for C772T were further resolved on 1% w/v standard agarose gel. Conditions were optimised to ensure specific cutting of the variant genotype by RsaI (New England Biolabs) producing two fragments of 364 & 673bp, before analysis.
8.2.4 Statistical Analysis of PTPRC Variations

Data was collected and descriptive statistics interpreted using SPSS standard package (SPSS, Inc., Chicago, IL). Analysis entailed the use of a combination of the SPSS package, Microsoft Excel, and the Quantitative Skills Tables package (Uitenbroek 1997).

Genotype and allele frequencies were collated and Hardy-Weinberg Equilibrium (HWE) assessed (by $X^2$ goodness-of-fit) to check for genotyping accuracy. Linkage disequilibrium (LD) analysis was carried out using the methodology of Zhao and colleagues, using the 2LD computer program (Zhao 2004). The G-Power statistical package was used to extrapolate study power a-priori; it was determined that the current statistical model would return 90% power at $\omega=0.25$ (small to medium effect size), at $\alpha=0.05$ (see Table and Figure 8-1 below) (Faul, Erdfelder et al. 2007). Assumption free Chi-square ($X^2$) goodness-of-fit tests were carried out to determine if significant differences exist in allele frequencies between cases and controls ($\alpha=0.05$). Analysis of male cases versus male controls and female cases versus female controls was also carried out to test for significant differences by gender.
Table 8-1  *A-priori* Statistical Power Calculations

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<th>A priori: Compute required sample size</th>
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<td></td>
<td>Effect size w</td>
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<tr>
<td></td>
<td>α err prob</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Power (1−β err prob)</td>
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<tr>
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<td>Output</td>
<td>Noncentrality parameter λ</td>
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<td></td>
<td></td>
<td>Critical $\chi^2$</td>
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<tr>
<td></td>
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<td></td>
<td>Actual power</td>
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</tbody>
</table>

Table 8-1 shows statistical power calculation (*a-priori*) summary for the current study design. The table summarises that the current statistical model would return 90% power at $w=0.25$ (small to medium effect size), at $\alpha=0.05$

![Figure 8-1 A-priori Statistical Power Calculation Power Curve](image)

Figure 8-1 shows the sample size necessary (y-axis) to reach given statistical powers (x-axis) for the current study design as calculated using the G-Power package.
8.3 Results for PTPRC Variations

Real-time PCR cycling and melt results were collated for C77G. Figure 8-2 below shows real-time PCR amplification cycling prior to melting. Figure 8-3 shows the melt identities of G77G homozygotes, C77C homozygotes, and C77G heterozygotes. Figure 8-4 shows an example of the PTPRC C77G HRM discrimination plot used for genotyping the marker (G77G, C77C, and C77G heterozygote melts curves). Figure 8-5 shows real-time PCR amplicons of PTPRC C77G (144bp), and no-template controls (NTCs) as resolved by 2% w/v standard agarose gel electrophoresis. Genotype and Allele Frequencies for PTPRC C77G are shown in Table 8-2. For the C772T marker Figure 8-6 shows the undigested PCR amplicons (1,037bp) and no-template controls (NTCs) as resolved by 2% w/v standard agarose gel electrophoresis after PCR. Following PCR, restriction enzyme digest was undertaken and Figure 8-7 shows PTPRC C772T Rsal restriction enzyme digest products by 1% w/v agarose gel electrophoresis. A summary of allele and genotype frequencies for PTPRC C772T are shown in Table 8-3.

Hardy Weinberg equilibrium (HWE) held for C772T (Cases HWE P=0.27, Controls HWE P=0.10) but not for C77G (HWE P<0.05) which is most likely the result of the low number of observations for one of the G allele for this marker. Subsequently, we undertook Linkage disequilibrium analysis between C77G and C772T as outlined in the methods section. Figure 8-8 shows the haplotype
frequency estimation (which was first necessary to calculate linkage disequilibrium between the two markers; C77G and C772T), and C77G-C772T Linkage Disequilibrium (LD) Analysis Results are shown in Table 8-4. The two tested markers were found to be in linkage disequilibrium (D'=0.9970, SD=0.0385).

Standard Chi-square ($\chi^2$) analysis was used to determine p-values for genotype and allele frequencies. Independent Chi-square analysis returned no statistically significant difference (all P>0.05) for genotype frequencies between MS and control samples for C77G ($\chi^2=0.65$, P=0.72) or C772T ($\chi^2=1.06$, P=0.59). Similarly, no statistically significant difference was observed for allele frequencies for the C77G marker ($\chi^2=0.48$, P=0.49) or C772T ($\chi^2=0.20$, P=0.66). Analysis of male cases versus male controls and female cases versus female controls also showed no significant difference in genotype or allele frequencies with all P>0.05. Similarly, no significant result was detected within the different MS clinical subtypes (all P>0.05, data not shown). The observed allele frequencies were also checked against the National Centre for Biotechnology Information (NCBI) SNP, Ensembl, and Weizmann Institute of Science online databases and found to be consistent with previously published findings.
Figure 8-2 PTPRC C77G HRM Real-Time PCR Amplification (Cycling)

Figure 8-2 shows (A) real-time PCR amplification cycling for the PTPRC gene prior (prior to melt curve genotyping).

Figure 8-3 PTPRC C77G HRM Melt Profiles

Figure 8-3 shows the PTPRC C77G HRM melt identities of the tested samples (A) G77G homozygotes, (B) C77C homozygotes, and (C) C77G heterozygotes.
Figure 8-4 PTPRC C77G HRM Discrimination Plot (Melt)

Figure 8-4 shows an example of the PTPRC C77G HRM discrimination plot used for genotyping (A) G77G homozygotes, (B) C77C homozygotes, and (C) C77G heterozygotes.

Figure 8-5 PTPRC C77G Real-Time PCR Amplicons by AGE

Figure 8-5 shows real-time PCR amplicons of (A) PTPRC C77G (144bp), and (B) no-template controls (NTCs) as resolved by 2% w/v standard agarose gel electrophoresis.
Table 8-2 Genotype and Allele Frequencies for PTPRC C77G

<table>
<thead>
<tr>
<th></th>
<th>Genotype Frequencies</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>CC (97%)</td>
</tr>
<tr>
<td>Control</td>
<td>171</td>
<td>165</td>
</tr>
<tr>
<td>MS</td>
<td>155</td>
<td>147</td>
</tr>
</tbody>
</table>

\[ X^2 = 0.65, \ P = 0.72 \]

\[ X^2 = 0.48, \ P = 0.49 \]

Table 8-2 shows allele and genotype frequencies for PTPRC C77G genotyping. No significant differences were detected between cases and controls (all \( P > 0.05 \)).

Figure 8-6 shows the (A) undigested PCR amplicons (1,037bp) and (B) no-template controls (NTCs) as resolved by 2% w/v standard agarose gel electrophoresis.
Figure 8-7 PTPRC C772T Rsal Restriction Enzyme Digest Products by AGE

Figure 8-7 shows PTPRC C772T Rsal restriction enzyme digest products by 1% w/v agarose gel electrophoresis. (A) represents digested heterozygotes (1037, 673, 364bp), while (B) represents no-template controls (NTCs).

<table>
<thead>
<tr>
<th>Genotype Frequencies</th>
<th>Allele Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td><strong>CC</strong></td>
</tr>
<tr>
<td>Control</td>
<td>180</td>
</tr>
<tr>
<td>MS</td>
<td>181</td>
</tr>
</tbody>
</table>

$X^2 = 1.06, P = 0.59$

$X^2 = 0.20, P = 0.66$

Table 8-3 shows allele and genotype frequencies for PTPRC C772T genotyping. No significant differences were detected between cases and controls (all $P > 0.05$).
Estimates of Gene Frequencies (Assuming Independence)

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<th>2</th>
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</thead>
<tbody>
<tr>
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<td>0.0276</td>
</tr>
<tr>
<td>2</td>
<td>0.8896</td>
<td>0.1104</td>
</tr>
</tbody>
</table>

# of Typed Individuals: 326

There are 4 Possible Haplotypes of These 2 Loci. They are Listed Below, with their Estimated Frequencies:

<table>
<thead>
<tr>
<th>Allele at Locus 1</th>
<th>Allele at Locus 2</th>
<th>Haplotype Frequency Independent</th>
<th>w/Association</th>
</tr>
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<tbody>
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<tr>
<td>H1: Allelic Associations Allowed</td>
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**Figure 8-8 Haplotype Estimation for PTPRC C77G & C772T**

Figure 8-8 shows the estimated haplotype frequencies (which are first necessary to calculate linkage disequilibrium between the two markers) between all possible alleles at the two genetic loci for PTPRC C77G and C772T. This data was generated by analyzing the genotypes and then allele combinations present for each patient at both loci.
Table 8-4 PTPRC C77G-C772T Linkage Disequilibrium Analysis

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<td>Chi-squared statistic</td>
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<td>(Pearson Chi-squared statistic of haplotype table)</td>
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<td>Global disequilibrium statistics and their standard errors:</td>
<td></td>
</tr>
<tr>
<td>Phi coefficient W</td>
<td>(2.28 / 652)^0.5 = 0.0592, SD = 0.0010</td>
</tr>
<tr>
<td>Cramer's V</td>
<td>W/[min(2,2)-1]^0.5 = 0.0592, SD = 0.0081</td>
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<tr>
<td><strong>D' coefficient</strong></td>
<td><strong>0.997048, SD = 0.0385</strong> (Var = 0.001485)</td>
</tr>
<tr>
<td>Kullback-Leibler information</td>
<td>0.002305</td>
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Table 8-4 shows the results obtained from linkage disequilibrium analysis after haplotype estimation, for the two markers PTPRC C77G and C772T, using the case-control populations from this study. The D' coefficient (0.997048) indicates strong evidence for linkage disequilibrium (LD) between the two tested markers. The standard deviation (SD) observed for the calculated D' coefficient was 0.0385.
8.4 Discussion of PTPRC Variation Studies

MS is a complex neurological disease (Lassmann, Bruck et al. 2007). Recent studies have defined that CD45 is crucial for lymphocyte signalling and could potentially play a role in MS susceptibility (Hennig, Fry et al. 2008). We set out to determine whether genotype and allele frequencies are different between MS and matched healthy control DNA for the C77G and C772T variants in PTPRC, in an Australian population. High resolution melt analysis (HRM) was selected as the method for genotyping C77G as it allows a high throughput and more efficient analysis over traditional methods (Erali, Voelkerding et al. 2008). However, not all variants can be detected using this method and in this study a traditional RFLP method was used for C772T. Hardy-Weinberg equilibrium (HWE) analysis showed that C77G was not in HWE (P<0.05). Subsequently, Linkage disequilibrium (LD) analysis was carried out and the two tested markers were found to be in strong linkage disequilibrium (D'=0.9970, SD=0.0385) indicating that although the C77G marker may be relatively uninformative in this investigation, the other tested marker C772T constitutes a good surrogate for the former.

The findings of this study do not support the notion that these markers play a significant role in MS susceptibility in the tested population because no significant difference could be detected between MS patients and healthy matched controls by either the genotype or allele frequencies for the investigated markers (all
Although previous associations with other autoimmune diseases have been reported, our findings suggest no association in the tested Australian Caucasian MS population. These findings suggest that either a larger population is required to detect modest effects that these markers may be contributing, or that these variants do not contribute to susceptibility in the Australian population. Given that both positive and negative associations continue to be reported for the same genetic marker (C77G) in different studies, it is plausible to assume that the contribution of the tested polymorphism to MS may depend on the genetic background of the population being studied (Jacobsen, Schweer et al. 2000).

Other variants within the PTPRC gene may still be associated with MS because variations within this highly functional gene have been previously shown to be associated with diseases with autoimmune mechanisms, such as Graves’ disease, type 1 diabetes, and susceptibility to hepatitis infections (Boxall, Stanton et al. 2004; Ward, Hennig et al. 2006). Other interesting candidates include the C59A mutation, which results in alternative splicing and alters the amino acid sequence (H to Q) that then interferes with antibody binding to the CD45RA domain, and A138G, which is also known to alter splicing resulting in an amino acid substitution (Thr-47 to Ala) that has been detected in the Japanese population (Jacobsen, Hoffmann et al. 2002; Stanton, Boxall et al. 2003). These markers are known to reside within a large LD block within Caucasian populations (Consortium 2003). Given the importance placed on investigating the mechanisms of T-cell autoreactivity, and the possible implications variants could
have on the response to interferon-beta therapy (a commonly used therapeutic agent in MS) (Zafranskaya, Oschmann et al. 2007), further studies of other PTPRC variations are justified.
Chapter 9

Genetic Variation Studies in TLR3

9.1 Introduction to Variation Studies in TLR3

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of uncertain aetiology. Associations between genetically determined childhood susceptibility to viral infections and the risk of developing MS in early adulthood have been described (Hernan, Zhang et al. 2001). Predispositions to autoimmunity appear to play an important role central to the evolution of the pathology of the disease (Pender and Greer 2007). Autoreactive T cells directed against myelin basic protein and proteolipid protein appear to contribute to pathogenesis, with viruses being possible triggers in individuals with a susceptible predisposition (Cirone, Cuomo et al. 2002). The role of human endogenous retroviruses in autoimmune diseases has received increasing attention in recent times (Christensen 2005). Toll-like receptor 3 (TLR3) plays a fundamental role in pathogen recognition, and human neurons express TLR3 in order to facilitate innate immune responses to viral dsRNA (Lafon, Megret et al. 2006). TLR3 is known to be expressed by human astrocytes in order to trigger the production of neuroprotective mediators and anti-inflammatory cytokines including interleukins -9, 10, and 11 (IL-9, IL-10, and IL-11) (Bsibsi, Persoon-Deen et al. 2006).

Polymorphisms in TLR3 have previously been investigated in other diseases in the context of altered innate immune response (Ueta, Sotozono et al. 2007). A recent study by Jack and colleagues found that activation of human microglia via
TLR3 signalling changes the profile of local central nervous system (CNS) immune response by translation of T\textsubscript{H}1 polarizing signals to CD4 T-cells (Jack, Arbour et al. 2007). The importance of TLR3 in both innate and adaptive immune responses has been repeatedly demonstrated (Heinz, Haehnel et al. 2003). Mounting evidence suggests that the ability of individuals to respond to toll-like receptor (TLR) ligands may be impaired by variations such as single nucleotide polymorphisms (SNPs) within TLR genes resulting in altered susceptibility and course of infection, particularly in inflammatory diseases (Schroder and Schumann 2005). Immunohistochemical analysis of MS brains and spinal cords by Bsibsi et al. have shown that enhanced expression of TLR3 (and TLR4) occurs in inflamed CNS tissues (Bsibsi, Ravid et al. 2002). Furthermore, animal models have recently reported that infections with viruses, such as Theiler’s murine encephalomyelitis virus (TMEV), are sufficient to induce TLR3-mediated MS-like pathologies (So, Kang et al. 2006).

We set out to investigate an 8bp Adenine insertion/deletion [-/A]	extsubscript{8} (rs3081264) and a single-nucleotide polymorphism (SNP); C1236T (rs3775291) to determine frequencies of these variants within an Australian Caucasian population, comparing MS affected and matched healthy control DNA by capillary gel electrophoresis, and TaqMan genotyping assay methods. Identifying TLR3 variations associated with MS susceptibility may lead to a better understanding of the aetiology of MS.
9.2 Experimental Procedure

9.2.1 Subjects

The Griffith University Ethics Committee approved research involving human experimentation (Approval HSC/09/03/HREC). Informed consent was obtained from all participants. The case-control populations consisted of 205 MS patients and 196 healthy controls for [-/A]_B (rs3081264) and 199 MS patients and 198 healthy controls for C1236T (rs3775291), which had been matched for age (±5 years), gender, and ethnicity (Caucasian). The MS population was recruited from the Multiple Sclerosis Clinic at the Royal Brisbane & Women’s Hospital, Brisbane, Queensland, Australia; all samples were procured from patients residing in the South East Queensland region. The population consisted of approximately 75% females and 25% males, and was subcategorised based on clinical course: Relapsing-Remitting (RR), Secondary-Progressive (SP), and Primary-Progressive (PP). Frequencies were: RR=51%, SP=24% and PP=25% for [-/A]_B (rs3081264) and RR=40%, SP=35%, and PP=25% for C1236T (rs3775291). The matched control samples were obtained via the Genomics Research Centre Clinic, Southport, Gold Coast, Australia. Genomic DNA was extracted from peripheral blood using a standard salting-out procedure.
9.2.2 TLR3 [-/A]₈ Genotyping Assay

An 8bp Adenine insertion/deletion [-/A]₈ (rs3081264) was chosen for investigation. This variation is the only known insertion-deletion within the TLR3 gene, to date. DNA concentrations were adjusted to 10ng/µL for each sample. Specific forward and reverse polymerase chain reaction (PCR) primers were designed F – 5’ GAGCTGAGATTGC ACTGCTG -3’ and R – 5’ CTTCCAATTGCGTGAAAACA – 3’. The reverse primers were labelled with the fluorescent reporter dye FAM. Primers were ordered from GeneWorks, Australia. Genomic DNA was used as a template to generate PCR products for subsequent genotyping by capillary gel electrophoresis. Assay conditions were empirically determined. Efficient thermal cycling consisted of 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds, with a final extension of 72°C for 3 minutes. A total of 40ng of genomic DNA was amplified using 10X PCR buffer, 3mM MgCl₂, 0.2mM dNTP, 10µM total primers (5µM forward, 5µM reverse) and 0.2µM Taq polymerase in a 25µL final volume on a Corbett (Sydney, Australia) PC-960 thermocycler. PCR products were electrophoresed on a 2% w/v normal agarose gel to confirm the presence of DNA fragments (either 175bp or 183bp in size). As agarose gel electrophoresis lacks the sensitivity to resolve an 8bp difference, the PCR products were genotyped using ABI PRISM® 310 Genetic Analyser (Applied Biosystems, USA). Appropriate DNA dilutions were determined with regards to relative fluorescence from the GeneScan-350 Size Standard marker. For capillary gel electrophoresis
equimolar concentrations of PCR product were combined with Hi-Di Formamide (Applied Biosystems) and ABI310 Genetic Analyser Performance Optimised Polymer-4 (POP-4) (Applied Biosystems) according to the manufacturer’s instructions and then run at 60V for approximately 20 minutes per sample. Collection of data and analysis were carried out on the attached software platform (ABI-310, Applied Biosystems, USA).

9.2.3 TLR3 C1236T TaqMan Genotyping Assay

Nearby base sequence variations to the marker of interest (TLR3 C1236T rs3775291) influenced the selection of the current assay. A specific TaqMan Pre-Designed Genotyping Assay was ordered from Applied Biosystems, Australia (Part Number 4351379). The TaqMan SNP assay incorporates probes labelled with FAM or VIC dyes at the 5’ end and a minor-groove binder (MGB) and non-fluorescent quencher at the 3’ end. Reactions consisted of 2XTaqMan Universal PCR Master Mix, 20XTaqMan SNP Genotyping Assay Mix, dH₂O, and 10ng starting template (DNA) in a total volume of 20μL. Thermal conditions consisted of 95°C for 10 minutes once, followed by 92°C for 15 seconds and 60°C for 1 minute repeated for 40 cycles. Reactions were performed on a Corbett Life Science Rotor-Gene 6000 real-time quantitative cycler collecting fluorescence on green (470nm source, 510nm detect) and yellow (530nm source, 555nm detect).
9.2.4 Statistical Analysis of TLR3 Variations

Data for the 8bp Adenine insertion/deletion [-/A]₈ (rs3081264) was collected on the ABI PRISM® 310 Genetic Analyser (Macintosh) platform. Each genotype was determined based on distinct peak sizes (bp) for each sample. Descriptive statistics were assessed using SPSS Standard Package with follow-up analysis using Microsoft Excel. Genotype distributions were checked for Hardy-Weinberg equilibrium (HWE). Observed genotype and allele frequencies were investigated using Chi-square independence test with α-level set at 0.05.

Results for the TLR3 C1236T marker (TaqMan Assay) were resolved on the Corbett Rotor-Gene 6000 real-time cycler software (Corbett Life Science, Australia) collecting fluorescence on two separate channels (yellow and green) providing cycling information. A common threshold was applied for analysis and CT values were tabulated into discrimination plots to determine genotypes.
9.2.5 Genotyping Summary

For the TLR3 marker [-/A]₈ (rs3081264) 205 (99%) cases and 196 (94%) controls were genotyped successfully. For the marker TLR3 C1236T 199 (96%) cases and 198 (95%) matched controls were genotyped successfully. The remaining samples proved difficult to amplify efficiently or were not able to be definitively categorised during analysis.

9.3 Results for TLR3 Variations

For TLR3 [-/A]₈, agarose gel electrophoresis (AGE) was carried out (after PCR) to qualitatively check for PCR amplification. Figure 9-1 below shows PCR amplicons of the TLR3 [-/A]₈ Insertion-Deletion (175 and 183bp) and no-template controls (NTCs). The 2 alleles of the variant are not distinguishable (8bp difference) by AGE, therefore capillary gel electrophoresis (GeneScan) was carried out to facilitate genotyping. Figure 9-2 shows the TLR3 [-/A]₈ Deletion Homozygote (175/175bp)., Figure 9-3 shows the TLR3 [-/A]₈ Insertion-Deletion Heterozygote (175/183bp), and Figure 9-4 shows the TLR3 [-/A]₈ Insertion Homozygote (183/183bp). A summary of allele and genotype frequencies for TLR3 [-/A]₈ genotyping are shown in Table 9-1. The Hardy-Weinberg equilibrium held for all analyses with all P>0.05 ([-/A]₈ Cases HWE P=0.12, Controls HWE P=0.06; C1236T Cases HWE P=0.12, Controls HWE P=0.15).
Figure 9-5 shows the Discrimination Plot for TLR3 C1236T TaqMan Genotyping (Cases), whilst Figure 9-6 shows the Discrimination Plot for TLR3 C1236T TaqMan Genotyping (Controls). Table 9-2 shows a summary of allele and genotype frequencies for C1236T genotyping by TaqMan genotyping assay.

Independent Chi-square tests returned no statistically significant difference (all \( P>0.05 \)) between genotype frequencies between MS and control samples for TLR3 \([-/A]_8\) (\( \chi^2=1.03, P=0.60 \)) or C1236T (\( \chi^2=0.35, P=0.84 \)). Similarly, no statistically significant difference was observed for allele frequencies for \([-/A]_8\) (\( \chi^2=1.09, P=0.30 \)) or C1236T (\( \chi^2=0.31, P=0.58 \)). For controls the deletion was more common (51%) than the insertion (49%) whereas a converse trend was observed in MS cases, with the insertion being more common (53%) than the deletion (47%). For the C1236T marker the C allele was more common than the T allele for both cases (66%) and controls (68%). Previously determined allele frequencies were not available for TLR3 \([-/A]_8\). Previous frequencies were available for TLR3 C1236T online at both the NCBI SNP database and the Ensembl database; upon comparison both the case and control frequencies for the C and T alleles were similar to the previously published findings.
Figure 9-1 TLR3 [-/A]₈ Insertion-Deletion PCR Amplicons by AGE

Figure 9-1 shows (A) PCR amplicons of the TLR3 [-/A]₈ Insertion-Deletion (175 and 183bp) and (B) no-template controls (NTCs). Note that the two alleles, which differ by only 8bp, cannot be resolved by AGE. This AGE step is used as a qualitative check.

Figure 9-2 TLR3 [-/A]₈ Deletion Homozygote

Figure 9-2 shows (A) MW marker and (B) the TLR3 [-/A]₈ Deletion Homozygote (175/175bp).
Figure 9-3 TLR3 [-/A] Insertion-Deletion Heterozygote

Figure 9-3 shows (A) MW marker and (B) the TLR3 [-/A] Insertion-Deletion Heterozygote (175/183bp).

Figure 9-4 TLR3 [-/A] Insertion Homozygote

Figure 9-4 shows (A) MW marker and (B) the TLR3 [-/A] Insertion Homozygote (183/183bp).
Table 9-1 shows allele and genotype frequencies for TLR3 [-/A]₈ genotyping. No significant differences were detected between cases and controls (all P>0.05).
Figure 9-5 shows the collated results of TLR3 C1236T TaqMan genotyping for cases. Relative fluorescence of VIC (C allele) is plotted on the x-axis, while relative fluorescence of FAM (T allele) is plotted on the y-axis. (A) shows T1236T homozygotes, (B) shows C1236T heterozygotes, and (C) represents C1236C homozygotes.
Figure 9-6 shows the collated results of TLR3 C1236T TaqMan genotyping for controls. Relative fluorescence of VIC (C allele) is plotted on the x-axis, while relative fluorescence of FAM (T allele) is plotted on the y-axis. (A) shows T1236T homozygotes, (B) shows C1236T heterozygotes, and (C) represents C1236C homozygotes.
Table 9-2 Genotype and Allele Frequencies for TLR3 C1236T

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<th>Genotype Frequencies</th>
<th>Allele Frequencies</th>
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<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>Control</td>
<td>87(44%)</td>
<td>95(48%)</td>
</tr>
<tr>
<td>MS</td>
<td>82(41%)</td>
<td>99(50%)</td>
</tr>
</tbody>
</table>

\[X^2 = 0.35, \ P = 0.84\]

Table 9-2 shows allele and genotype frequencies for C1236T genotyping. No significant differences were detected between cases and controls (all \(P > 0.05\)).
9.4 Discussion of TLR3 Variation Studies

Human neurons are known to express TLR3 during the immune response to viral infections (Prehaud, Megret et al. 2005). Toll-like receptors play a key role in pathogen recognition and studies have shown that susceptibility to infections and inflammatory diseases may be regulated in part by genetic variations within the gene that codes for the receptor (Schroder and Schumann 2005).

TLRs are known to be activated by several triggers, including viruses and bacteria upon presentation of pathogen associated molecular patterns (PAMPs) with distinct receptors being activated by specific antigens. In general, toll-like receptors participate in modulating a T_H2 (anti-inflammatory) to T_H1 (inflammatory) shift, which involves the increased expression of pro-inflammatory cytokines. This process is thought to drive CNS inflammation, whilst interaction with T- and B-cells, and macrophages is thought to contribute to CNS demyelination (see Figure 9-7 below). Infections leading to autoimmune disease have been under scrutiny in recent years (Bach 2005). It is well established that MS is a complex inflammatory T-cell-mediated autoimmune disease (Weiner 2004). The complex mechanisms underlying the processes by which autoimmunity occurs and the nature of the contributing genetic variations remain undefined (Lassmann 2006).
In this study we set out to determine whether genotype and allele frequencies differ between MS and matched control DNA for the TLR3 \([-/A]_8\) and C1236T markers in our population. We found no evidence for these markers in the tested population. No significant differences could be detected between MS patients and controls by either genotype or allele frequencies (all \(P>0.05\)).

The importance of toll-like receptors in the CNS is well established (Bsibsi, Ravid et al. 2002). Furthermore, important roles for TLR3 have been proposed in mechanisms relating to expression of neuroprotective mediators and in mounting innate immune response to infections (Prehaud, Megret et al. 2005; Bsibsi, Persoon-Deen et al. 2006). Other variations within TLR3 may still play a role in susceptibility to MS given the role and function of this receptor. Human microglia also thought to be key components in the MS pathogenesis cascade, also express toll-like receptors in response to T cell stimulus (Jack, Arbour et al. 2007).

Recent studies have by Martino and colleagues have proposed that the interaction between inflammatory mediators and neural precursor cells may have important consequences for CNS homeostasis and repair (Martino and Pluchino 2007). Recent genetic studies have implicated the pathways involving toll-like receptors and their associated transcription factors (such as IRF5) as MS susceptibility candidates (Kristjansdottir, Sandling et al. 2008) whilst other efforts focus on haplotype analysis involving targets such as TLR4 (Urcelay, Blanco-
Kelly et al. 2007) Other members of the toll-like family such as TLR9 still remain topical and uninvestigated in MS (Prinz, Garbe et al. 2006). Whilst the precise role of toll-like receptors in inflammatory neurobiology remains complex, further genetic analyses investigating the variations within the toll-like receptor family that are likely to contribute to susceptibility may prove insightful.
Toll-Like Receptors (B) are known to be activated upon presentation of Pathogen Associated Molecular Patterns (A). TLRs are thought to participate in modulating the $T_{H2}$ (anti-inflammatory) to $T_{H1}$ (inflammatory) shift. Inflammatory cytokines including TNF-$\alpha$ and IFN-$\gamma$ are increased (C), whilst anti-inflammatory cytokines such as IL-9, 10, and 11 are decreased (D). This mechanism drives CNS inflammation, whilst subsequent interactions with T- (T) and B-cells (B) as well as macrophages (M) facilitate cell-mediated processes of CNS demyelination (E).
Chapter 10

General Discussion
10.1 Introduction

10.1.1 The MS Paradigm

Although our understanding of MS has improved in recent years, much of the complexity associated with the genetics of the disease remains unexplained. Multiple sclerosis is the most common idiopathic inflammatory autoimmune disease involving the central nervous system. Multifocal immune-mediated destruction of the myelin sheath coupled with axonal degeneration results in variable neurological deficits including dysarthria, ataxia, tremor, and extrapyramidal signs (Noseworthy, Lucchinetti et al. 2000; Compston and Coles 2008). Approximately 85% of patients suffer the relapsing-remitting form of the disease whereby they experience acute attacks of neurological dysfunction that persist for days to weeks that then remit suddenly. Most patients progress to the secondary-progressive form of the disease (from the relapsing-remitting) within 10 years of onset. The secondary-progressive stage is characterised by the accumulation of progressive neurological sequelae without recovery. This stage of the disease often involves axonal deterioration and neuron loss, and is generally considered the neurodegenerative phase of the disease (Lucchinetti, Bruck et al. 2001; Steinman 2001). Primary-progressive MS accounts for only approximately 15% of all MS cases and is similar to the secondary-progressive phase but for that these patients do not experience a relapsing-remitting course beforehand.
10.1.2 Aetiology of Multiple Sclerosis

The exact cause or causes of MS remain unknown. It is widely accepted that both environmental and genetic factors contribute to the phenotype (Noseworthy 1999). Strong evidence from twin studies supports the notion that genes and genetic variations play key roles in susceptibility to development of the disease (Steinman 1996; Weiner 2004; Ebers 2008; Kantarci 2008). The risk of developing MS between individuals appears to increase proportionally with the amount of shared genetic information (degree of relatedness). Indeed, the highest rates of MS are seen between monozygotic twins (approximately 30% concordance). The rates of concordance with first degree relatives drops to approximately 5%, and even lower yet to approximately 0.1% for the general population (Sadovnick, Yee et al. 2005).

Whole-genome studies in the mouse model, experimental allergic encephalomyelitis (EAE) have shown that several loci are involved in the animal model (Ebers, Kukay et al. 1996; Haines, Ter-Minassian et al. 1996; Butterfield, Sudweeks et al. 1998; Sawcer, Maranian et al. 2002). However, to date only the class II major histocompatibility complex haplotypes HLA-DR and HLA-DQ are demonstrated to overlap with (some) human cases, and specific candidate genes that can be shown as causative (either alone or in combination) are yet to be elucidated.
10.1.3 Pathogenesis of Multiple Sclerosis

The predominant theory regarding MS pathogenesis purports that formation of abnormal helper T-cells precipitates autoreactivity against myelin self-antigens. Activated T-cells are inherently motile and readily cross the blood-brain barrier (BBB). This extravasation is facilitated by adhesion molecules such as very-late antigen 4 (VLA-4) and stimulated by myelin antigens presented by antigen presenting cells (APCs). These cells differentiate into T-helper type 1 cells (T\textsubscript{H}1). T-helper type 1 cells are known to secrete proinflammatory cytokines such as interferon-gamma (IFN-\gamma) and tumour necrosis factor-alpha (TNF-\alpha). These events along with the activation of B-cells, cytotoxic T-cells, and activation of macrophages, culminate to result in the inflammation mediated demyelination seen in MS (Bar-Or 2008; Dittel 2008; Lassmann 2008). The rate limiting step in this pathogenesis is the initial development of abnormal myelin specific T-cells.

Animal models (EAE) have demonstrated that myelin specific T-cell clones isolated from one animal can be adoptively transferred to another, furthermore transgenic mice (Rag1\textsuperscript{-/-} with myelin basic protein specific T-cell antigen receptor) with only T-cell clone specific for myelin basic protein (MBP) readily develop spontaneous EAE (Ben-Nun, Wekerle et al. 1981; Ben-Nun and Cohen 1982; Zamvil, Nelson et al. 1985; Zamvil, Nelson et al. 1985; Lafaille, Nagashima et al. 1994; Mendel, Kerlero de Rosbo et al. 1995). Myelin basic protein-specific T-helper 2 cells (T\textsubscript{H}2) have been shown to cause experimental autoimmune
encephalomyelitis in immunodeficient hosts, rather than protect them from the
disease (Lafaille, Keere et al. 1997). It is becoming increasingly evident that both
T- and B-cells are involved in MS pathogenesis. An intriguing point recent
experiments raise is that whilst B-cells contribute to the development and
severity of the phenotype, they are not in themselves able to initiate or sustain
the disease (Archelos and Hartung 2000).

10.1.4 Multiple Sclerosis Research Aims

The ultimate aim of MS research is to assist in the development of interventions
that will eventually improve the lives of sufferers of the disease. Defining the
molecular pathogenesis of the disease remains central to understanding the
processes leading to disability. Our current understanding of the disease does
not yet facilitate the accurate prediction of the effectiveness of the therapeutic
interventions currently in use. Current therapies do not include a cure for MS.
Research involving pathological changes underlying the natural course of the
disease; mechanisms of neuronal, oligodendroglial, and astrocytic damage and
repair; identification of the genes involved in pathogenesis; and the investigation
of potential triggers for the disease are warranted. The potential benefits of these
approaches extend beyond MS research alone, as basic scientific research in
this field drives the development of other modalities and disciplines such as the
advances seem recently in medical imaging, pharmacology, and molecular
biology (Furlan, Pluchino et al. 2003; Nakashima, Fujihara et al. 2003; Pluchino, Quattrini et al. 2003; Friese and Fugger 2005; Gilmore, Geurts et al. 2008).
10.2 Thesis Development

10.2.1 Theoretical Underpinnings

This thesis set out to investigate the molecular genetics of MS by studying gene expression and genetic variation in candidate genes implicated in biological processes and pathways involving neuroinflammation and myelination. The significance of the illness is well established, with MS being a paradigm of complex disease that disables sufferers indiscriminately and unpredictably. The rationale underlying investigation of the disease is that furthering basic scientific understanding of the molecular mechanisms leading to the pathogenesis of MS will likely afford new insights into more streamlined diagnosis, targeted treatment options, and better prognostication of the currently unpredictable (and variable) clinical course. As our understanding of the natural history of MS is expanded new realisations regarding complex diseases (disease with significant interaction between environmental, genetic, and behavioural components) are unravelling. The two main aims of this research were to (1) accurately quantify novel candidate genes thought to be involved in MS using post-mortem brain tissue RNA from MS sufferers versus health controls by relative quantitative real-time polymerase chain reaction methods (qRT-PCR) and (2) examine genomic variations in genes that may be associated with MS using blood-derived lymphocytic DNA from MS sufferers versus age, gender, and ethnicity matched healthy controls.
10.2.2 Methodological Approach

In the first part of this project we undertook case-control gene expression studies of 5 genes (CLDN11, GDNF, NRG1, PTPRC, and TLR3) implicated in biological processes involved in CNS inflammation and demyelination by qRT-PCR. In the second part of this project we undertook case-control association studies of genetic variations within 4 genes (MTHFR, MTRR, PTPRC, and TLR3) by genomic methods (RFLP, HRM, Capillary Gel Electrophoresis, and TaqMan Genotyping Assay).
10.3 Gene Expression Studies

10.3.1 Gene Expression Studies Overview

In the first part of the thesis gene expression studies were undertaken in normal-appearing white matter (NAWM) and normal-appearing grey matter (NAGM) of secondary progressive frontal and temporal tissues of MS brains versus healthy matched controls. The defining histological characteristic of MS are widespread lesions in the central nervous system (CNS) termed plaques. Venocentric focal inflammatory demyelinating plaques with perivascular lymphocytes and plasma cells predominantly form in the NAWM (Compston 1999; Barnett and Sutton 2006). It has been shown that plaques can also develop in the peripheral gyri of the brain and deep grey matter nuclei, and the NAGM of MS brains has become a specific tissue of interest in recent years (Ceccarelli, Rocca et al. 2007; Gilmore, Geurts et al. 2008; Gilmore, Donaldson et al. 2008). The gene expression studies carried out in this thesis focused on candidate genes involved in central nervous system myelination, the survival and differentiation of neurons, the growth and differentiation of neuronal and glial cells, B- and T-cell receptor antigen signalling, and pathogen recognition and activation of innate immunity.
10.3.2 Gene Expression Studies Background

To date, two methods have been predominantly used to investigate multiple sclerosis gene expression; comparative gene expression microarray, and quantitative polymerase chain reaction (quantitative PCR). Numerous variants of these techniques have been developed and continue to be refined. The microarray platform has been used in other diseases to identify biological pathways involved in skin and breast cancers (Perou, Sorlie et al. 2000; Ridley 2000). Quantitative PCR methods have recently been developed to allow sensitive quantification of specific targets (Kelleher, Leck et al. 2001; Nolan, Hands et al. 2006). In an attempt to determine which genes are uniquely expressed in MS brain tissues, Becker et al. undertook experiments investigating a normalised complementary DNA (cDNA) library of MS lesions from a single case in 1997 (Becker, Mattson et al. 1997). The key findings of that study were that 16 genes involved in immunity were found to be differentially expressed in the MS lesion, 7 of those genes had also previously been shown to be involved in systemic lupus erythematosus (SLE), and 2 were previously associated with insulin-dependent diabetes mellitus (IDDM).

Subsequent experiments reporting gene expression differences in white matter using cDNA microarrays were then published in 1999 by Whitney and colleagues (Whitney, Becker et al. 1999). Sixty-two differentially expressed genes were identified, including the Duffy chemokine receptor, interferon regulatory factor-2,
and tumour necrosis factor alpha receptor-2. Those initial studies paved the way for further gene expression investigations in MS as laboratories around the world set out to replicate the findings and define the remaining genes involved in the disorder. Recent advances in real-time technology have improved the modality and real-time PCR is now promising to revolutionize some aspects of diagnosis and treatment (Lindberg and Kappos 2006). These last few years, gene expression techniques and expertise have become more common in molecular biology laboratories. Microarray experiments in MS have implicated hundreds of genes with significantly altered expression profiles (Tajouri, Fernandez et al. 2007). As we move towards interrogating known susceptibility genes, quantitative real-time PCR (qRT-PCR) remains, and continues to become, an increasingly precise method for the sensitive quantification of mRNA from candidate genes (Wong and Medrano 2005; Nolan, Hands et al. 2006). The real-time reverse transcription polymerase chain reaction addresses the evident requirement for quantitative data analysis in molecular medicine, biotechnology, microbiology and diagnostics and has become the method of choice for the quantification of mRNA (Nolan, Hands et al. 2006).
10.3.3 NAWM Gene Expression Studies Discussion

10.3.3.1 NRG1 in NAWM Findings

In the studies in normal-appearing white matter (NAWM) (Chapter 5) we found that the expression of neuregulin-1 (NRG1) was increased in the secondary-progressive white matter tissue of multiple sclerosis brains compared to healthy neuropathology free controls. Quantitative real-time investigations showed that the expression of NRG1 was increased in both the overall analysis approximately 20-fold (↑19.72-fold, P=0.004) and the frontal analysis also 20-fold (↑19.94-fold, P=0.021) in MS NAWM compared controls. Immunoblot was also used to confirm the qualitative presence of the NRG1 protein.

Increased NRG1 expression in chronically affected MS brains may represent gliosis or attempts by oligodendrocytes at remyelination (Bieber 2008; Lucchinetti 2008). Changes in the expression of NRG1 have been shown to regulate transmembrane proteolipid proteins (predominantly myelin proteins). The proteolipid protein 1 gene (PLP1) is known to play an important role in compaction, stabilization, and maintenance of myelin sheaths and mutations leading to a primary-progressive MS phenotype have been recently described (Warshawsky, Rudick et al. 2005). A recent study by Nave and colleagues reports that in the peripheral nervous system, neuregulin-1 type III (SMDF) regulates cell-membrane growth and adjusts myelin sheath thickness to match axon calibre. The authors go further to state that it is currently unclear whether
this system operates in the central nervous system, but express that it is a question of major importance which may have substantial ramifications for demyelinating diseases (Nave and Salzer 2006). Studies in the EAE mouse model have shown that the GGF2 isoform of NRG1 promotes the proliferation and survival of the oligodendrocyte, the myelinating cell of the CNS (Cannella, Hoban et al. 1998). Hence, NRG1 may constitute a valuable marker of remyelination in demyelinating neuropathies. A recent study by Edwards and colleagues investigated this phenomenon finding that NRG1 growth factors regulate the proliferation but not the apoptosis of neuronal progenitor cells (Edwards and Bottenstein 2006). NRG1 is also documented as having important roles in development and plasticity in the human brain. A recent investigation published by Law et al found widespread expression of NRG1 in the adult human brain, including, but not limited to, brain areas and cell populations implicated in schizophrenia (Law, Shannon Weickert et al. 2004). The investigators concluded that it may be beneficial to ascertain whether NRG1 expression alters in the disease affected brain. NRG1 is an attractive target for genetic expression studies in MS owing to its involvement with neural development and its potential role in susceptibility to neurological disorders. Expression of NRG1 in MS seems significantly different in the affected brain compared to healthy neuropathology free controls.
10.3.3.2 TLR3 in NAWM Findings

The expression of the toll-like receptor 3 gene (TLR3) was found to be significantly increased in our studies of MS affected NAWM brain tissues. The TLR3 gene was found to be increased 543-fold in the overall analysis (↑543, P=0.0007) and 230-fold (↑230-fold, P=0.0112) in MS frontal white matter compared to healthy matched neuropathology free controls. Immunoblot analysis was also used to confirm the qualitative presence of the TLR3 protein.

Toll-like receptors (TLRs) are proteins that are involved in the linking of the innate and acquired divisions of the immune system (Akira, Takeda et al. 2001). Distinct TLRs recognise pathogens via pattern-recognition receptors. TLR3 specifically recognises double-stranded RNA (dsRNA) viruses (Oda and Kitano 2006). Akira and colleagues conducted studies in knockout mouse models (Akira and Takeda 2004) and have found that activation of TLRs may protect hosts from viral infections (Akira, Takeda et al. 2001). A recent study by So et al reports that the Theiler’s murine encephalomyelitis virus (TMEV) model uses TLR3 specifically to mediate the expression of cytokines and chemokines in astrocytes (So, Kang et al. 2006). Since infectious triggers have been postulated for the development of MS, investigations into the possible role of TLRs in the human MS brain are warranted. We conducted qRT-PCR experiments to accurately ascertain whether TLR3 gene expression differs in MS brain tissues compared to healthy matched controls. We found consistent increased gene expression of
TLR3 in the NAWM of MS tissue samples. Increased TLR3 expression has been previously associated with proinflammatory cytokine expression and it is well established that those cytokines are increased in MS (Kahl, Kruse et al. 2002). TLR3 has been shown to interact with interferon-regulatory factors (such as interferon regulatory factor 1; IRF1) and domain containing adaptor proteins (TIRAPs) such as toll-interleukin receptor 1 (TIR1). The toll-like interleukin 1 receptor (TIR) is known to be responsible for the signal transduction and activation of the TLR4 immune pathway which leads to transcriptional activation of proinflammatory genes (Henneke and Golenbock 2001). It is known that TLRs partake in activation of adaptive immune responses (Schnare, Barton et al. 2001; Barton and Medzhitov 2002). A study by Bsibsi and colleagues in 2002 showed that in vivo expression of TLR3 and TLR4 in the brain and spinal cord from both control and multiple sclerosis brains revealed enhanced expression of these TLRs in inflamed CNS tissues (Bsibsi, Ravid et al. 2002). In the same study, the authors concluded that there is broad and regulated expression of TLRs, both in vitro and in vivo by human glial cells. In a more recent study, Cameron et al. report that TLR3 functions autonomously in neurons to regulate axonal growth, which advances a novel hypothesis that this class of receptor may contribute to neurological injury repair and regeneration (Cameron, Alexopoulou et al. 2007).
10.3.3.3 NAWM Non-Significant Results

Although expression changes were also seen in CLDN11 in overall (↑4.56-fold), frontal (↑1.75-fold) and temporal lobe white matter (↑31.12-fold), GDNF overall (↓1.52-fold), frontal (↓2.46-fold) and temporal lobe (↑2.38-fold), NRG1 temporal (↑19.29-fold), PTPRC overall (↓3.18-fold), frontal (↓3.49-fold) and temporal (↓2.46-fold) samples; those results did not reach statistical significance (all P>0.05).

10.3.4 NAGM Gene Expression Studies Discussion

10.3.4.1 GDNF in NAGM Findings

In the studies in normal-appearing grey matter (NAGM) (Chapter 6) we found that the expression of glial cell derived neurotrophic factor (GDNF) was increased in the secondary-progressive grey matter tissue of multiple sclerosis brains compared to healthy matched controls. Quantitative real-time analysis showed that the expression of GDNF was increased approximately 156-fold in the overall analysis (↑155.82, P=0.002) and approximately 55-fold (↑54.55-fold, P=0.0009) in MS frontal white matter compared to healthy controls. Immunoblot was also used to confirm the qualitative presence of the GDNF protein.
Glial cell line-derived neurotrophic factor (GDNF) plays an important role in the differentiation of neurons during normal development, and animal studies have demonstrated its importance in survival and recovery of populations of mature neurons after traumatic brain injury (Rahimi-Movaghar, Yan et al. 2005). GDNF was originally discovered as a trophic factor for midbrain dopaminergic neurons by Lin and colleagues in 1993 (Lin, Doherty et al. 1993). Since that time it has received much attention as a potential therapeutic target for the treatment of neurodegenerative diseases (Lapchak, Gash et al. 1997; Olson 1997; Grondin and Gash 1998). GDNF is known to promote the survival of numerous types of neurons including sub-populations of peripheral autonomic and sensory, as well as central motor neurons (Henderson, Phillips et al. 1994; Trupp, Ryden et al. 1995; Heuckeroth, Lampe et al. 1998). In our investigations, expression of GDNF was found to be significantly increased in the NAGM of multiple sclerosis brains compared to neuropathology free controls. Increased expression of GDNF in conjunction with brain-derived neurotrophic factor (BDNF) have been shown to alter the expression of neuronal nitric oxide synthase (NOS), c-Jun, and p75, which can prevent motor-neuron death in rats (Wu, Li et al. 2003). A recent study by Wu and colleagues has also demonstrated that increased expression of GDNF can induce histone deacetylase inhibitors to up-regulate astrocytes to induce protection of dopaminergic neurons (Wu, Chen et al. 2008). Studies of the human condition amyotrophic lateral sclerosis (ALS) have shown that GDNF expression is upregulated at both the mRNA and protein levels in degenerating motor neurons (Mitsuma, Yamamoto et al. 1999). Those investigators comment
that GDNF may constitute a good candidate for new therapeutic approaches. A study by Heuckeroth and colleagues reports that GDNF can promote proliferation and survival of enteric neurons and glial progenitors in culture (Heuckeroth, Lampe et al. 1998). Another study by Grondin et al has previously shown that GDNF can also induce neuroprotective changes in dopamine neurons in Parkinson's disease (Grondin and Gash 1998). The increased expression of GDNF in MS NAGM constitutes an interesting finding which may warrant further investigation. The various isoforms of GDNF are now becoming well defined and the role of those variants in MS is still unknown.

10.3.4.2 NRG1 in NAGM Findings

In our investigations neuregulin-1 (NRG1) was found to be increased in the secondary-progressive grey matter tissue of MS brain tissues compared to healthy neuropathology free controls. Quantitative real-time analysis showed that the expression of NRG1 was increased approximately 142-fold in the overall analysis, in both frontal and temporal samples (↑142.19, P=0.022). Immunoblot was also used to confirm the qualitative presence of the NRG1 protein.
Neuregulin, also known as heregulin or NEU differentiation factor (NDF), was first identified by Holmes et al. in 1992 (Holmes, Sliwkowski et al. 1992). In 1998 Cannella and colleagues examined the effect of recombinant human glial growth factor 2 (an alternative name for neuregulin; rhGGF2) on clinical recovery rates and repair to damaged myelin in chronic relapsing experimental allergic encephalomyelitis (EAE) mice (Cannella, Hoban et al. 1998). Treatment with rhGGF2 delayed the signs of demyelination and decreased severity of the phenotype, and resulted in statistically significant reductions in relapse rates. Furthermore, animals treated with rhGGF2 demonstrated increased remyelination over controls. They also showed that these effects were well correlated with mRNA expression of myelin basic protein exon 2 (a remyelination marker), and also an increase in IL-10 (a cytokine with important inflammatory and immunomodulatory effects) at both the RNA and protein levels. Recent studies by Taveggia et al have shown that increased NRG1 expression correlates with oligodendroglial remyelination noting that regulation of NRG1 in the CNS is an important determinant of the extent of myelination (Taveggia, Thaker et al. 2008). A recent meta-analysis by Li and colleagues has shown that there is a strong positive association between NRG1 and schizophrenia (Li, Collier et al. 2006). That result confirms several earlier associations (Petryshen, Middleton et al. 2005; Sullivan 2005). While the complete role of NRG1 in the brain is not fully understood, it has been shown that NRG1 can enhance remyelination in models of MS (Cannella, Hoban et al. 1998). In our studies,
increased expression of NRG1 was seen in both NAGM (↑142.19) and NAWM (↑19.94-fold), with the former being more pronounced.

Given its role in oligodendroglial myelination, NRG1 and its various isoforms remain important targets for future research. Rather than the current immunosuppressive therapies available, neurotrophic factors could constitute excellent targets for future targeted therapies, however efforts would first have to be made to better understand these factors and how they are targeted to axons and glia (Loeb 2007).

10.3.4.3 PTPRC in NAGM Findings

In Chapter 6, protein tyrosine phosphatase receptor type C (PTPRC) gene expression was also found to be increased in both the overall analysis (↑31.15-fold, \( P=0.0011 \)) and the frontal lobe samples (↑22.60, \( P=5.34\times10^{-5} \)) in the secondary-progressive grey matter tissue of multiple sclerosis brains compared to healthy matched neuropathology free controls. Immunoblot was also used to confirm the qualitative presence of the PTPRC protein.
The protein tyrosine phosphatase receptor type C gene (PTPRC) codes for the leukocyte common antigen (LCA) CD45. The LCA is a major high molecular mass leukocyte cell surface molecule that is known to be extensively involved in immune response and regulation. The CD45 molecule is required for both lymphocyte activation and development (Trowbridge and Thomas 1994). In this study, PTPRC expression was found to be significantly increased in the NAGM of MS brain tissues. Increased PTPRC expression has previously been associated with regulatory events that facilitate pathological cascades allowing autoreactive lymphocytes to initiate immune responses in the brain (Compston and Coles 2008). Failure of effector cells to suppress regulatory lymphocytes in the MS brain has also been shown (Viglietta, Baecher-Allan et al. 2004). An area of MS research that is currently generating much interest is the investigation of the balance between T-cells and their regulators (T-regulatory cells). In a T-cell-mediated autoimmune response, such as that seen in multiple sclerosis, CD4+ T-cells are thought to orchestrate immune responses that result in inflammation within the brain and spinal cord. In addition to promoting inflammation, CD4 T-cells also have the ability to prevent and reduce inflammation. (Dittel 2008). Frasca and colleagues have reported that CD4+ T-cells have the ability to both amplify and delete CD8+ (cytotoxic) T-cells (Frasca, Piazza et al. 1998). Studies in the mouse model EAE have previously shown that T-regulatory cells can limit the levels of the proinflammatory cytokine interferon-gamma (IFN-γ) (O'Connor, Malpass et al. 2007).
The role of T-regulatory (T-reg) cells in the control of autoaggressive immune responses is the subject of intense investigation and recent studies in the EAE mouse model have sparked interest in the development of T-reg targeted immunotherapies (O'Connor and Anderton 2008). Those therapies may be directed against immune cells or their receptors, such as CD45. PTPRC may be playing a key role in coding for the receptor that mediates those responses. Tackenberg and colleagues conducted isoform expression studies in PTPRC in another autoimmune condition Myasthenia gravis and concluded that various isoforms of the gene might be responsible for different T-cell responses (Tackenberg, Nitschke et al. 2003). T-cells are known to attack the brain and spinal cord in MS. The common leukocyte antigen receptor coded for by the gene PTPRC is an important determinant of immune responses in MS. The increased gene expression of PTPRC that was found in our investigations suggests that further studies into this gene may provide valuable insights into the role of the common leukocyte antigen receptor in MS pathogenesis.

10.3.4.4 TLR3 in NAGM Findings

The toll-like receptor 3 gene (TLR3) was found to be increased in the overall analysis (↑101.29, P=0.0004) in the secondary-progressive grey matter tissue (NAGM) of multiple sclerosis brains compared to healthy matched controls. Immunoblots were also carried out to test for the TLR3 protein.
TLR3 was originally mapped to chromosome 4q35 by Rock and colleagues in 1998 (Rock, Hardiman et al. 1998). Toll-like receptors are known to participate in activation of adaptive immune responses (Schnare, Barton et al. 2001; Barton and Medzhitov 2002). In this study, TLR3 gene expression was found to be significantly increased in the NAGM (as well as the NAWM) of the tested MS brain tissues. Immunohistochemical studies of TLR3 in rat brain have shown that the gene is highly expressed in lesions but not in normal, healthy controls (Bsibsi, Ravid et al. 2002; Bsibsi, Persoon-Deen et al. 2006). In a more recent study, Cameron and colleagues found that toll-like receptor 3 (TLR3) is expressed in neurons and is concentrated in areas they refer to as growth cones (Cameron, Alexopoulou et al. 2007). They found that activation of TLR3 with poly I:C rapidly induced growth cone collapse and inhibited neuronal outgrowth. Furthermore, poly I:C injections resulted in impaired axonal development and sensory-motor deficits. Those findings indicate that TLR3 activation negatively regulates axonal growth and might provide a future target for injury-repair strategies. Given that TLR-mediated activation of innate immunity controls host defence our findings suggest that TLR3 may be an important marker involved in the development of MS that requires further investigation.
10.3.4.5 NAGM Non-Significant Results

Although expression changes were also seen in CLDN11 in overall (↓1.00-fold), frontal (↓1.44-fold) and temporal lobe grey matter (↑1.77-fold), NRG1 frontal (↑19.75-fold), PTPRC temporal (↑79.34-fold) and TLR3 frontal (↑26.26-fold) samples; those results did not reach statistical significance (all P>0.05).

10.3.5 Gene Expression Results Summary

Overall, our gene expression studies showed statistically significant increased gene expression differences in the genes NRG1 and TLR3 in both the NAWM and NAGM. Additionally, statistically significant increased gene expression of the genes GDNF and PTPRC were observed in the MS samples compared to healthy matched controls but those changes were confined to the NAGM samples.
10.4 Genetic Variation Studies

10.4.1 Genetic Variation Studies Overview

In the second part of this thesis we undertook genetic variation studies of MTHFR A1298C and MTRR A66G by restriction fragment length polymorphism (RFLP) methods (Chapter 7), PTPRC C77G by high resolution melt (HRM) analysis; and C772T by RFLP (Chapter 8), and TLR3 [-/A]_8 by capillary gel electrophoresis; and C1236T by TaqMan genotyping assay (Chapter 9). The study population consisted of genomic DNA (gDNA) samples from 205 cases and an equal number of age, gender, and ethnicity matched controls (Caucasians) from an Australian population. Our investigations did not yield a statistically significant result for any of the genomic case-control association studies undertaken (all P>0.05).

10.4.2 Genetic Variation Studies Background

Previous investigations have shown that MS harbours a significant genetic component, however it is not yet known exactly what genes or their particular variants are involved (Sawcer 2008). A fundamental premise of this research is that particular genetic variations unpin the susceptibility to developing MS. Although the presence of heritability in MS is well established, the genes involved
in the numerous biological processes that contribute to the overall phenotype currently remain undefined.

In the second part of this thesis genetic investigations were undertaken in selected candidate genes that have been implicated in fundamental biological processes involved in MS. Previous approaches in other research have included investigation of animal models (such as EAE and TMEV), familial investigations, and more recently, genome-wide association (GWA) studies. Although genetic association studies have some limitations, many benefits have also emerged from these investigations over the years (Moutou, Gardes et al. 2004).

To date, several studies have implicated numerous, potentially valuable associations between susceptibility and MS in particular populations; interleukin-7 receptor gene IL7R (Gregory, Schmidt et al. 2007), the kinesin family member 1B gene KIF1B (Aulchenko, Hoppenbrouwers et al. 2008), and most notably the MHC alleles DR15 and DQ6 with the corresponding DRB1*1501, DRB5*0101, DQA1*0102, and DQB2*0602 genotypes being most notable (Compston and Coles 2008). The degree of applicability of these types of findings to other populations is not always immediately evident. Positive genomic associations in other diseases such as epilepsy (Scheffer and Berkovic 2003), Alzheimer’s disease (Hernandez-Charro, Moreno et al. 2004), diabetes mellitus (Marrosu, Motzo et al. 2004), and breast cancer (Cheng, Chen et al. 2005) have in the past
sometimes gone on to show limited benefit in populations other than the initial study cohort.

Conflicting results have also been reported in MS research in the genes coding cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (Rasmussen, Kelly et al. 2001; Kantarci, Hebrink et al. 2003), protein tyrosine phosphatase receptor type C (PTPRC) (Jacobsen, Schweer et al. 2000; Cocco, Murru et al. 2004), and the human endogenous retrovirus K113 (HERV-K113) (Moyes, Martin et al. 2005; Moyes, Goris et al. 2008). Statistically speaking, large populations are required to demonstrate high statistical power and detect small effect sizes, however it is a cruel irony that large populations are also clinically heterogeneous and that small effects and complex interactions may be lost in those analyses (Lewis 2002).

The genetic variation studies carried out as part of this research focused on candidate genes involved in homocysteine remethylation (MTHFR), one-carbon metabolism (MTRR), common human leukocyte antigen receptor signalling (PTPRC), and recognition of pathogen-associated molecular patterns (PAMPs) (TLR3). The rationale behind these investigations was that these processes are involved in molecular mechanisms that may be dysregulated leading to the development of MS.
10.4.3 Genetic Variation Studies Discussion

10.4.3.1 MTHFR & MTRR Genetic Variation Studies

In Chapter 7 we undertook genotyping in the genes MTHFR and MTRR. The genes MTHFR and MTRR code for 5,10-methylenetetrahydrofolate reductase, and 5-methyltetrahydrofolate-homocysteine methyltransferase reductase, respectively. Methionine synthase reductase is an enzyme that, under normal conditions, catalyzes the remethylation of homocysteine (Hcy) to methionine via cobalamin and folate dependant reactions. Cobalamin acts as an intermediate methyl carrier between methylenetetrahydrofolate reductase and Hcy and thus 5-methyltetrahydrofolate-homocysteine methyltransferase reductase plays an important role in maintaining cobalamin in an active form. In this way total plasma Hcy (pHcy) concentrations can be regulated. Elevated intracellular pHcy levels have previously been implicated as having a role in CNS dysfunction, neurodegenerative, and cerebrovascular diseases through inhibition of the methylation of myelin basic protein (MBP) and membrane phospholipids, and also disruption of biogenic amine metabolism (Troen 2005). The total case-control population consisted of 140 cases and 140 age, gender, ethnicity (Caucasian) matched controls. Of the cohort of 140 cases and controls, 10 cases and 9 matched controls (MTHFR) and 17 cases and 16 matched controls (MTRR) were excluded due to genotyping difficulties, leaving 130 cases and 131 matched controls (MTHFR) and 123 cases and 124 matched controls (MTRR) for final analysis.
It is thought that the central nervous system may be particularly sensitive to pHcy increases as this process promotes excitotoxicity by stimulation of N-methyl-D-aspartate (NMDA) receptors which may result in damage to neuronal DNA, subsequently triggering apoptosis in neuronal cells (Kruman, Culmsee et al. 2000; Ho, Ortiz et al. 2002). Although previous studies report positive associations for other markers within MTHFR, such as the C66T variant with Schizophrenia (Susser, Brown et al. 1998; Muntjewerff, Kahn et al. 2006; Muntjewerff, Gellekink et al. 2008), and also MTRR A66G with increased pHcy levels (Gaughan, Kluijtmans et al. 2001) and neuropsychiatric disorders (Lindenbaum, Healton et al. 1988; Lindenbaum, Healton et al. 1995), we found no significant allelic frequency difference between cases and controls for either of the tested markers (MTHFR $X^2=1.15$, $P=0.28$, MTRR $X^2=0.005$, $P=0.95$) in our MS population.

10.4.3.2 PTPRC Genetic Variation Studies

In Chapter 8 we undertook investigation of two genetic markers in the gene PTPRC. The study population consisted of 205 cases and 205 matched healthy controls however, the total number of successfully genotyped cases and controls were 155 cases and 171 matched healthy controls for the C77G marker and 181 cases and 180 healthy matched controls for the C772T marker. The remaining samples proved difficult to amplify efficiently or were not able to be definitively categorised during analysis.
Autoreactive T-lymphocytes and their associated antigens have long been presumed important features of MS pathogenesis. The Protein tyrosine phosphatase receptor type C gene (PTPRC) encodes the T-cell receptor CD45 (also designated the leukocyte common antigen; LCA). Variations within PTPRC have been previously associated with diseases of autoimmune origin such as type 1 diabetes mellitus, Graves' disease, and hepatitis infection susceptibility (Boxall, Stanton et al. 2004; Ward, Hennig et al. 2006). We set out to investigate two variants within the PTPRC gene, C77G and C772T in patients with MS and matched healthy controls to determine whether significant differences exist in these markers in an Australian population. We employed high resolution melt analysis (HRM) and restriction length polymorphism (RFLP) techniques to determine genotypic and allelic frequencies. Our study found no significant difference between case and control frequencies for PTPRC C77G by either genotype ($X^2=0.65, P=0.72$) or allele ($X^2=0.48, P=0.49$). Similarly, we did not find evidence to suggest an association between PTPRC C772T by genotype ($X^2=1.06, P=0.59$) or allele ($X^2=0.20, P=0.66$). Hardy-Weinberg equilibrium (HWE) analysis showed that C77G was not in HWE ($P<0.05$), therefore we undertook linkage disequilibrium (LD) analysis, which showed that strong linkage disequilibrium exists between the two tested markers ($D'=0.9970, SD=0.0385$). This finding indicates that although the C77G marker may be relatively uninformative, the other tested marker C772T constitutes a good surrogate for the former. Previous studies investigating C77G have reported positive associations in some studies (Jacobsen, Schweer et al. 2000; Vyshkina, Leist et
al. 2004) and negative findings in others (Barcellos, Caillier et al. 2001; Cocco, Murru et al. 2004). This study reveals no evidence to suggest that these markers are associated with MS in the tested Australian Caucasian population. Although the PTPRC gene has a significant role in regulating CD4$^+$ and CD8$^+$ autoreactive T-cells, interferon-beta (IFN-β) responsiveness, and potentially other important processes, our study did not find evidence for a positive association in the tested MS population.

10.4.3.3 TLR3 Genetic Variation Studies

In Chapter 9 we undertook investigations of the [-/A]$_8$ insertion-deletion and C1236T genetic variations within the human toll-like receptor 3 gene (TLR3) for association with multiple sclerosis in an Australian population. For the [-/A]$_8$ marker 205 (99%) cases and 196 (94%) controls were genotyped successfully. For the C1236T marker 199 (96%) cases and 198 (95%) matched controls were genotyped successfully. The remaining samples proved difficult to amplify efficiently or were not able to be definitively categorised during analysis.

Toll-like receptor 3 is coded by the gene TLR3 and is recognised as an important factor in virus recognition and is known to be involved in expression of neuroprotective mediators (Bsibsi, Persoon-Deen et al. 2006). We set out to investigate 2 variations within the TLR3 gene, an 8bp insertion-deletion [-/A]$_8$ and a single base-pair variation C1236T in subjects with MS and matched healthy
controls to determine whether significant differences exist in these markers between cases and matched, healthy controls. We used capillary gel electrophoresis and TaqMan genotyping assay techniques to resolve genotypes for the markers, respectively. Our study found no significant difference between frequencies for TLR3 [-/A] by genotype ($X^2=1.03, P=0.60$) or allele ($X^2=1.09, P=0.30$) between cases and controls. Similarly, we found no evidence for association for TLR3 C1236T by genotype ($X^2=0.35, P=0.84$) or allele frequency ($X^2=0.31, P=0.58$).

Recent studies report that activation of human microglia via TLR3 mediated signalling can change the profile of local central nervous system immune response by translation of $T_{H1}$ polarizing signals to $CD4^+ T$-cells (Jack, Arbour et al. 2007). TLR3 is a key determinant in innate and adaptive immune responses to viral infections (Heinz, Haehnel et al. 2003). A Japanese study reveals a positive association in that population for TLR3 polymorphisms with Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), which result from disordered innate immune response (Ueta, Sotozono et al. 2007). Other studies have emphasized the role of TLR3-signaling, which can induce the initial inflammatory cytokine responses that are seen in some forms of encephalitis and also multiple sclerosis (So, Kang et al. 2006).

Although TLR3 remains both an interesting and important gene in terms of gene expression and regulation, this genomic investigation revealed no evidence to
suggest that the TLR3 [-/A]₈ insertion-deletion or the C1236T variation are positively associated with MS in the tested population.
10.5 General Conclusions

10.5.1 Current Knowledge

It is not clear why some people are more susceptible to multiple sclerosis than others. This is a conundrum that has faced MS researchers for many decades, since approximately the 1830s. Genetic investigations in MS have focused on large cohorts of families including MS patients, and in particular the investigation of twins. Such studies have used genomic screening approaches to identify regions of interest that may contribute to the risk of developing MS; however we still do not have a definitive list of causative genes. Amongst the only unambiguous genetic associations to date is the HLA Class II region located on chromosome 6p21.3 (Lincoln, Montpetit et al. 2005; Ramagopalan, Herrera et al. 2008).

MS is generally considered to be a T-cell-mediated autoimmune demyelinating disease and it is therefore hypothesised that antigen recognition through human leukocytes antigens (HLAs) plays an important role in the disease process. Investigations of MS epidemiology have shown that cases are not evenly distributed around the world, lending credence to the notion that MS Susceptibility is both environmentally and genetically determined.
We now know that individuals acquire risk levels of new areas of residence if they migrate in early adulthood, MS is less common at the equator, and it affects approximately twice as many women as men (Ebers and Sadovnick 1994). The equatorial gradient has been linked to sunlight exposure and thus vitamin D status over the years, but studies have produced spurious findings with some reporting positive associations (Fukazawa, Yabe et al. 1999) whilst others failed to demonstrate the same association (Niino, Kikuchi et al. 2002). More research needs to be carried out into the role of vitamin D before its true role can be clearly deduced.

A persistent notion in MS research is that infectious agents may trigger MS in susceptible individuals. Indeed, viruses have been used to induce animal models of the disease (Dal Canto, Kim et al. 1996), and the link between viral infections and autoimmune disease has been intently investigated over the years (Cirone, Cuomo et al. 2002; Bach 2005; DeLorenze, Munger et al. 2006). A causal relationship between MS and a pathogen not yet been identified.
10.5.2 Findings & Implications

10.5.2.1 Gene Expression Studies Synopsis

For the gene expression studies 5 genes were investigated; claudin 11 (CLDN11), glial cell derived neurotrophic factor (GDNF), neuregulin 1 (NRG1), protein tyrosine phosphatase receptor type C (PTPRC), and toll-like receptor 3 (TLR3).

In the first part of this thesis we undertook gene expression studies in normal-appearing white matter (NAWM studies, Chapter 5) and normal-appearing grey matter (NAGM studies, Chapter 6) of multiple sclerosis (MS) affected brain tissues to determine if significant differences in gene expression exist in the MS brain compared to normal, healthy, age, gender, ethnicity (Caucasian), and anatomically matched controls. The genes investigated included candidates involved in central nervous system myelination (CLDN11), the survival and differentiation of neurons (GDNF), the growth and differentiation of neuronal and glial cells (NRG1), B- and T-cell receptor antigen signalling (PTPRC), and pathogen recognition and activation of innate immunity (TLR3). Frontal and temporal brain tissue samples from secondary-progressive MS sufferers were compared to healthy matched neuropathology free controls. The studies in NAWM were focused on the role of the candidate genes in the white matter tissue of the brain which is predominantly neuroglia, whilst the NAGM studies
were focused on the candidate genes relative to their role in the grey matter of the brain, which are predominantly neurons.

Increased expression of NRG1, and TLR3 in the NAWM MS brain tissues portray a gene expression pattern consistent with gliosis and attempted remyelination in the tested samples. Neuregulins are known to exist in a number of isoforms. Given their role in growth and differentiation of neuronal and glial cells, more investigations of this gene are justified.

The increased expression of TLR3 heralds T-cell activity (T-helper and T-reg cell activation) and possibly transcriptional activation of proinflammatory genes leading to the expression of proinflammatory cytokines such as TNF-α and IL-1. These observations suggest that further studies in TLR3 in MS may yield additional important findings.

We undertook studies in normal-appearing grey matter (NAGM) in Chapter 6 and found that the expression of GDNF, PTPRC, NRG1, and TLR3 genes were increased in the NAGM of the tested samples.

Increased expression of GDNF, NRG1, PTPRC, and TLR3 suggest gene expression patterns primarily consistent with astrocytes attempting to induce protection of dopaminergic neurons (↑GDNF), attempted oligodendroglial myelination (↑NRG1), autoreactive lymphocytes driving the process of induction
of proinflammatory cytokines (↑PTPRC), and the activation of TLR3 negatively regulating axonal growth (↑TLR3).

The increased expression of NRG1 and TLR3 in both NAWM and NAGM suggest significant systemic widespread changes of these genes in relation to MS and could be plaque inflammation triggered. The other two genes that were found to be significantly different in the NAGM (GDNF and PTPRC) may indicate secondary changes in response to disease effects from plaque inflammation and damage. The upregulation of these genes may also represent other significant pathological processes in MS and their exact contributions still remain to be thoroughly investigated. Further studies of these genes may help define their roles in the pathogenesis of MS.

Whilst every effort was made to match the case and control brain tissue samples used in this research (matched for age, gender, ethnicity, anatomical location, and autolysis time), it is possible that some of the variation observed between gene expression differences between cases and controls may have been due to the origin of those tissues (cases were collected from the Human Brain and Spinal Fluid Resource Center, Los Angeles, USA, whilst the controls were collected from National Health and Medical Research Brain Bank, University of Queensland, Australia). The equatorial gradient has been associated with incidence of MS (MS occurs more frequently in higher latitudes than in places close to the equator) in some studies, which predict that lack of sunlight spurs
chronic Vitamin D deficiencies that initiate the cascade of immune events causing MS (Hayes, Cantorna et al. 1997; Hayes 2000). However, other studies have been unable to reproduce the association, instead stating that reduced exposure to sunlight in higher latitudes alters normal secretion of melatonin by the pineal gland, creating an immunosuppressant effect that sets the stage for developing the disease (Sandyk and Awerbuch 1994; Sandyk 1995). It remains unclear as to whether any of the genes tested in this research are directly affected by variations in exposure to sunlight, and whether Vitamin D related genes influence or interact with the tested genes potentially confounding the analyses. Latitude-matched samples could be used in future studies to investigate this effect.
10.5.2.2 Genetic Variation Studies Synopsis

For the genetic variation studies 4 genes encompassing 6 variants were investigated; 5,10-methylenetetrahydrofolate reductase (MTHFR A1298C), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR A66G), protein tyrosine phosphatase receptor type C (PTPRC C77G and C772T), and toll-like receptor 3 (TLR3 [-/A] and C236T). Our findings do not support a positive association between any of the tested markers and MS in our population with all P>0.05; (MTHFR χ²=1.15, P=0.28); (MTRR χ²=0.005, P=0.95); (PTPRC C77G χ²=0.65, P=0.72); (PTPRC C772T χ²=1.06, P=0.59); (TLR3 [-/A] χ²=1.03, P=0.60); (TLR3 C1236T χ²=0.35, P=0.84).

Although our findings do not suggest a positive association between MS and the tested markers, modest effects within markers of these genes should not yet be discounted. Larger sample sizes and other markers within these genes may yield still yield significant results. Given the roles of these genes in other neurological disorders such as MTHFR & MTRR in Schizophrenia, PTPRC in immune activation and response, TLR3 in proinflammatory cytokine secretion, further studies investigating variants affecting gene expression and/or regulation within these genes, may yield valuable results in future studies. Pathological heterogeneity is well established in multiple sclerosis (Lucchinetti, Bruck et al. 2004; Morales, Parisi et al. 2006; Lucchinetti 2008) and alludes to the importance
of defining the genetic variations that interact with environmental factors in disease development.
10.5.3 Future Directions

Compelling evidence suggests that MS is a complex genetic disorder. The identification of MS susceptibility genes remains a significant challenge. The detection of definitive susceptibility loci would contribute greatly to our understanding of the aetiology of the disease. Genetic advances are likely to contribute to a better knowledge of the heterogeneity, clinical course, prognosis, and response to therapy for sufferers. Methods of rapid high-throughput screening should be considered, and statistical methods allowing for the extrapolation of modest effects could be developed. The study of non-Caucasian populations could provide interesting and valuable new insights into the genetics of the disease. Genomic and clinical information could be better combined to facilitate more informative study populations. Pharmacogenomic studies, investigations of the response patients with specific genotypes demonstrate with particular therapies, could also provide valuable insights. The detection of an MS pathogen/s remains elusive. Investigation of infectious triggers could yield important findings even though the methods used to date have not yet clearly implicated a causative agent. Research investigating genes involved in molecular pathways such as inflammation and myelination remain fundamental to understanding the disease. Finally, if research efforts are to yield any benefit at all, the need to validate whether the results obtained from investigations are a result of cause or the effect of the disease cannot be over emphasized.
Chapter 11

References


McAlpine, D. (1961). "The benign form of multiple sclerosis. A study based on 241 cases seen within three years of onset and followed up until the tenth year or more of the disease." *Brain* **84**: 186-203.


Appendices

Appendix A - Candidate Genes Investigated

Appendix B - Brain Tissue Localisations

Appendix C - qRT-PCR Primers and Transcriptional Targets

Appendix D - qRT-PCR Conditions
Appendix A – Candidate Genes Investigated

CLDN11

Name: Claudin 11 (oligodendrocyte transmembrane protein)

Cytogenetic Location: 3q26.2-q26.3

Transcript Investigated: NM_005602 (lone transcript of interest)

Overview: The protein encoded by this gene belongs to the claudin family of tight junction associated proteins and is a major component of central nervous system myelin that is necessary for normal CNS function. There is growing evidence that the protein determines the permeability between layers of myelin sheaths via focal adhesion and, with its expression highly regulated during development, may play an important role in cellular proliferation and migration. In addition, the protein is a candidate autoantigen in the development of autoimmune demyelinating disease.

GDNF

Name: Glial cell derived neurotrophic factor

Cytogenetic Location: 5p13.1-p12

Transcript Investigated: NM_000514 (iso-1 preprotein)

Overview: This gene encodes a highly conserved neurotrophic factor. The recombinant form of this protein was shown to promote the survival and differentiation of dopaminergic neurons in culture, and was able to prevent
apoptosis of motor neurons induced by axotomy. The encoded protein is
processed to a mature secreted form that exists as a homodimer. The mature
form of the protein is a ligand for the product of the RET (rearranged during
transfection) protooncogene. In addition to the transcript encoding GDNF, two
additional alternative transcripts encoding distinct proteins, referred to as
astrocyte-derived trophic factors, have also been described. Mutations in this
gene may be associated with Hirschsprung disease.

MTHFR

Name: 5,10-methylenetetrahydrofolate reductase (NADPH)

Cytogenetic Location: 1p36.3

Overview: Methylenetetrahydrofolate reductase (EC 1.5.1.20) catalyzes the
conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a
cosubstrate for homocysteine remethylation to methionine.
MTRR

**Name:** 5-methyltetrahydrofolate-homocysteine methyltransferase reductase

**Cytogenetic Location:** 5p15.3-p15.2

**Overview:** Methionine is an essential amino acid required for protein synthesis and one-carbon metabolism. Its synthesis is catalyzed by the enzyme methionine synthase. Methionine synthase eventually becomes inactive due to the oxidation of its cob(I)alamin cofactor. The protein encoded by this gene regenerates a functional methionine synthase via reductive methylation. It is a member of the ferredoxin-NADP(+) reductase (FNR) family of electron transferases. Patients of the cbl-E complementation group of disorders of folate/cobalamin metabolism are defective in reductive activation of methionine synthase. Alternative splicing of this gene results in multiple transcript variants encoding distinct isoforms.

NRG1

**Name:** Neuregulin 1

**Cytogenetic Location:** 8p21-p12

**Transcript Investigated:** NM_013959 (lone transcript of interest)

**Overview:** Neuregulin 1 (NRG1) was originally identified as a 44-kD glycoprotein that interacts with the NEU/ERBB2 receptor tyrosine kinase to increase its phosphorylation on tyrosine residues. It is known that an extraordinary variety of different isoforms are produced from the NRG1 gene by alternative splicing. These isoforms include heregulins (HRGs), glial growth factors (GGFs) and
sensory and motor neuron-derived factor (SMDF). They are tissue-specifically expressed and differ significantly in their structure. The HRG isoforms all contain immunoglobulin (Ig) and epidermal growth factor-like (EGF-like) domains. GGF and GGF2 isoforms contain a kringle-like sequence plus Ig and EGF-like domains; and the SMDF isoform shares only the EGF-like domain with other isoforms. The receptors for all NRG1 isoforms are the ERBB family of tyrosine kinase transmembrane receptors. Through interaction with ERBB receptors, NRG1 isoforms induce the growth and differentiation of epithelial, neuronal, glial, and other types of cells.

PTPRC

**Name:** Protein tyrosine phosphatase, receptor type, C

**Cytogenetic Location:** 1q31-q32

**Transcript Investigated:** NM_080921 (isoform-2 precursor; in conserved region)

**Overview:** The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. This PTP contains an extracellular domain, a single transmembrane segment and two tandem intracytoplasmic catalytic domains, and thus belongs to receptor type PTP. This gene is specifically expressed in hematopoietic cells. This PTP has been shown to be an essential regulator of T- and B-cell antigen receptor signaling. It functions through either direct interaction with components of the antigen receptor
complexes, or by activating various Src family kinases required for the antigen receptor signaling. This PTP also suppresses JAK kinases, and thus functions as a regulator of cytokine receptor signaling. Four alternatively spliced transcripts variants of this gene, which encode distinct isoforms, have been reported.

TLR3

**Name:** Toll-like receptor 3

**Cytogenetic Location:** 4q35

**Transcript Investigated:** NM_003265 (lone transcript of interest)

**Overview:** The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. They recognise pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression. This receptor is most abundantly expressed in placenta and pancreas, and is restricted to the dendritic subpopulation of the leukocytes. It recognises dsRNA associated with viral infection, and induces the activation of NF-kappaB and the production of type I interferons. It may thus play a role in host defense against viruses. Use of alternative polyadenylation sites to generate different length transcripts has been noted for this gene.
### Appendix B - Brain Tissue Localisations

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Localisation/s</th>
<th>Pathology</th>
<th>Hemisphere</th>
<th>Gender</th>
<th>Age</th>
<th>Autolysis</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anterior Intraventricular Foramen Radiata Corporis Callosi</td>
<td>Chronic Inactive</td>
<td>Left</td>
<td>F</td>
<td>76</td>
<td>9.5</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>Anterior Frontal Lentiform Thalamus Radiata Corporis Callosi</td>
<td>Chronic Active</td>
<td>Right</td>
<td>F</td>
<td>64</td>
<td>9.0</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>Posterior Thalamus Radiata Corporis Callosi</td>
<td>Active</td>
<td>Left</td>
<td>F</td>
<td>79</td>
<td>12.0</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>Superior Frontal Gyrus Anterior Genu Corpus Callosum</td>
<td>Chronic Active</td>
<td>Left</td>
<td>M</td>
<td>61</td>
<td>10.0</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>Anterior Caudate Nucleus Gyrus Frontalis Medius</td>
<td>Chronic Active</td>
<td>Left</td>
<td>F</td>
<td>53</td>
<td>23.6</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>Anterior Intraventricular Foramen Radiata Corporis Callosi</td>
<td>Chronic Active</td>
<td>Left</td>
<td>F</td>
<td>51</td>
<td>19.5</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>Cornu Posterius Inferior Gyrus Temporalis Medius</td>
<td>Chronic Active</td>
<td>Right</td>
<td>F</td>
<td>57</td>
<td>19.8</td>
<td>14</td>
</tr>
</tbody>
</table>

1. Microscopic pathology adjacent to normal appearing white matter determined by H&E, LFB, and CD68 stains
2. Years at time of death
3. Hours to histopathology
4. Years of disease duration
## Appendix C - qRT-PCR Primers and Transcriptional Targets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target Transcript/s</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLDN11</td>
<td>NM_005602</td>
<td>CGGTGTGGCTAAGTACAGGC</td>
<td>CAAAGCTCACGATGGTGCTT</td>
<td>Lone Transcript&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>GDNF</td>
<td>NM_000514 (iso-1 preprotein)</td>
<td>GAGCAGTGACTCAAATATGCCA</td>
<td>CCTCTCCGACCTTTTCTCTG</td>
<td>Product Conserved Across NM_199231, NM_199234</td>
</tr>
<tr>
<td>NRG1</td>
<td>NM_013959 (SMDF)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>TGAGGCATAACACTTCACCTGT</td>
<td>AAATACGATTCTCGGTGGCG</td>
<td>Product Conserved Across NM_013958, NM_013957, NM_004495, NM_013961, NM_013964, NM_013960, NM_013956, NM_013956</td>
</tr>
<tr>
<td>PTPRC</td>
<td>NM_080921</td>
<td>ATTACCTGGAATCCCCCTCAA</td>
<td>TTGTGAAATGACACATTGCAGC</td>
<td>Product Conserved Across NM_002838, NM_080922, NM_080923</td>
</tr>
<tr>
<td>TLR3</td>
<td>NM_003265</td>
<td>AAGAGTTTTCTCCAGGGTGTTT</td>
<td>CAGATTCCGAATGCTTTGTTTG</td>
<td>Lone Transcript&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Sensory-Motor Derived Factor

<sup>2</sup>Entrez Gene Database National Centre for Biotechnology Information

<sup>3</sup>Intron-spanning PCR primers
## Appendix D - qRT-PCR Conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cycling Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLDN11</td>
<td>(95°C-5min)1;(95 °C-30s,57 °C-30s,72 °C-60s)40-45;(72 °C-3min)1</td>
</tr>
<tr>
<td>GDNF</td>
<td>(95°C-5min)1;(95 °C-30s,56 °C-30s,72 °C-60s)40-45;(72 °C-3min)1</td>
</tr>
<tr>
<td>NRG1</td>
<td>(95°C-5min)1;(95 °C-30s,58 °C-30s,72 °C-30s)40-45;(72 °C-3min)1</td>
</tr>
<tr>
<td>PTPRC</td>
<td>(95°C-5min)1;(95 °C-30s,57 °C-30s,72 °C-60s)40-45;(72 °C-3min)1</td>
</tr>
<tr>
<td>TLR3</td>
<td>(95°C-5min)1;(95 °C-30s,57 °C-30s,72 °C-45s)40-45;(72 °C-3min)1</td>
</tr>
</tbody>
</table>

Appendix C shows the thermal cycling conditions used for the real-time PCR gene expression experiments in NAWM and NAGM (Chapters 5 and 6, respectively). The same thermal cycling conditions were used for both case and control experiments.