A CLINICAL AND BIOCHEMICAL ANALYSIS OF THE PROGNOSTIC RELEVANCE OF BIOMARKERS IN MULTIPLE SCLEROSIS.

A thesis submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy (PhD).

April 2011

Dr Gillian Ingram, MBChB, MRCP(UK).

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

The work in this thesis was initiated by the development of an extensive serum, plasma, cerebrospinal fluid (CSF) and deoxyribonucleic acid (DNA) multiple sclerosis (MS) disease and control biobank, with samples linked to extensive longitudinal clinical information. The biobank was designed to be utilised with the aim of identifying serum, plasma, CSF and genetic biomarkers for MS. Further, to examine the relevance of potential biomarkers to disease ignition, disease course and progression, response to treatment and ability to predict prognosis in patients with MS, comparing markers to clinical phenotype and non-neurological controls. I elected to investigate various components, fragments and regulators of the complement (C) system based on work showing C to be an important player in MS and in other neuroinflammatory diseases.

An initial pilot study examined serum C regulator factor H (fH) in MS disease subgroups and showed differential expression of fH in patients with progressive disease compared to patients with relapsing remitting disease and controls. These initial results were expanded demonstrating fH as a potentially sensitive and specific biomarker of disease course subgroups. Further, this thesis examines other complement proteins, fragments and regulators in plasma so as to build patient complement profiles specific to, and therefore biomarkers of, disease states.

In an attempt to further knowledge in relation to the expression and extent of C activation in MS, the thesis examines C in CSF and then immunohistochemically in

brain and spinal cord tissue showing up-regulation of C in relation to MS plaques throughout disease course.

This work has identified a novel serum biomarker of MS disease course and patterns of C markers which provide biomarkers of MS and disease state. Up-regulation of C in MS and deposition within white matter plaques, implies a pathogenic role for C in MS.

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed ..... **(candidate)** **Date** 14-04-11....

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree PhD

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STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references.

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STATEMENT 3

I hereby give consent for my thesis to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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To John, Olivia and Jacob

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This work was initiated by the devolvement of a large clinical database of MS patients built up primarily through the hard work of Professor Neil Robertson over the 10 years prior to my developing the biobank. The work involved in setting up and maintaining this resource can not be underestimated and many thanks also go out to Dr Claire Hirst who spent vast amounts of time to improving the quality of the data. I would also like to thank both Neil and Claire who put ethics in place for my work making the start of my research period much easier. I would also like to thank Dr Huw Morris for allowing the use the Cardiff Neurological Disease Biobank and Neurogenetic Research Study for collection of control samples. This thesis would not have been possible without the help and support of University colleagues, Dr Mark Cossburn and Dr Katherine Baker, and NHS staff including Dr T Pickersgill and all of the MS nurses and neurology ward staff in the University Hospital of Wales. Their continual support and enthusiasm allowed collection of samples, once established, to proceed with only guidance. Additional thanks goes to Emma Colley, a medical student who assisted with data collection for Chapter 4. I would also like to thank our patients who continue to generously provide samples and data when-ever required. I am also grateful to the MS Society (UK) who partly funded this research.

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TABLE OF CONTENTS

ABBREVIATIONS

AD	Alzheimer 's disease
ADEAE	Antibody-mediated demyelinating experimental autoimmune encephalomyelitis
AMD	Age-related macular degeneration
APC	Antigen presenting cells
APP	Amyloid precursor protein
A-RRMS	Acute-relapsing remitting multiple sclerosis
BBB	Blood-brain barrier
Blocking buffer	1% bovine serum albumin in phosphate buffer solution
BCB	Blood-CSF barrier
BSA	Bovine serum albumin
C	Complement
CDMS	Clinically definite multiple sclerosis
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nervous system
CPMS	Clinically probable multiple sclerosis
CRP	C-reactive protein
CR1	Complement receptor 1
CSF	Cerebrospinal fluid
CV%	Coefficient of variation
CVF	Cobra venom factor
C1-inh	C1 inhibitor
C4bp	C4 binding protein
C9neo	C9 neoantigen
DAB	Diaminobenzidine
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EBNA	Epstein-Barr Virus nuclear antigen
EDSS	Expanded disability status scale
ELISA	Enzyme-linked immunosorbant assay
GFAP	Glial fibrillary acidic protein
FB	Factor B
FH	Factor H
FHL-1	Factor H-like protein-1
FI	Factor I
GM	Grey matter
GWAS	Genome wide association studies
ICC	Intraclass correlation coefficient
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
LFB	Luxol fast blue
LSDMS	Laboratory supported definite multiple sclerosis
LSPMS	Laboratory supported probable multiple sclerosis
IQR	Interquartile range
mAb	Monoclonal antibody
MAC	Membrane attack complex
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
MCP	Membrane cofactor protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSSS	Multiple sclerosis severity score
Mθ	Macrophages
NAMW	Normal appearing white matter
NF	Neurofilament
NMO	Neuromyelitis optica
NO	Nitric oxide
OCB	Oligoclonal bands
OGD	Oligodendrocyte
OPD	Orthophenylenediamine
OR	Odds Ratio
O_3	Oxygen free radicals
PBS	Phosphate buffer solution
PLP	Proteolipoprotein
PPMS	Primary progressive multiple sclerosis
PQ	Postal questionnaire
ROC	Receiver operating characteristic
RRMS	Relapsing remitting multiple sclerosis
sCR1	Soluble complement receptor 1
SD	Standard deviation
SNP	Single nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
SPSS	Statistical package for Social Sciences
S-RRMS	Stable-relapsing remitting multiple sclerosis
TCC	Terminal complement complex
Th	T helper
TI	Telephone interview
TNF	Tumor necrosis factor
VEP	Visual evoked potential
VLA-4	Very Late Antigen-4
Washing Buffer	Phosphate buffer solution/0.1% Tween 20

CHAPTER SUMMARIES

Chapter 1: Introduction

MS is a complex and heterogeneous disease of the central nervous system (CNS) in which clinical features, including presentation, disease course, and rates of accumulation of disability, demonstrate high degrees of individual variation. This makes the prediction of disease course and outcome by clinical measures difficult. Currently, available markers of disease necessary for accurate diagnosis, prediction of disease course and monitoring of treatments are limited, and for patient management, reliable and practical biomarkers are essential. Chapter 1 reviews the clinical features, aetiology, pathogenesis, and current diagnostic and prognostic markers of MS and examines potential candidates for disease biomarkers.

Chapter 2: Complement in multiple sclerosis: its role in disease and potential as a biomarker

MS has a poorly defined and complex immunopathogenesis; although initiated by reactive T cells, persistent inflammation is evident throughout the disease course. A contribution from C has long been suspected, based on the results of pathological and functional studies which have demonstrated C activation products in MS brain and biological fluids. However, the extent and nature of C activation, and its contribution to disease phenotype and long term outcome, remain unclear. Furthermore, functional polymorphisms in components and regulators of the C system which cause

dysregulation and are known to contribute to other autoimmune inflammatory disorders, have not to date been investigated in MS in any detail. In this chapter I review evidence from pathological, animal model and human functional and genetic studies, implicating activation of C in MS. I also evaluate the potential of C components, fragments and regulators as biomarkers of disease, and suggest appropriate directions for research.

Chapter 3: Methods

For analysis of potential biomarkers, it is essential to have a comprehensive, well-maintained biobank with appropriately processed plasma, serum, CSF and DNA samples linked to clinical data. Here I describe the development of the biobank used for my research and outline the standards for sample collection and processing which ensure reliability of samples for analysis.

Chapter 4: Validity of patient derived disability and clinical data in multiple sclerosis

The South Wales MS clinical database provides detailed information on over 2200 patients; however, given a disease course of over 30 years, longitudinal data, essential for accurate biomarker studies, is often incomplete. The objectives of this study were to determine validity of patient derived historical data and describe the utility of a locally relevant, patient administered questionnaire designed to ascertain current disability and other important disease milestones, allowing retrieval of retrospective missing patient

data. A well-described cohort of 99 patients was identified for whom comparable detailed prospective longitudinal clinician derived data was available. Patient derived data was collected by completion of a standardised questionnaire or telephone interview for comparison.

Reliability analysis for current expanded disability status scale (EDSS) demonstrated an intraclass correlation coefficient (ICC) of 0.79 between questionnaire and clinician derived data in 79 patients with complete agreement in 75.9%. ICC for year of disease onset, diagnosis and onset of secondary progression was 0.86, 0.91 and 0.78 respectively. Time to EDSS >4.0, 6.0 and 8.0 all had ICC of >0.9. Less robust agreement was observed for current disease course (Kappa coefficient 0.71), initial relapse rate (ICC 0.37) and clinical features at disease onset (Kappa 0.25).

In conclusion, self-reported questionnaires can provide reliable current and retrospective data on time to disability milestones with high levels of correlation observed for some additional elements, supporting the use of selected components of patient derived data in clinical practice and for epidemiological, genetic and biomarker studies.

Chapter 5: Complement regulator factor H as a serum biomarker of multiple sclerosis disease state

C regulator fH and its Tyr402His polymorphism have recently been implicated as potential biomarkers of disease in chronic inflammatory CNS conditions such as

Alzheimer's disease (AD) and age-related macular degeneration (AMD). Given the known association of MS and C, regulator fH was examined to determine if it might identify or predict specific pathological processes and outcomes in MS.

Employing novel assays, fH and its His402 variant was measured in serum from 350 MS patients classified according to disease course and relapse status. Serum fH levels were significantly higher in progressive disease ($p<0.001$) compared to controls and relapsing patients, after controlling for variables including disease duration, age, gender, disability and treatment. Serum fH levels were capable of distinguishing secondary progressive from relapsing remitting disease (excluding patients in clinical relapse) with a sensitivity of 89.41%, specificity of 69.47% and a positive predictive value of 72.38%. Acute relapse was also associated with transiently increased fH levels ($p=0.009$) compared to stable relapsing disease. In clinically stable patients, fH levels remained constant over one year (coefficient of variation% (CV%, standard deviation (SD)/mean) = 6.8%), however, in patients in transition from relapsing to progressive disease, fH levels significantly increased over a period of 2 years ($p=0.007$). Concentration of the His402 variant in heterozygotes was significantly higher in secondary progressive ($p<0.01$) and primary progressive ($p<0.05$) disease, suggesting altered expression or consumption of variants when fH is up-regulated.

Serum fH may be an effective indicator of progression and a practical and accessible biomarker and stratifying tool in determining disease course, providing objective evidence to help guide therapeutic decisions.

Chapter 6: Elevated plasma C4a levels in multiple sclerosis correlate with disease activity

C plays a pivotal role in the pathogenesis of MS and C4a, an activated fragment of C component C4, has recently been linked to disease activity. Plasma C4 and plasma and CSF C4a were correlated with clinical disease in a well-characterised cohort of patients and controls.

Plasma C4 levels weakly correlated with plasma C4a ($r=0.27$, $p=0.001$). Plasma C4 was non-significantly ($p=0.056$) elevated overall in patients compared to controls. Plasma C4a levels were significantly elevated in patients sampled during acute relapse compared to both controls ($p<0.001$) and patients with stable relapsing remitting disease ($p=0.004$); decreasing in convalescent post-relapse samples over 2 months ($p=0.04$). CSF C4a was significantly ($p=0.003$) elevated in MS patients compared to controls and furthermore showed correlation with a raised immunoglobulin (Ig) G CSF:plasma ratio, indicating intrathecal production.

Dynamic changes in C4a levels were demonstrated in patients in acute relapse, implying a systemic humoral inflammatory component at relapse ignition; however, because of low sensitivity and specificity between patient groups it is unlikely that C4 or C4a could be employed as reliable clinical diagnostic or disease state biomarkers. In addition, results demonstrate intrathecal and systemic activation of C, reflected in

changes in CSF and plasma C4a. These data support a role for C activation in MS pathogenesis and suggests a systemic component to disease.

Chapter 7: Systemic complement profiles in multiple sclerosis are useful as biomarkers of disease state

Chapters 5 and 6 demonstrate a significant and dynamic systemic and local activation and up-regulation of C evident from C components fH, C4 and C4a, detectable in serum/plasma and CSF samples. The source of C proteins involved in these processes remains unclear and although the majority of C synthesis is hepatic, local production may also contribute to tissue injury. In this chapter analysis of local and systemic C proteins, activation products and regulators is extended in a well phenotyped cohort of MS patients to more completely characterise C activation in MS and to examine complotype profiles as potential biomarkers of disease state.

Using in-house and commercially available assays, C proteins C3, fB, C4 and C1s, activation products Bb, C4a and TCC, and regulators C1 inhibitor (C1-inh), fH and clusterin were measured in plasma from 194 carefully phenotyped patients with MS, together with 35 healthy controls. Additional analysis of CSF for a similar array of C components was performed in a further 37 patients with MS, 5 with CIS and 24 neurological controls. Results were inserted into a logistic regression analysis to develop predictive models for disease state.

Increased plasma levels of C3 ($p=0.004$), C4 ($p=0.001$), C4a ($p<0.001$), C1-inh ($p<0.001$), and fH ($p<0.001$) and reduced levels of C9 ($p<0.001$) were observed in MS patients compared to controls. A combined profile of these C proteins and regulators resulted in a model which differentiated cases from controls with a predictive value of 97%. In addition, independent increases in levels of C9, C4a and fH were observed in acute relapse which in combination with objective clinical markers differentiated patients in acute relapse from those in stable remission with a predictive value of 75%. It was possible to measure C1s, fB, C9, fH, clusterin and TCC concentrations in the CSF; however, there were no significant differences seen between subgroups. CSF plasma correlations suggest the principal source of synthesis for fH, C1s and clusterin is systemic whilst fB and C9 is both systemic and local.

In this chapter, further evidence is provided of alterations in both local and systemic expression of C in MS. C activation occurred via both classical and alternative pathways in acute and chronic disease, suggesting that C targeted therapies may be of use in addition to therapies targeting adaptive immune responses. Furthermore, combined models of systemic C levels with objective phenotypic characteristics seem to be useful biomarkers of disease state and may inform response to treatment.

Chapter 8: An immunohistochemical analysis of complement in multiple sclerosis post-mortem tissue.

There remains some controversy as to the contribution of inflammation to axonal degeneration in MS and the extent and nature of C activation and regulation in relation to pathological processes. The aim of this study was to evaluate a broad range of C proteins, activation products and regulators in MS brain and spinal cord tissue to determine the relationship of C to inflammation and axonal degeneration.

Thirteen plaques from 9 cases of late-stage progressive MS and 3 control patients were examined immunohistochemically with quantification of antibodies in different tissue areas. Extensive deposition and synthesis of C was found predominantly in plaque and periplaque areas specific to astrocytes, with staining of neurons in periplaque grey matter, indicating C synthesis. The data also demonstrate variable myelin staining, predominantly of C regulators, in periplaque and normal appearing white matter (NAWM) areas, co-localizing with APP positive damaged axons. Activation of C was evident even in the centers' of inactive plaques from patients with long disease duration; whereas control patients were consistently negative for cellular C staining.

C immunostaining of MS plaques in this study was specific and was interpreted as direct evidence of C contributing to pathogenic processes even in late-stage disease.

The data suggest that anti-C therapies should be considered in addition to traditional T-cell mediated treatments for preventing disease progression.

Chapter 9: Inflammatory disease associated complement polymorphisms do not contribute to disease phenotype in multiple sclerosis.

Dysregulation of the C system caused by polymorphisms in C components and regulators has been shown to contribute to disease process in a number of neuro-inflammatory disorders including AMD, AD, schizophrenia and vascular disorders. Chapter 9 assesses 8 SNPs in C genes, known to be associated with other neuro-inflammatory diseases, and examines genotype-phenotype correlations in 887 comprehensively characterised MS patients.

After correcting for multiple tests, significant associations were demonstrated between a SNP in C receptor 1 (CR1) and age at onset, and a SNP in fH and typical magnetic resonance imaging (MRI) at disease onset. In addition, association was seen for 3 SNPs in C5 and C3 for reduced time to disability milestones; however, after correcting for multiple tests only one of these remained significant. No association was seen for other phenotypic variables including time to secondary progression. Previous studies examining phenotype-genotype correlations in MS have been limited and none have been convincingly replicated. It is therefore difficult to determine the appropriate number of patients needed to power such a study and it maybe that replication of this

work in larger, similarly phenotyped populations, will provide a clearer picture of relevant associations.

CHAPTER 1: MULTIPLE SCLEROSIS

1.1 Definition and history

MS was first described in 1838 by the pathologist Robert Carswell, then later, in 1841, by Jean Cruveilhier, a Parisian anatomist. However, the first complete description of the disease was presented to the Société Médicale des Hôspitaux in March 1865 and in May 1866 by a French Neurologist, Jean Martin Charcot. Charcot's description of "la sclérose en plaque disseminées" included the clinical and pathological features taken from 3 cases and included insights into the duel nature of myelin loss and axonal damage seen in MS (Sherwin 1957). Since that time MS has become one if the commonest causes of neurological disability in young adults in northern European, Caucasian populations with an approximate life-time risk of 1 in 400 (Compston et al. 2002). MS is a chronic inflammatory demyelinating disease in which lesions are disseminated in time and space. It is characterised by recurrent episodes of focal neurological disability within the CNS, which occur at an unpredictable frequency and remit to varying extents over many years (Confavreux et al. 2006b).

1.2 Epidemiology and aetiology

The prevalence of MS world wide varies, with most studies showing prevalence between 84 (Ford et al. 1998) and 203 (Rothwell et al. 1998) per 100,000 population. The UK lies in a region of high prevalence and has recorded one of the highest disease frequencies in the world at 258 per 100,000 population (Poskanzer et al. 1976); however a recent study in South Wales showed a prevalence of 146 per 100,000

population which had risen over 20 years from 101 per 100,000 population (Swingler et al. 1988; Hirst et al. 2009). This study suggests that the increase in prevalence is predominantly a result of a rising incidence in females, with an increasing sex ratio of incident patients from 1.8:1 to 4.3:1 female:male in the 20 year period; the majority of previous studies have shown female:male ratios of 2:1 (Kurtzke et al. 1979). MS typically effects young adults, the mean age at onset ranges from 28.8-32.6 years (Panelius 1969; Confavreux et al. 1980) for females and 28.7 -31.6 years for males (Panelius 1969; Poser et al. 1979); however, approximately 7-13% of patients present before age 20 years (Panelius 1969; Phadke 1990) and 10% after the age of 50 years (Noseworthy et al. 1983).

The aetiology of MS is likely to be a combination of unidentified environmental challenges and genetic factors. Epidemiological studies have long recognised the important role of genetic factors in the susceptibility to MS with classical twin studies demonstrating a monozygotic concordance rate of 25-38% compared to 3-5% in dizygotic twins (Mumford et al. 1994), a background population risk for non-biological relatives of adoptees compared to biological relatives (Ebers et al. 1995) and increased offspring recurrence in conjugal pairs (Robertson et al. 1997). In addition, a decreasing recurrence risk with genetic distance from the proband in both population-based and clinic cohort studies has been modelled to suggest involvement of multiple genetic loci, each exerting a modest individual effect (Kalman et al. 2002).

Early studies of gene candidates recognised the importance of the major histocompatibility complex (MHC), located on chromosome 6p21 (Bertrams et al. 1972; Jersild et al. 1972; Winchester et al. 1975; Compston et al. 1976). Since that time, nearly all populations studied have shown association with allele DRB1*1501, (Compston et al. 2006) the few exceptions to this being studies in which the frequency of DRB1*1501 is too low or high in the local population making the studies underpowered (Poskanzer et al. 1980). Other DR alleles have also been shown to influence susceptibility - DR08 adds extra risk, whereas DR14 essentially nullifies the risk from DR1501 (Dyment et al. 2005; Barcellos et al. 2006). Other work has confirmed a residual association beyond that attributable to the DRB1 gene, in the HLA-C gene; specifically, the HLA-C*05 allele (or a variant within the area of high linkage disequilibrium (LD)) showed a protective effect ($p = 3.3 \times 10^{-5}$) (Yeo et al. 2007). MHC genes have been suggested by a number of studies to influence disease type and severity as well as susceptibility (Hensiek et al. 2002; Barcellos et al. 2003; DeLuca et al. 2007; Smestad et al. 2007); however, none has been conclusively replicated.

Identification of risk alleles outside of the MHC region has proven more difficult with an increasing realisation of the sample sizes required for definitive genetic studies (Sawcer et al. 2005). More recently, advances in molecular genetic technologies together with increased power from collaborative clinical resources have led to identification and confirmation of single nucleotide polymorphisms (SNPs) within the interleukin-7 receptor (IL7R) (rs6897932; $p=5.45 \times 10^{-20}$) and interleukin-2 receptor

(IL2R) (rs2104286; $p=9.59x10^{-29}$) genes, in which the major allele of each polymorphism confers susceptibility to MS (Gregory et al. 2007; Hafler et al. 2007; Lundmark et al. 2007; IMSGC 2008). The degree of risk for developing MS conferred by these polymorphisms is small (odds ratios: 1.17 IL7R, 1.22 IL2R); however, the SNP variation in these genes causes subtle changes in regulation of these inflammatory mediators including reduction of IL7Rα expression and dysfunction of CD25 regulatory T cells (Puel et al. 1998; Roifman et al. 2000; Shevach et al. 2001; Gregory et al. 2007). Further to this, SNPs rs34536443 from the tyrosine kinase 2 gene ($p=2.7x10^{-6}$), rs12708716 from the CLEC16A gene ($p=1.6x10^{-16}$) and rs763361from the CD226 gene ($p=5.4x10^{-8}$) (the later two both also associated with type 1 diabetes) have also shown association with MS. Further SNPs are likely to be identified with more genome wide association studies (GWAS). Understanding the pathological implications of this expanding set of disease related polymorphisms will give vital disease information for MS and for other autoimmune disorder in which these genes have also been implicated (Vella et al. 2005; Brand et al. 2007).

1.3 Pathogenesis

Overview

The fundamental concept underlying the perceived pathogenesis of MS is of wide arrays of inflammatory mediators interacting to cause both demyelination and axonal damage which in turn results in a variety of neurological deficits (Compston 2004) (fig 1.1).

T-cell activation

T cells are initially activated in the systemic circulation via foreign antigens presented by antigen presenting cells (APCs) with interaction of CD40 on APCs and CD40L on T cells. They are then reactivated within the CNS at times of inflammation or relapse. Unfortunately, the story in MS is not this simple; myelin auto-reactive T cells are present in both MS patients and healthy individuals and normally are suppressed in the immune system by regulatory T cells (Costantino et al. 2008). However, in the MS patient this immune regulation is broken down possibly by impaired functioning of T regulatory cells, which has recently been linked to expression of IL-7Rα (Michel et al. 2008). The failure of regulation leads to proliferation and activation in the systemic circulation of auto-reactive T cells.

Another widely accepted hypothesis in autoimmune disease is the concept of molecular mimicry – if an invading microbe has a component immunologically indistinguishable from a part of the human body (in the case of MS, a component of the oligodendrocyte (OGD) -myelin unit), the immune system can attack "self" by mistake; hence some individuals with auto-reactive T cells develop disease whilst other individuals have high levels of auto-reactive T cells and no autoimmune dysfunction (Libbey et al. 2007). Relapses of MS are often thought to be triggered by a viral infection or immunization. Many common viruses such as Influenza, Measles and Epstein-Barr virus (EBV), have genetic sequences that mimic those found in the myelin sheath (Olson et al. 2001; Olson et al. 2005). The concept of molecular mimicry would explain

how T cell activation by common viruses can precipitate a demyelinating event (Libbey et al. 2007).

Blood-brain barrier breakdown

The CNS is separated from the systemic circulation by a blood-brain barrier (BBB) which consists of a layer of endothelial cells interspersed with tight junctions abutting a basement membrane; a continuous covering to this is provided by the extended foot processes of astrocytes (Persidsky et al. 2006; Correale et al. 2007). In inflammatory episodes of MS, activated T cells express integrins such as Very Late Antigen-4 (VLA-4), which bind to up-regulated cell adhesion molecules on the BBB endothelial surface and allow T cells to adhere to and "walk through" the tight junctions. They then pass through the extracellular matrix which is broken down by various inflammatory molecules including matrix metalloproteinases (MMPs), thereby allowing diapedesis of T cells into the CNS (Rosenberg 2009). Once in the CNS, T cells reencounter antigen and activate microglia, which in turn re-present antigen to T cells setting up a pro-inflammatory loop.

Conduction block

The pathology most clearly responsible for conduction block in MS is segmental demyelination of varying lengths along an internode. However, inflammatory mediators have been recognized to independently cause conduction block; for example, nitric oxide has been shown in animal studies to cause reversible conduction block in central and peripheral demyelinated axons (Redford et al. 1997) and is thought to be responsible for reversible symptoms in MS such as Uhthoff's phenomenon

(symptomatic deterioration with increases in body temperature) and transient worsening of old symptoms at times of infection. Other mechanisms of conduction blockage are unproven but are thought to be due to either impaired function of ion channels (seen in experimental autoimmune neuritis) (Novakovic et al. 1996) or disturbance of metabolic functions of glial cell (Wolswijk et al. 2003).

Demyelination

Demyelination in MS is dependent on multiple factors and the extent of involvement of various mediators is thought to differ in individuals. Activated T cells bind directly to myelin epitopes leading to activation of macrophages which can directly phagocytose myelin or, along with T cells and glial cells, can release toxic products such as proteases (Cuzner et al. 1999) or cytotoxic mediators. These include pro-inflammatory cytokines e.g. tumour necrosis factor (TNF) -α, Interferon (IFN) -γ (Selmaj et al. 1988; Navikas et al. 1996), soluble reactive oxygen and nitrogen intermediate mediators (Smith et al. 1999; Smith et al. 2002), free radicals causing oxidative stress, and glutamic acid causing excitotoxicity (Bruck 2005b). The presence of extensive inflammatory infiltrates such as TNF-α can result in oligodendrocyte death through activation of an apoptotic cascade ultimately involving release by damaged oligodendrocytes, of death ligands or receptors such as Fas, FasL or Trail (Cannella et al. 2007). Further attack to myelin comes from antibodies and C components, mainly the terminal C complex C5b-9 released from infiltrating B cells (Morgan et al. 1984).

Initial studies in MS supported the concept of a T-helper (Th) 1 driven autoimmune condition with the predominant secretion of IFN-γ (Sospedra et al. 2005). However,

more recent work suggests a more complex model including different T-cell populations including Th-17 cells which predominantly secrete IL-17 (Harrington et al. 2005). Evidence from animal models has demonstrated that IL-17 deficient mice are protected from experimental autoimmune encephalomyelitis (EAE) (Komiyama et al. 2006); also Th-1, Th-9 and Th-17 cells have been shown to induce EAE, although each population of T cells induced a different phenotype suggesting that the predominant T-cell population may contribute to disease heterogeneity (Jager et al. 2009). Two papers have now translated this work into humans and established the importance of Th-17 in MS, showing increased numbers of Th-17 cells in blood (Durelli et al. 2009) and CSF (Brucklacher-Waldert et al. 2009) of patients with active disease. Despite this work, the precise contribution of the T-cell subsets and their associated cytokines to disease remains unclear.

Remyelination

At the end of a functional relapse, resolution of inflammation and loss of conduction block leads to variable clinical recovery. Axonal remyelination has been shown to contribute to clinical recovery, evidenced by MRI studies showing up to 40% of sclerotic plaques with signs of remyelination (Barkhof et al. 2003) and pathological studies showing remyelination in some, but not all, areas (Lucchinetti et al. 1996). Remyelinated areas are also known as shadow plaques, inflecting that the remyelination is incomplete with thinner myelin and shorter internodal lengths more susceptible to conduction block at times of recurrent inflammation. The extent to which axons remyelinate is dependent on a number of factors including: the extent of axonal injury,

the survival of OGDs and recruitment of OGD precursor cells, as well as the presence of a conducive environment (Chari et al. 2002).

Axonal damage

Axonal loss, initially attributed to acute episodes of demyelination, has traditionally been felt to play a larger role in the later progressive stages of disease (Bitsch et al. 2000). However, axonal damage has been demonstrated in active and inactive demyelinated plaques (Ferguson et al. 1997; Bitsch et al. 2000; Kornek et al. 2000), as well as in normal appearing white matter outside inflammatory foci (Bjartmar et al. 2001). Axonal damage has also been shown to be present in very early disease, possibly representing an independent primary physiological anomaly (Ferguson et al. 1997; De Stefano et al. 2001; De Stefano et al. 2010). This persistent slow axonal loss is thought to be caused by chronic inflammation but also loss of trophic support from growth factors for neurons and axons (Weiner 2008). The degree to which inflammation and degenerative processes contribute to axonal loss is not clear; however, axonal and neuronal losses are now recognized to be central features of MS occurring throughout all parts of the CNS without the need for prior demyelination (Bruck 2005a).

Relapsing to progressive disease

At initiation, disease is driven by the adaptive immune response, with antigen primed T cells migrating into the CSF where Th1 and Th17 cells release pro-inflammatory cytokines to mount an inflammatory attack (Gocke et al. 2007; Kebir et al. 2007; McFarland et al. 2007). However, it has been suggested that as patients move through their disease course into the progressive phase, the innate immune system (consisting of

macrophages, dendritic cells, mast cells and C) plays a larger role. Progressive disease has been characterised by chronic inflammation associated with activation of peripheral dendritic cells evidenced by higher concentrations of interleukin-12 and 18 released from dendritic cells in progressive patients compared to relapsing patients, with some correlation seen with disease duration (Weiner 2008). Adding to this, a recent pathological study of 67 post-mortem cases has demonstrated inflammatory cells in both early RRMS and progressive patients with high correlation between the amount of inflammation and degree of axonal injury. Interestingly, the authors noted the nature of inflammation changed, with T cells and B cells dominating in RRMS and plasma cells in progressive disease (Frischer et al. 2009).

1.4 Immunopathology

The first detailed histological descriptions of MS describing the pathogenesis of the demyelinating process were documented in the middle of the 19th century. In 1868 Charcot observed: '...the long persistence of the axis cylinders deprived of medullary sheathing, in the midst of the foci of sclerosis, probably play an important part here. Transmission of voluntary impulses would still proceed by means of the denuded axis cylinders, but it would be carried on irregularly, in a broken or jerky manner'.

Central to the pathology of MS is the demyelinating plaque in which, macroscopically, lesions are round, greyish areas with finger-like extensions, centred on medium-sized vessels and accumulating either periventricularly or near the outer surfaces of brain or spinal cord (Bruck 2005b). Microscopically, plaques show axons devoid of myelin

sheaths, embedded in astroglial scar tissue and surrounded by a heterogeneous array of inflammatory infiltrates mainly consisting of lymphocytes and macrophages; but also adhesion molecules, cytokines, chemokines, antibodies, C and other toxic substances. The inflammatory response, located around vessels, is not restricted to areas of demyelination but can affect areas of normal looking white and grey matter (Lovas et al. 2000; Bjartmar et al. 2001) where axonal injury correlates with the degree of residual inflammation (Kornek et al. 2000).

Lesions can be further classified into active demyelinating, inactive demyelinated (distinguished by the extent of destruction of myelin sheaths and myelin debris within macrophages) or remyelinated (so-called shadow plaques in which areas have a reduced density of myelinated fibres associated with thin myelin sheaths) (Lassmann 1983). The active demyelinating plaque typically shows myelin sheaths in the process of dissolution and can be classified further into "acute" or "chronic active". An acute plaque will show heterogeneous demyelination whereas the chronic active plaque has a demyelinated centre surrounded by a zone of active demyelination. Active plaques typically show dense macrophage and T cell infiltration with C and immunoglobulin deposition (Prineas et al. 2001). One study has also suggested that in early MS there is heterogeneity of lesions with some patients showing a pattern with predominant oligodendrocyte apoptosis (Lucchinetti et al. 1996). This is discussed in further detail in Chapters 2 and 8.

1.5 Clinical features and disease course

MS is a highly variable condition in which clinical features are determined by the anatomical location of demyelination. Classical clinical presentations and features include optic neuritis, weakness or sensory loss in arms or legs, ataxia or vertigo. In the presence of classical features in relapsing disease, a firm diagnosis can often be easily confirmed; however, patients can present atypically with progressive disease, or prominent cortical signs such as early dementia, seizures, apraxia or fatigue; in these situations diagnosis can be more complicated.

Natural history studies suggest that about 80% of patients present with a relapsing remitting course (RRMS) (Confavreux et al. 2006a) in which relapses are defined as the first occurrence, recurrence or worsening of symptoms representing neurological dysfunction, marked by a sub-acute onset followed by variable recovery. The whole relapse must last at least 24 hours and there must be at least a month period between 2 relapses (Schumacker et al. 1965; Lublin et al. 1996). The frequency with which relapses occur is highly variable, but clinic based cohorts suggest relapse rates to be around 0.6-1.1 per year (Fog et al. 1970; Patzold et al. 1982), decreasing over time (Panelius 1969; Patzold et al. 1982).

Of the patients presenting with RRMS, less than 20% have a benign disease course with little accumulation of disability EDSS (typically less than 4 over a 20 year period) (Amato et al. 2007; Hirst et al. 2008). However, even within this benign group the majority go on to disability in the long term (Hirst et al. 2008). The term malignant

disease, or aggressive MS, has been historically somewhat poorly defined. A recent well accepted definition states malignant or aggressive disease to be "disease with a rapid progressive course, leading to significant disability in multiple neurological systems or death in a relatively short time after disease onset" (Lublin et al. 1996). Identifying individuals within these specific disease subgroups is becoming more important with the advent of newer therapies with higher side effect profiles which are currently being reserved for patients with more aggressive disease.

At around 10 years from onset approximately 80% of patients with RRMS go on to develop secondary progressive MS (SPMS) in which relapses abate and there is a slow accumulation of disability over a number of years (Lublin et al. 1996). Studies suggest between 5 and 20% of patients present with a primary progressive course (PPMS) which often develops as an upper motor neurone myelopathy affecting the legs, which slowly deteriorates (Cottrell et al. 1999).

At disease onset clinical information offers limited evidence when predicting disease course. It has been suggested that patients with benign disease have a younger age of onset and are more likely to present with sensory symptoms, whilst patients with ataxia and high pyramidal scores on the EDSS, will have a more aggressive disease (Poser et al. 1986; Weinshenker et al. 1991; Hirst et al. 2008). However, at best this information gives us clues as to which patients will benefit from early aggressive treatment, and no firm clinical prognostic markers are available.

1.6 Diagnosis

Classification

Classification of MS has traditionally focussed on a level of probability with the use of paraclinical markers to enhance the likelihood of disease. There have been a number of different classification systems which have been refined over the years including the Allison and Millar classification system in 1954 (Allison et al. 1954), the diagnostic criteria of Schumacher in 1965 (Schumacker et al. 1965) and the McAlpine system in 1972 (McAlpine et al. 1972). One of the most commonly used systems has been the Poser criteria, first established in 1983 (Poser et al. 1983). This has 4 categories – clinically definite (CDMS) or probable (CPMS), and laboratory supported definite (LSDMS) or probable (LSPMS) MS. Using the most recent diagnostic system, the McDonald criteria (McDonald et al. 2001), a definite diagnosis, after excluding differentials, is based on history, examination and, if necessary, the use of additional investigations, to establish dissemination of inflammatory lesions in time and space.

The differential diagnosis of MS is broad and on presentation, investigations such as MRI, CSF examination and blood tests are used to exclude mimicking conditions such as structural lesions, central nervous system vasculitis or vascular malformations. These tests however, are limited in their diagnostic capacities and must be interpreted correctly. The use of visual evoked potentials (VEPs) is helpful in some cases when diagnosis is uncertain or to clarify risk of progression from clinically isolated syndrome (CIS) to CDMS (Gronseth et al. 2000).

Magnetic resonance imaging

T_2 weighted MRI studies of the brain and spinal cord are now used routinely to verify diagnosis according to dissemination in time and space, especially useful in the patient with a CIS in whom MRI can be repeated at intervals to assess accumulation of lesions (Miller et al. 1998). The use of serial MRI scans to monitor disease progression and response to treatment is less reliable. Gadolinium enhancement in T_1 weighted MR images reflects active inflammation, and these enhanced lesions can be present for 4-6 weeks. This can be useful in assessment of a patient's disease activity and is often used as a surrogate marker when considering disease modifying treatments (NICE 2007). There is only weak to moderate correlation between disability and MRI parameters, in particular T2 lesion load (Li et al. 2006); however, more recent studies are showing MRI to be more predictive of outcome, especially when looking at quantitative techniques such as volume measurement (Rashid et al. 2008) and combined lesion and atrophy measures (Bakshi et al. 2008). When considering MR scans, it is important to remember that significant CNS pathology may be present in the absence of signs and symptoms, and changes in MRI are not always consistent with clinical deterioration in the patient (Isaac et al. 1988).

Cerebrospinal fluid

The examination of lumbar puncture fluid has been used for over 70 years in the diagnosis of MS. In the 1960's CSF protein electrophoresis to detect oligocolonal bands (OCB), bands in the gammaglobulin region representing IgG, not paired in serum samples; was first used to increase the sensitivity, if not specificity, of diagnosis in MS (Link 1967). Their role remains important in some more complex cases in which

verification of an immunological mechanism of disease is necessary (Link et al. 2006). Despite their use in diagnosis, OCBs have not reliably demonstrated correlation with clinical phenotype (Koch et al. 2007), although more recently a better prognosis has been reported in OCB negative patients (Joseph et al. 2009).

1.7 Biomarkers in MS

A biomarker may be defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to therapeutic intervention" (Bielekova et al. 2004). In order to be clinically useful, a biomarker must be easy to acquire with minimally invasive techniques, and measurement should be simple, reproducible to a standardized level and cost effective.

In MS the main biomarkers currently used are clinical details and MRI; however, these have limitations as described above. The main biological specimen types deemed to be useful are CSF, serum, plasma, urine, sputum and DNA. Although CSF is likely to provide higher concentrations of relevant biomarkers and reflect alterations in the brain more accurately, it can only be acquired through invasive techniques and hence sample numbers and subgroups are limited for testing and recurrent sampling is impractical. Serum, plasma, sputum and urine are easy to collect in significant quantities and large numbers; however, the levels of markers may be lower and can be altered by sample processing factors such as time to separation, separation techniques and storage (Bielekova et al. 2004). The use of serum markers in MS is not novel; indeed, the well-

known serum marker, oligoclonal IgG, reflects systemic immune disturbance and is associated with elevated intrathecal IgG synthesis in MS (Zeman et al. 1996). Serum markers may exist in MS if there is a systemic component to the disease, or if peripheral changes mimic central disease.

When considering MS, a clinically useful biomarker would provide information on disease ignition, progression and response to treatment, as well as on clinical subtype of disease. In this way it would be easier to predict prognosis and allow rational therapeutic decisions. In view of the two distinct pathological processes of demyelination and axonal loss seen in MS, a single surrogate marker is unlikely to be universally informative and multiple markers reflecting the balance between different pathological processes may be more useful.

Candidate disease markers

Potential biomarkers for investigation in MS may be discovered from either a screening approach using proteomics, or data gained from GWAS or a candidate approach. Candidate biomarkers may be generated from epidemiological, pathological, physiological or animal model studies. For example, sero-epidemiological studies have demonstrated association between MS and prior EBV infection and it has been hypothesised that intermittent peripheral EBV reactivation may drive continuing central inflammation. Recent small scale investigation has shown significant differences in median serum levels of EBV nuclear antigen-1 (EBNA-1) IgG between disease subgroups and positive correlation with disease activity reflected by number of Gd-

enhancing lesions and T2 lesion volume (Farrell et al. 2009). These data have led to hopes that EBNA-1 may be useful as an easily accessible and effective biomarker of disease activity; however, results are yet to be replicated or shown to be clinically applicable (Ingram et al. 2010).

Bielekova et al (Bielekova et al. 2004) have suggested a classification of biomarkers in MS, split into seven categories according to their pathological relevance and / or function:

i. Alteration in the immune system.
ii. Blood-brain barrier disruption.
iii. Demyelination.
iv. Oxidative stress and excitotoxicity.
v. Axonal / neuronal damage.
vi. Gliosis.
vii. Remyelination and repair.

Markers of inflammation

To date, most research has focused on biomarkers involved in the immune process including cytokines, chemokines, antibodies, C, adhesion molecules and other activation markers (table 1.1). Most current research is limited to small studies of CSF and therefore necessarily reflects changes in early phase disease; research has also been restricted by lack of phenotypic analysis of disease in comparison to the potential biomarker.

Markers of axonal damage

The major constituents of the axon cytoskeleton are light, intermediate and heavy chain neurofilaments (NF). NF-light forms the backbone to which NF-intermediate and NF-heavy co-polymerise to form the NF. The NFs are phosphorylated and the extent of phosphorylation determines the diameter of the axon and the velocity of axonal transport (Teunissen et al. 2005). In active MS lesions there is increased immunoreactivity of non-phosphorylated NFs (Mancardi et al. 2001), therefore, NFs with different levels of phosphorylation are potential biomarkers for axonal degeneration. Several studies have shown increased concentrations of NF-light in the CSF of MS patients in comparison to healthy controls (Lycke et al. 1998; Semra et al. 2002; Malmestrom et al. 2003; Haghighi et al. 2004; Norgren et al. 2004). Concentrations of NF-light are also higher in relapse than remission with concentrations peaking at 3 weeks (Malmestrom et al. 2003); however, correlation with disability has been variable (Lycke et al. 1998; Semra et al. 2002; Eikelenboom et al. 2003; Malmestrom et al. 2003). More recently however, NF-light in CSF of 99 patients with MS has shown correlation not only with disability but also prognosis; NF-light levels >386ng/L increased the risk for severe MS fivefold, and conversion to SPMS was more likely in cases with NF-light levels >386 ng/L (Salzer et al. 2010). There is a pressing need for this work now to be replicated to confirm this potentially exciting biomarker.

Other possible markers of axonal damage are tau protein; a phosphorylated microtubule-associated protein mainly found in neuronal axons (Binder et al. 1985;

Kapaki et al. 2000; Sussmuth et al. 2001; Jimenez-Jimenez et al. 2002; Bartosik-Psujek et al. 2004; Martinez-Yelamos et al. 2004; Brettschneider et al. 2005); amyloid precursor protein (APP) - induced in glial cells in response to injury such as inflammation (Gehrmann et al. 1995; Ferguson et al. 1997; Kornek et al. 2000) and 24S-hydroxycholesterol - an oxysterol exclusively derived from the brain and reported to be acutely raised at times of demyelination (Leoni et al. 2002; Teunissen et al. 2003). Although having potential for surrogacy, none have yet been established as biomarkers of disease.

1.8 Conclusion

MS is a disease of immense complexity, with heterogeneity shown in clinical, pathological and immunological features leading to recent debate into whether it is a single disease or a syndrome with different aetiologies (Confavreux et al. 2006a). Pathological separation of neuromyelitis optica (NMO) by clinical features and biomarkers of MRI and NMO IgG has enhanced this discussion (Wingerchuk et al. 2006).

The heterogeneity of disease course and the lack of objective clinical markers of prognosis make treatment decisions difficult for both clinician and patient. The advent of newer therapies, with higher side effect profiles, makes distinguishing aggressive, benign and progressive patients more important. Simple, easily repeatable, sensitive biomarkers would help with disease diagnosis, predicting prognosis as well as in monitoring of treatments. This thesis focuses on the C system and its components, fragments and regulators, as potential biomarkers in MS. There is a large body of

evidence (detailed in Chapter 2) demonstrating a contribution from C to the pathogenesis of MS; however, there is little previous work specifically examining individual or combined elements of the C system as potential biomarkers. Together with the departmental expertise available, the C system was viewed as a good potential target for investigation.

Figure 1.1: The pathogenic cascade in MS

The initiation of inflammation central to the pathogenesis of MS depends on the peripheral activation of T cells via foreign antigens. Activated T cells pass through the BBB into the CNS where they re-encounter specific antigen, are reactivated and bind directly to myelin epitopes leading to activation of macrophages (Mθ). These can directly phagocytose myelin or, along with T cells and glial cells, can release cytotoxic products and mediators. Further myelin attack comes from antibodies and C components. *MBP = myelin basic protein. MOG = myelin oligodendrocyte glycoprotein. NO = nitric oxide. O_3 = oxygen free radicals. Th = T helper cells.*

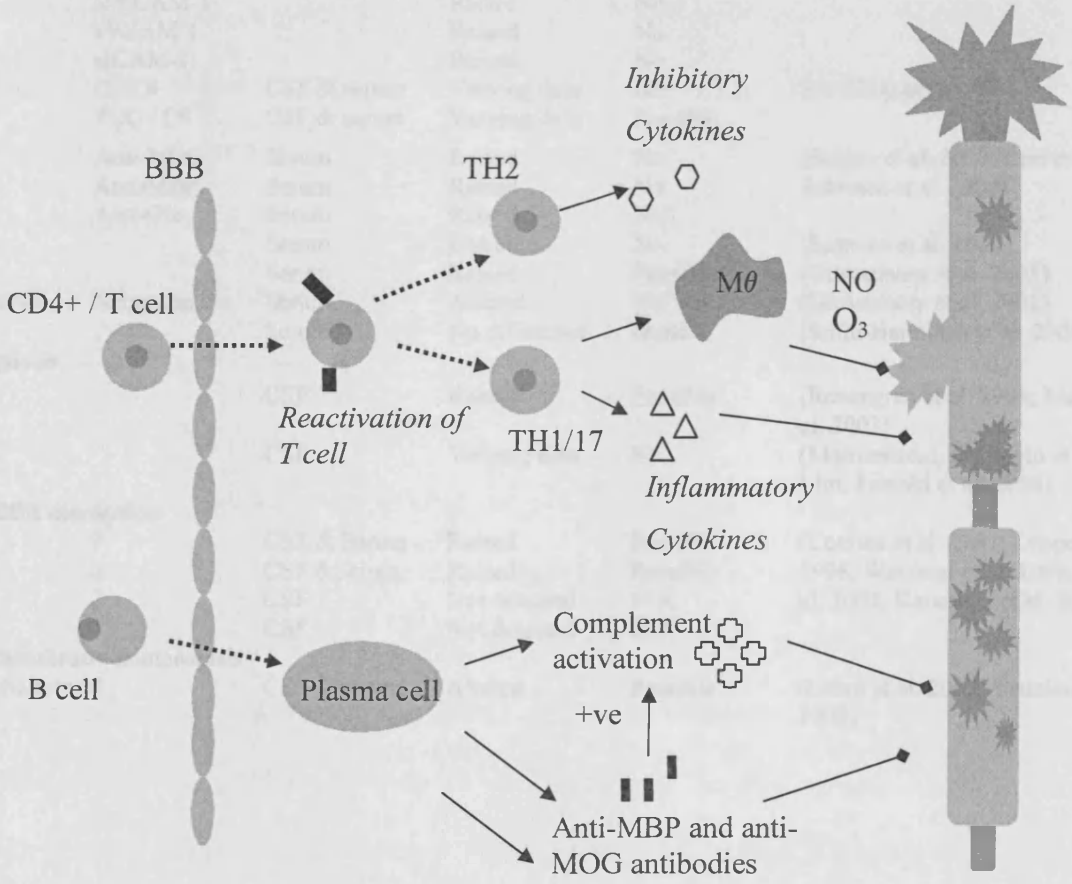


Table 1.1: Candidate inflammatory biomarkers in MS

Potential Biomarker	Examples	Type of specimen used	Level in MS compared to controls	Correlation to activity or progression	Recent evidence
Immune markers					
Cytokines	IL6	CSF & plasma	Raised	No	(Frei et al. 1991; Hautecoeur et al. 1997; Filion et al. 2003)
	IL10		Reduced	?	
	IL12		Raised	?	
	TNF alpha		Raised	Possible	
Chemokines and their receptors	CCL2 CCL5 CXCL10	CSF and blood	Varying data	N/A	(Trebst et al. 2001; Sorensen et al. 2004)
Adhesion molecules	sE-selectin	Serum	Raised	No	(Giovannoni et al. 2001; Kuenz et al. 2005)
	sP-selectin,		Raised	No	
	sPECAM-1		Raised	No	
	sVCAM-1		Raised	No	
	sICAM-1		Raised	No	
Complement	C3/C4	CSF & serum	Varying data	No	See Chapter 2
	TCC / C9	CSF & serum	Varying data	Possible	
Antibodies	Anti-MOG	Serum	Raised	No	(Berger et al. 2003; Lim et al. 2005; Schwarz et al. 2006)
	Anti-MBP	Serum	Raised	No	
	Anti-Glc	Serum	Raised	N/A	
Uric acid		Serum	Lowered	No	(Rentzos et al. 2006)
Nitric oxide		Serum	Raised	Possible	(Giovannoni et al. 2001)
Activation marker	Neopterin	Serum	Altered	No	(Giovannoni et al. 2001)
CRP		Serum	No difference	None	(Soilu-Hanninen et al. 2005)
Markers of gliosis					
GFAP		CSF	Raised	Possible	(Rosengren et al. 1995; Malmestrom et al. 2003)
S-100		CSF	Varying data	No	(Malmestrom, Haghighi et al. 2003; Lim, Petzold et al. 2004)
Markers of BBB disruption					
MMP	9	CSF & Serum	Raised	Possible	(Cossins et al. 1997; Leppert et al. 1998; Waubant et al. 1999; Williams et al. 2002; Kanesaka et al. 2006)
	3	CSF &Serum	Raised	Possible	
	2	CSF	Not detected	N/A	
	7	CSF	Not detected	N/A	
Markers of membrane homeostasis					
24s-hydroxycholesterol		CSF & serum	Altered	Possible	(Leoni et al. 2002; Teunissen et al. 2003)

CHAPTER 2: COMPLEMENT IN MULTIPLE SCLEROSIS: ITS ROLE IN DISEASE AND POTENTIAL AS A BIOMARKER

2.1 Introduction

Understanding of the role of C in the CNS and in systemic inflammatory disorders is now evolving at a considerable pace. Although first suggested to play a role in the pathogenesis of MS in the early 1970's (Lumsden 1971), the extent of C activation in subgroups of MS patients, the mechanisms of activation and the role of C in inflammation, axonal damage and neuroprotection all remain unclear. In particular the involvement of inflammatory mediators, including C, to the progressive phase of disease caused by axonal injury remains a point for debate and, without good animal models of axonal injury, remains difficult to investigate.

In part because of limited access to pathological human tissue, the animal model of MS, EAE, has proved useful in developing insights into the role of C in MS. Indeed, in a recent review it has been suggested that animal models of MS may provide the best and broadest understanding of underlying disease processes and should be the primary focus of research (Barnum et al. 2005). However, there are many well-rehearsed problems in extrapolating models of animal disease to the human context, and pathological, functional and genetic studies in man will continue to provide a valuable contribution that should not be overlooked.

Although C activation is not specific to MS, patterns of activation, assessed in combination with other inflammatory and immune markers, may be of value as biomarkers in MS subgroups. This chapter reviews the evidence from pathological work, animal model studies and human functional and genetic studies, implicating C in the pathogenesis of MS, and discusses the potential use of C components, fragments, activation products, regulators and their polymorphic variants as biomarkers of disease.

2.2 Complement activation and regulation

C plays a central role in the innate immune system, providing an important defence against infection and immune complex disease. The system consists of approximately 30 circulating and membrane expressed proteins which collaborate to provide protection from infection (Walport 2001b; Walport 2001a). The physiological actions of C are mediated through production of opsonins (molecules that enhance the ability of macrophages and neutrophils with C receptors to phagocytose material - C3b, iC3b, C4b etc.), anaphylatoxins (peptides that induce local and systemic inflammatory responses, increasing the permeability of blood vessels and attracting neutrophils through their chemotactic properties - C3a, C4a and C5a), and through direct killing of organisms by the terminal C complex (TCC) (C5b-9) which disrupts and forms pores in the phospholipid bilayer of a target cell. Although the C components in plasma are synthesised mainly by hepatocytes in the liver, within the CNS it is clear that glial cells and neurons can produce the majority of C proteins and expression is increased in

response to inflammation (Levi-Strauss et al. 1987; Morgan et al. 1996; Gasque et al. 2000).

The C system is activated via three initiating pathways (classical, alternative and lectin), all of which converge on a common effector pathway with formation of the TCC (fig 2.1). Inappropriate activation of C leading to depletion of the C components is normally prevented by C inhibitors present either in the fluid-phase or membrane bound (Morgan et al. 1994; Morgan et al. 1999) (fig 2.1). Regulation in the alternative pathway is particularly important because the pathway is characterised by a constant 'tick over' activation of C3 which can be amplified in pathology. Regulation in plasma is provided by fH and deficiency of fH allows unregulated amplification with breakdown of C3 leading to C3 deficiency.

2.3 Humoral immunity in neuromyelitis optica and MS

NMO is an inflammatory demyelinating disease predominantly affecting the optic nerves and spinal cord. Traditionally seen as a variant of MS, it has recently been redefined according to new criteria using a combination of phenotypic sub-typing along with a newly developed biomarker of disease, NMO-IgG (reported sensitivity of 58–76% and a specificity of 85–99% for NMO) (Giovannoni 2006; Wingerchuk et al. 2006). The clinical differences between NMO and MS are mirrored by pathological differences. NMO is typified by abundant Ig and C deposits surrounding blood vessels in a distinctive rim or rosette fashion (corresponding with aquaporin-4 expression) (Jarius et al. 2008) and thought to be driven by humoral processes (Wingerchuk 2006).

Therapeutic plasma exchange, known to non-selectively deplete plasma proteins, has been reported as having a beneficial role in NMO (Keegan et al. 2002; Watanabe et al. 2007) and thought to exert its therapeutic effect by reducing circulating levels of relevant auto-antibodies.

Until recently, studies of MS pathogenesis and treatment have focussed on the role of T cells, however, it is now clear that humoral immune mechanisms also play an important role which has recently been reassessed (Franciotta et al. 2008; Racke 2008). Circumstantial evidence for a B cell contribution to disease initially came from the demonstration of central clonal expansion of B cells and production of oligoclonal cerebrospinal fluid IgG (used routinely as a diagnostic biomarker in MS) (Link et al. 2006). More recently, several studies have demonstrated antibodies to myelin and other CNS autoantigens in MS patients, but without convincing disease specificity, and the specific antigens responsible for initiating the immune response in MS remain unidentified (Berger et al. 2007). Although Ig and C deposits are described in MS lesions, the localization of infiltrate is different from that in NMO (Breij et al. 2008) and has been suggested to only be present in a histological subgroup of patients (pattern II lesions) (Lucchinetti et al. 1996).

Therapies targeting humoral mechanisms, such as plasma exchange (Weinshenker et al. 1999; Weinshenker 2001; Keegan et al. 2002) and rituximab (a B cell depleting mAb) (Hauser et al. 2008), have been tested with variable success in acute demyelinating events and it has been suggested that patients with pattern II histological lesions,

showing prominent Ig and C deposition, are more likely to have a favourable response to this intervention (Keegan et al. 2005). The recognition of specific biomarkers for MS patients with extensive C activation may therefore be important in identifying patients who may benefit most from therapies targeting humoral immunity (Oh et al. 2008).

2.4 Complement in MS – evidence from pathology

A role for C in MS was initially proposed following the demonstration of C component C3 deposited in the brains of MS patients (Lumsden 1971). Immunocytochemical localization of the TCC in the brains of 5 of 7 MS patients was reported in 1989; TCC was demonstrated in association with capillary endothelial cells, predominantly within plaques and adjacent white matter (Compston et al. 1989). Localization of C activation to areas of active myelin destruction was demonstrated in a case report showing TCC deposited exclusively in such areas (Storch et al. 1998). In a study of 23 cases of SPMS, the opsonin C3d was shown deposited in association with short segments of disrupted myelin in plaques with low grade active demyelination and provided evidence for C contribution to disease progression as well as acute inflammation (Prineas et al. 2001).

More recently, a complex and heterogeneous pathological model for MS has been proposed by Luchinetti et al, who have described four distinct patterns of demyelination in a group of 83 patients, with specimens collected either at autopsy (n = 32) or biopsy (n = 51) (Lucchinetti et al. 1996; Lucchinetti et al. 2000). The pathological classification was based mainly on the presence of Ig and activated C (pattern II only),

remyelination (patterns I and II); and primary OGD damage (pattern III and IV). The authors suggested that the four different patterns of disease provided evidence for different mechanisms of immune-mediated demyelination, with C playing a role in a distinct but large subgroup of patients.

When considering the pathological subgroups and their relation to disease phenotype it must be noted that the frequencies of the different patterns seen may be heavily skewed by patient selection for biopsy (possibly more aggressive disease). Further, the specimens collected in the study by Luchinetti et al were from a cohort of patients with very early disease (the mean disease duration before autopsy or biopsy was 39 and 9 months respectively) and therefore may not accurately represent a global picture of MS. In addition the study showed C and Ig depositions only in pattern II lesions, represented exclusively in over 50% of the patients and in all subgroups of disease (relapsing remitting, secondary and primary progressive). A more recent study has failed to demonstrate this heterogeneity, showing C and Ig deposition consistently present in areas of demyelination (Breij et al. 2008). These authors have suggested that the heterogeneity described by Luchinetti et al may have been a phenomenon of early disease and was therefore not seen in their samples in which the mean disease duration was 22 years. Serum or CSF biomarkers that accurately identify any pathological subgroups would help considerably with clarifying these issues and would provide direction for future research.

2.5 Complement in MS – animal models

EAE, most commonly in rodents, has long been used as an animal model for MS (Mackay et al. 1973; Lassmann 2008), but was initially constructed to examine the monophasic illness; acute disseminated encephalomyelitis (Kabat et al. 1949). However, since its introduction numerous variations to the initial concept have evolved. Disease induction with a known CNS antigen (such as MBP (Zamvil et al. 1985), proteolipid protein (PLP) (Yamamura et al. 1986), MOG, myelin associated glycoprotein (MAG) and S-100 protein (Kojima et al. 1997)) causes a paralysis in animals with varying degrees of demyelination depending on the method of EAE induction and species used (Lassmann 2007). The two main models employed are the MBP model (resulting in a monophasic, non-demyelinating disease) and the antibody-mediated demyelinating EAE model (ADEAE) (where MBP immunisation is followed by administration of anti-MOG antibodies). Although the MBP induced model of EAE is thought by some to bear the closest resemblance to MS, it is likely that different models will have relevance in the understanding of different immunopathological subtypes or phases of disease. Information gained from these studies must be tempered by the knowledge that EAE, like most animal models of disease, is an incomplete and not entirely accurate reflection of the human situation. Models reflecting the axonal damage seen in MS have yet to be used to analyse disruption of the CNS but may yet give a clearer understanding of progressive disease (Bjartmar et al. 2003). Despite all these problems, much information on C pathology, potential C biomarkers and therapeutics has been obtained from EAE studies in normal animals and transgenic

mouse models (Steinman et al. 2006), and these model have provided a test bed for studies of treatment with C suppressors (Oh et al. 2008).

Anti-complement Agents in EAE

The first studies to examine the role of C in EAE used the C activator cobra-venom factor (CVF) as a treatment to reduce serum C; markedly reducing the severity of EAE (Abrahamson 1971; Pabst et al. 1971; Linington et al. 1989; Hinman et al. 1999). Despite initial success, it was soon realised that response to this treatment was transient and repeated injections were highly immunogenic; further, CVF-induced C activation led to production of vast quantities of the pro-inflammatory anaphylatoxins C3a and C5a, sufficient to cause a shock syndrome in recipients. It was also reported that, although CVF treatment in MBP induced EAE caused suppression of disease, in the ADEAE model CVF had no effect on disease severity despite C9 deposition being abolished (Piddlesden et al. 1991).

To combat the problems of CVF, sCR1 (which blocks the action of C3 and C5 convertases) was trialled as a treatment to decrease C activity and reduce the severity of EAE (Piddlesden et al. 1994). Treatment with sCR1 worked to some extent but showed much less reduction in clinical severity scores when compared to treatment with CVF, perhaps reflecting an incomplete blockade of C activation (Vriesendorp et al. 1997).

Gene Deletion Models in EAE

Gene deleted (knock-out) animal models have been used to enhance our understanding of the roles of the pathways of C activation in EAE. A study of C3 and factor B (fB) knock-out mice (the former unable to activate C, the latter alternative pathway deficient) demonstrated normal incidence of MOG-induced EAE but attenuated severity of chronic disease in both mouse strains, with decreased numbers of infiltrating macrophages and T cells (Nataf et al. 2000). This work showed unequivocally that C contributed to the development of EAE, and also that the alternative pathway was vital in the development of disease. The role of C3 has since been questioned, with C3 knock-out mice showing similar disease to wild type mice in one study (Calida et al. 2001), but attenuated disease in others (Barnum et al. 2006; Szalai et al. 2007). The differences here may be explained by the use of different mouse models. Evidence that the alternative pathway is the main player is supported by studies in C4-deficient (classical pathway absent) animals, which showed EAE onset and progression virtually identical to wild-type animals (Morariu et al. 1978; Boos et al. 2005).

Once activated, C has multiple roles in the EAE inflammatory lesion, causing demyelination (Piddlesden et al. 1993a), axonal damage and OGD injury (Linington et al. 1989). The mechanisms by which this damage is mediated have been examined in detail. The membrane attack complex (MAC), the cytolytic end-product of the C cascade, is thought to play a significant role in demyelination, with evidence from pathological studies (as discussed), *in vitro* studies (deficiency of the MAC inhibitor CD59 was shown to be the cause of rat OGD susceptibility to lysis in culture

(Piddlesden et al. 1993b)), and *in vivo* studies. C9/MAC deposition was shown to correlate closely with demyelination in the spinal cord of rats in which EAE was induced with anti-MOG antibodies (Piddlesden et al. 1993a), while C6-deficient rats (unable to produce MAC), when immunised with MBP and given anti-MOG antibodies to induce ADEAE, failed to develop demyelination, axonal damage or paralysis, but when reconstituted with C6 went on to develop pathology and clinical disease similar to wild type rats (Mead et al. 2002). Despite these findings, the role of MAC has been questioned (Barnum et al. 2006); C5 knock-out mice were not protected from MBP-induced EAE, suggesting that the terminal pathway was not essential for disease (Weerth et al. 2003; Niculescu et al. 2004). The extent of the role of MAC in demyelination and remyelination in models and MS thus remains uncertain.

Anaphylatoxins C3a and C5a are important inflammatory mediators and were initially considered to play an important role in CNS inflammation, based predominantly on evidence showing the expression of their receptors (C3aR and C5aR) on neuronal cells (Gasque et al. 2002). C3aR expression on microglia and infiltrating macrophages was increased during EAE but neuronal expression was largely unchanged (Davoust et al. 1999). The expression of C5aR on microglia and hypertrophic astrocytes within the spinal cords of Lewis rats was highly up-regulated in EAE (Nataf et al. 1998), leading to the hypothesis that C5a is an important inflammatory mediator in EAE. Since these initial studies, it has been demonstrated that C5a plays no role in demyelination in EAE; C5aR deficient animals are fully susceptible to EAE (Reiman et al. 2005), and treatment with a C5aR antagonist failed to protect against development of EAE

(Morgan et al. 2004). In contrast to this, C3aR-deficient mice in which EAE was induced, exhibited an attenuated clinical course, and immunohistochemical analysis revealed reduced demyelination and infiltration of macrophages and T cells compared to control mice, indicating that anaphylatoxin C3a plays a role in inflammatory cell recruitment and demyelination (Boos et al. 2004). Interestingly, these effects were more apparent in the chronic phase of disease, corresponding with results from C3-deficient mice (Nataf et al. 2000). More recent work with C3aR and C5aR double knock out mice failed to demonstrate any protection against EAE severity although disease onset was delayed; the authors claim the data indicated a level of cross-modulation between the C3aR and C5aR during EAE (Ramos et al. 2009).

Summary

Animal models have been useful in clarifying the role of C in EAE. It is clear that the alternative pathway contributes to disease, while the roles of the classical and terminal pathways are less clear, with the latter reported to be vital for demyelination in some but not all EAE models. The anaphylatoxin C5a likely contributes little to disease, while C3a plays a larger part – predominantly through recruitment of inflammatory cells. The alternative pathway plays a dominant role in EAE, as has been shown in many other disease models (Abarrategui-Garrido et al. 2008; Fang et al. 2008), impacting particularly in chronic disease. While direct translation of these findings into human disease is not possible, they may suggest that the alternative pathway is important in disease perpetuation after initiation by a primary source. The roles of C in

mediating axonal damage need to be further investigated and require animal models that represent axonal damage and chronic disease more clearly.

2.6 Complement as a serological or CSF biomarker of MS

In light of the evidence implicating C in MS, various C proteins have been considered as biomarkers of disease activity. These are summarised in table 2.1 and discussed below.

C3 and C4

C3 and C4 are the most abundant of the C proteins and are routinely measured in many laboratories. Like most of the C components, both C3 and C4 are acute-phase reactants and increased synthesis in response to inflammation can mask even quite marked consumption. Perhaps, as a consequence, studies of C3 and C4 levels in MS serum and CSF have shown inconsistent and conflicting results (Jans et al. 1984; Jongen et al. 2000). Levels in serum and CSF from patients with neuroinflammatory diseases are likely to be influenced both by the presence of active inflammation and the integrity of the BBB, both highly fluctuant in MS. Indeed, positive correlation of C3 and C4 CSF:serum ratios with albumin CSF:serum ratios has been identified in controls but not in MS patients (Jongen et al. 2000).

Terminal Pathway Components and the Terminal Complement Complex

Measurements of the terminal component, C9, and of the TCC have been performed in MS, although results have again been inconsistent. Low levels of C9 in MS CSF have been demonstrated in two studies including one in which patients were sub-classified

into acute relapsing, remission and progressive with lowered C9 levels in all groups, implicating prior activation of the terminal C pathway (Morgan et al. 1984; Compston et al. 1989). Other studies have shown no difference in C9 levels compared to controls (Halawa et al. 1989; Rodriguez et al. 1990). Measurement of TCC in CSF in three studies has consistently shown raised levels in MS patients (Sanders et al. 1986; Mollnes et al. 1987; Sellebjerg et al. 1998), one study showing good correlation of CSF TCC concentration with disability (Sellebjerg et al. 1998). There were no changes in C9 or C5b-9 levels in plasma in any of these studies, suggesting that CSF changes were a much better indicator of CNS disease.

Complement Regulators

To date, little work has been conducted looking specifically at C regulators in MS. A recent study using proteomic analyses to examine C proteins in MS CSF found significant reduction in one unspecified isoform of fB, and of the alternative splice product of the fH gene, fH-like protein-1 (fHL-1), in MS CSF (Finehout et al. 2005). The authors suggested that measurement of specific C component and regulator protein isoforms would be most informative as biomarkers. Although there have been no follow-up studies, this work highlights the potential of C measurements as biomarkers in MS.

In one study, antibodies to two membrane C regulators (CD46 and CD59) were identified in MS serum, present in the acute phase of RRMS but not in chronic MS or control groups (Pinter et al. 2000). The antibodies were directed against the active site of the C regulator, inactivating their regulatory function and leading to excess

activation of C. The authors suggest this as a mechanism for damage caused during acute relapse but so far no one has either replicated this very small study performed with less than 20 patients in each subgroup or investigated these antibodies as possible biomarkers of disease. Nevertheless, the study does highlight the need for the sub-classification of MS on clinical grounds at the point of study design.

Summary

Although showing some variation in MS, C components and activation products have not been established as biomarkers of disease, in part because results obtained from different studies measuring the same C component have often been conflicting. These differences could be due to lack of standard techniques of measurement or to low patient numbers leading to underpowered studies; however, universally there has been poor stratification of study groups according to phenotype. In a relapsing immunologically heterogeneous inflammatory disease, clinical phenotype (such as aggressive or benign disease or inter-current relapse) is likely to have a large effect on levels of C components. It is the view of the author that much information can be gained from further analysis of C components and regulators in serum, plasma and CSF employing correlation of levels with clinical phenotype and other modalities of disease activity and severity such as MRI.

2.7 Role of complement in neuroprotection in MS

Neuroprotection from Sublytic MAC

The neurotoxic effects of MAC are concentration dependent and it has been suggested that partial deficiency of terminal components has evolutionary benefits (Ross et al. 1984; Wurzner et al. 1992; Tanhehco et al. 2000). Several groups have shown that a sublytic dose of MAC can be cytoprotective, with prior exposure leading to accumulated resistance to a subsequent lytic attack (Wurzner 2003; Rus et al. 2005).

In one study in EAE, C5 deficient mice showed greater inflammatory demyelination and axonal loss (Weerth et al. 2003), with more OGD apoptosis compared to controls (Niculescu et al. 2004). Studies *in vitro* strongly suggested that this was a consequence of sublytic levels of MAC conferring protection from OGD apoptosis (Soane et al. 1999; Soane et al. 2001; Cudrici et al. 2006a; Cudrici et al. 2006b).

It is known that OGD apoptosis is associated with activation of caspases 3, 8, 9 and 11 (Soane et al. 1999; Shibata et al. 2000; Hisahara et al. 2001; Cudrici et al. 2006b) and inhibition with caspase–3 and -8 inhibitor can prevent OGD apoptosis *in vitro* (Soane et al. 1999). Sublytic MAC has been shown *in vitro* to inhibit cytochrome c activation of caspase-3 and 9 and induce phosphorylation of BCL2-antagonist of cell death (Bad) which, when phosphorylated, can bind to cytoplasmic 14-3-3 protein and increase cell survival (Soane et al. 2001). Sublytic MAC has also be shown to inhibit Fas ligand (FasL) and TNF-α induced OGD apoptosis by inhibition of caspase-8 processing (Soane et al. 1999; Cudrici et al. 2006a). This provides *in vitro* and *in vivo* evidence of

neuroprotection mediated by low levels of MAC, acting primarily via regulation of caspases (Rus et al. 2006a; Rus et al. 2006b).

Neuroprotection by Anaphylatoxins

In vitro studies of anaphylatoxins C3a and C5a have shown potential neuroprotective effects. Pre-treatment of primary murine corticohippocampal neurons with human or mouse recombinant C5a reduced glutamate neurotoxicity and prevented glutamate induced neuronal apoptosis (Osaka et al. 1999); these events were signalled through mitogen-activated protein kinase (MAPK)-mediated regulation of caspase cascades (Mukherjee et al. 2001). C3a was neuroprotective against N-methyl-d-aspartate (NMDA) toxicity in the presence of astrocytes, but did not protect against serum deprivation-induced apoptotic neuronal death, or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate-mediated excitotoxicity (van Beek et al. 2001). In animal models, C5a-receptor deficient mice were more susceptible to apoptotic injury *in vivo* due to increased excitotoxicity (Mukherjee et al. 2008).

2.8 Genetic findings implicating complement in MS

Epidemiological studies have long recognised the important role of genetic factors in susceptibility to MS, with a number of largely unidentified genes contributing to disease risk (Ebers et al. 1986; Robertson et al. 1996; Sadovnick et al. 1996). More recently genome-wide association studies have confirmed that SNPs in inflammatory

cytokine receptors IL2Rα and IL7R are associated with susceptibility to MS (Gregory et al. 2007; Hafler et al. 2007; IMSGC 2008).

A more significant role for genetic variability influencing C in MS may be signaled by recent findings in other CNS disorders with a significant inflammatory component. Studies in AMD have recently identified a SNP in the locus encoding the C regulator fH (Tyr402His); homozygosity for the minor allele conferred a six-fold increased risk of disease (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005). This common polymorphism is located in short consensus repeat 7, a region implicated in binding multiple ligands, and has been suggested to alter affinity of fH for surfaces, thereby influencing capacity to inhibit C (Skerka et al. 2007). Other identified polymorphisms in the alternative pathway component fB and the classical pathway component C2 may further contribute to disease risk (Gold et al. 2006). These minor changes in the C proteins causing subtle dysregulation in C have been shown to be strongly associated with the disease and are suggested to be global markers for AMD (Kaur et al. 2006). The Tyr402His polymorphism in fH described in AMD has also been identified as a risk allele for AD in some but not all studies (Hamilton et al. 2007; Zetterberg et al. 2008).

Given the role of C in neuro-inflammation and the reports of C polymorphisms contributing to other inflammatory conditions, a candidate gene approach in MS to identify association with C components and regulators would seem logical. Although, no studies have yet identified susceptibility genes within C components or receptors

(table 2.2), evidence for linkage in MS in the region around the centromere of chromosome 5 (containing genes for C components C6, C7, and C9), has been identified in four independent linkage studies (Ebers et al. 1996; Haines et al. 1996; Sawcer et al. 1996; Kuokkanen et al. 1997).

All of the studies so far looking at candidate genes have been underpowered to detect significant differences where the risk proportioned to each identified allele is very small (odds ratios <1.3) (Sawcer et al. 2005), therefore negative studies can not be deemed reliable. Also, work to date has focused on disease susceptibility risk and has not examined phenotype, which may be more likely to be altered by C dysregulation. Further studies with large groups of patients are needed in order to uncover genetic biomarkers for MS within the C system, and to direct functional studies.

2.9 Complement activation and dysregulation in neurological disorders

C has been firmly implicated in the pathology of a number of diseases of the CNS and its role in NMO and AMD has been discussed. In addition, there have been reports of the alternative pathway regulator fH being a marker in AD, significantly elevated in AD patients compared to controls (Hye et al. 2006). Further evidence of a role for fH in AD pathology has been provided by Honda and co-workers who developed a mAb that specifically stained senile plaques and subsequently demonstrated that the mAb bound fH, present both in the senile plaques and in CSF in AD (Honda et al. 2000). The Tyr402His polymorphism in fH described in AMD has also been identified as a risk

allele for AD in some but not all studies (Hamilton et al. 2007; Zetterberg et al. 2008). More recently the GWAS in AD has revealed further SNPs in C regulator clusterin (rs11136000) and CR1 (rs6656401) with odds ratios for disease 0.81 and 1.21 respectively (Lambert et al. 2009). Further genetic and functional work is being done to establish these C regulators as clinically useful biomarkers in AD.

C activation is also described in several other CNS conditions including Guillain–Barré syndromes in which antibody and C-mediated mechanisms have been shown to be important in pathogenesis (Willison et al. 2008), and ischemia-reperfusion injuries such as trauma and stroke in which C inhibition has been shown to be neuroprotective (Arumugam et al. 2004; Arumugam et al. 2009) (reviewed in (Gasque et al. 2000)). These disorders demonstrate how, although not being the primary pathogenic factor, C dysregulation can significantly contribute to disease in the presence of other disease initiating mechanisms.

2.10 Conclusion

Chapter 2 summarises compelling evidence from pathological, animal model and functional studies, that C plays an important role in the immunopathogenesis of MS. Prior understanding of the immunology of MS and knowledge gained from animal studies leads us to conclude that C does not initiate disease, but propagates ongoing disease with increased contribution over the course of the illness. Severity may vary between individuals according to the extent of C dysregulation, contributed to possibly by functional polymorphisms in C components and regulators. Despite detailed

information from animal model studies regarding the activation and regulation of C in demyelination, questions still remain as to the role of the terminal pathway, the extent of the neuroprotective function of C in disease, the role of C in axonal damage and the translation of these studies into the more complex human disease of MS.

This chapter has highlighted the importance of clinical classification of disease subgroups at the point of study design for functional and genetic work. In particular, studies looking at concentrations of C in serum and CSF have often been complicated by the disease heterogeneity in which acute relapse, stable and chronic disease are all highly likely to have distinctly varying C contributions. In recent years advances in technology, in particular the use of proteomic and genetic analytical techniques, have led to better opportunities for finding sensitive and specific biomarkers of disease, as proven in other complex disorders such as AD, AMD and NMO. Use of these techniques, along with better clinical stratification, will bring us closer to finding useful biomarkers for MS; however, given the genetic, pathological, immunological and clinical heterogeneity of MS, it is highly unlikely there will be a single effective surrogate biomarker for disease and a combination of markers, possibly including C components and polymorphisms, are more likely to be informative.

Figure 2.1: Activation and regulation of the complement system

Activation through both classical and alternative pathways results in the formation of C5 convertases, cleaving C5 and eventually forming the MAC (C5b-9). They also result in the formation of anaphylatoxins which cause inflammation and chemotaxis. The C system is under tight regulation by soluble (in italics) and membrane bound (in bold) proteins. (C1-inh = C1 inhibitor, C4bp = C4 binding protein, fI = factor I, MCP = membrane cofactor protein (CD46), CR1 = C receptor 1, DAF = decay accelerating factor (CD55), fH = factor H, fB = factor B).

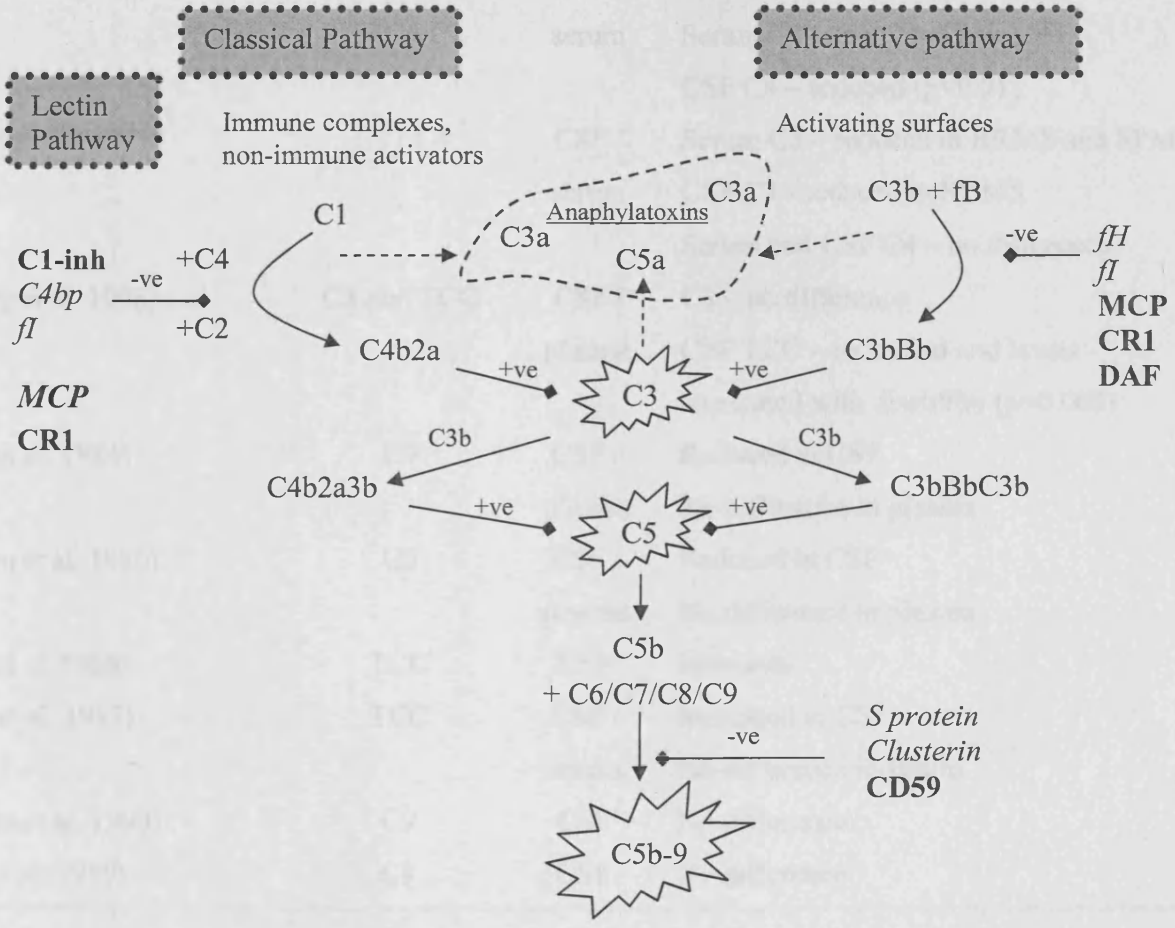


Table 2.1: Complement components in CSF and serum of MS patients

Reference	C component measured	Sample type	Significant alteration in concentration compared to controls
(Kuwert et al. 1965)	C3 / C4	CSF	Reduced
(Link 1972)	C3 / C4	CSF	No difference
(Dube et al. 1973)	C3 / C4	CSF	Increased
(Yam et al. 1980)	C3 / C4	CSF	C3 – increased C4 - no difference
(Price et al. 1980)	C3 / C4	CSF	In relapse – no difference In remission – decreased
(Jans et al. 1984)	C3 / C4	CSF / serum	Serum and CSF C3 – no difference Serum C4 – increased ($p=0.05$) CSF C4 – reduced ($p<0.01$)
(Jongen et al. 2000)	C3 / C4	CSF / serum	Serum C3 – reduced in RRMS and SPMS CSF C3 - reduced in RRMS Serum and CSF C4 – no difference
(Sellebjerg et al. 1998)	C3 and TCC	CSF / plasma	C3 – no difference CSF TCC – increased and levels correlated with disability ($p=0.003$)
(Morgan et al. 1984)	C9	CSF / plasma	Reduced in CSF No difference in plasma
(Compston et al. 1986)	C9	CSF / plasma	Reduced in CSF No difference in plasma
(Sanders et al. 1986)	TCC	CSF	Increased
(Mollnes et al. 1987)	TCC	CSF / serum	Increased in CSF No difference in serum
(Rodriguez et al. 1990)	C9	CSF	No difference
(Halawa et al. 1989)	C9	CSF	No difference

Table 2.2: Genetic studies of complement in MS

Study	Gene tested	No. SNPs tested from gene	Cohort of patients	Result
(Chataway et al. 1999)	C6	1	227 multiplex families	No association
	C7	3	466 affected siblings	No distortion of
			112 unaffected siblings	transmission
(Simon et al. 2007)	CR2	9	Not disclosed	No association
(Bulman et al. 1991)	C3	1 allele	129 patients	No association
			69 controls	

CHAPTER 3: METHODS

3.1 Patient ascertainment and sample collection

The University Hospital of Wales (UHW) is the major tertiary referral centre for neurology in South-East Wales, serving a local population of 1.2 million. The Department of Neurology has provided a network of MS clinics across South East Wales since 1999, with additional clinical data available on a subset of patients recruited in 1985. Approximately 1000 patient contacts are documented annually within this clinic network with detailed demographic and clinical data collected at presentation and following visits. Initial and subsequent complete datasets are available on 2388 patients with MS (including 2240 probands), comprising more than 90% of the local prevalent patients and may therefore be considered to be a representative sample of the prevalent population. All information collected is recorded on a secure NHS database so that longitudinal information and disability scores on individual patients can be tracked over the disease course. Ethical approval for the study was obtained from the South Wales ethics committee ref 05/WSE03/111.

Patients are classified according to diagnostic criteria. Some of the older patients on the data base are classified by the Poser criteria (Poser et al. 1983) where patients are defined according to clinically or laboratory supported definite or probable disease (table 3.1). However, more recently recruited patients are defined by the McDonald criteria (McDonald et al. 2001) which combines clinical and paraclinical evidence so as to demonstrate dissemination in time and space of lesions (table 3.2). Standardised

minimum current status data sets are collected at each clinic visit, including information on current disease course, relapse status, disability measured by the EDSS and therapeutic interventions, together with anatomical site and timing of relapses since last review. Patients with suspected relapses are seen within 5 days from initial contact and confirmed relapses are offered follow up at 2, 6 and 12 months. The EDSS (Kurtzke 1983) is a non-linear clinical scale from 0-10 with each point representing a different level of disability ranging from neurologically intact to death (table 3.3). EDSS 4 and less are compiled of a combination of functional scores based on different parts of the neurological examination. EDSS greater than 4 are based on various walking milestones, such as EDSS 6 is the ability to walk 100 metres with a single aid.

The MS biobank consists of serum, plasma and DNA samples collected at routine clinic visits so that longitudinal samples are matched with routine clinical data. A small number of samples were collected outside of these clinics, via home visits or through links with MS society groups with similar clinical information collected. Control plasma, serum and DNA (Ethics ref no. 09/MRE09/35) was sourced from patients' spouses and friends coming to routine clinics along with hospital and university employees. Control patients were excluded if they had a history or family history of any neurological disorder, or a history of other inflammatory conditions.

CSF samples were obtained from patients admitted to the neurology ward in the University Hospital of Wales, for investigation of either known MS or CIS. Control CSF samples were obtained from the investigated patients who subsequently turned out

to have an alternative diagnosis. All CSF samples were paired with serum, plasma and DNA samples.

3.2 Specimen processing and storage

CSF

CSF is a dynamic metabolically active substance which already has important functions in the diagnosis of neurological disorders. It is in direct continuum with the extracellular space of the CNS and thus is felt to hold advantages over serum as it is more likely to reflect the relevant central biological process. Unfortunately the specificity of available tests is often low as constituents can be similar in different pathological processes. CSF is obtained relatively easily by lumber puncture, but repeat sampling is difficult because of the invasiveness of the procedure.

To create a CSF biobank, storing CSF for use in different projects over many years, it is important that all samples are handled in a consistent fashion ensuring continuity. It is also important to aliquot samples into small volumes to avoid freeze-thaw cycles, which can degrade proteins. Proteomic studies have suggested that many proteins that are potential biomarkers for neuroinflammatory conditions degrade if not handled correctly, i.e. are not processed in sufficient time or stored at low enough temperatures (Bruegel et al. 2009). Our handling techniques are based on recommendations from such studies and from guidelines in a recent document by Teunissen et al (Teunissen et al. 2009).

CSF was taken atraumatically from between vertebral bodies L3/4 or L4/5. Bloody samples were discarded and samples were centrifuged (2000g/10mins) within 30 minutes of collection before being aliquoted and frozen at -80°c until use. CSF and paired serum samples were analysed in the routine laboratory for IgG, albumin and OCBs. IgG and albumin were measured on the BN 11 nephlometer and OCBs on the Sebia Hydrasys using Hydrogel 9 CSF Iso-electric focussing. Reference ranges for normal values were: serum IgG 5.2-15.5 g/L, CSF IgG <58 mg/L, serum albumin 35-50 g/L, CSF albumin 160-360 mg/L, albumin/IgG index 0.3-0.8.

Serum and plasma

When considering the storage of blood samples and establishing a biobank, one should allow for analysis of the most unstable product in all samples and treat samples uniformly. Handling time for serum and plasma has been shown in proteomic studies to be extremely important for some proteins and peptides (West-Nielsen et al. 2005). Various experiments have suggested optimal standards for sample processing (Baumann et al. 2005; Jimenez et al. 2007; Pieragostino et al. 2009). Based on these, and for practical reasons, serum and plasma samples were processed within 3 hours of retrieval and after centrifuge at 2000g for 10 minutes; supernants were frozen at -80°C in multiple aliquots until use; freeze-thaw cycles were avoided. Protocols for serum, plasma and CSF handling did not allow for the collection of cells which were not used in this thesis but may be useful in other work.

DNA

Venous blood was collected for DNA and extracted in the University Hospital of Wales medical genetics laboratory using the Autopure DNA Purification robot. Samples were suspended in DNA Hydration Solution from Qiagen (Product code 158916) and stored at -80°c. Saliva samples were collected using the Oragene DNA self-collection kit (DNA Genotek Inc, Ottawa, Ontario,Canada) and DNA extracted using the Oragene manual purification protocol (www.dnagenotek.com). Concentrations of DNA were measured using a NanoDrop Spectrophotometer ND1000.

All samples were labelled anonymously according to a unique patient identifier, date of sampling and sample type. The location within the freezer was documented on the described database.

3.3 Conclusion

Establishing a comprehensive biobank offers an important resource that can be valuable to many research studies for a number of years. However, for a useful biobank, one must consider many factors. Our biobank included samples from a range of sources (CSF, serum, plasma and DNA) and all samples were collected and stored in a rigorous fashion to allow their use for any potential project over the following years (patient consent included allowance for the use of samples in future projects not outlined in the patient information leaflet). Practical limitations restricted the quality of the handling techniques to some extent, and to optimize the biobank, accurate records should be held on the exact time to processing and time of day sample was collected (possibly important for fluctuations in circadian rhythms). Importantly for any biomarker

experiment, each sample collected was linked on a convenient and comprehensive clinical database with extensive accurate clinical information. These rigorous methods allowed accurate analysis of biomarkers in larger numbers of patients with extensive correlation to clinical phenotypic data.

Table 3.1: Poser criteria for multiple sclerosis

Category	Attacks	Clinical evidence	Paraclinical evidence	CSF OCB
CDMS	2	2		
	2	1	and 1	
LSDMS	2	1	or 1	+
	1	2		+
	1	1	and 1	+
CPMS	2	1		
	1	2		
	1	1	and 1	
LSPMS	2			+

CDMS: clinically definite multiple sclerosis. LSDMS: laboratory supported definite multiple sclerosis. CPMS: clinically probably multiple sclerosis. LSPMS: laboratory supported probable multiple sclerosis. Attack: the occurrence of neurological symptoms lasting at least 24 hours. Clinical evidence: neurological signs evident on examination. Paraclinical evidence: neurological dysfunction demonstrable by tests such as evoked potentials and tissue imaging studies. CSF OCB: cerebrospinal fluid oligoclonal bands. From (Poser et al. 1983).

Table 3.2: McDonald criteria for multiple sclerosis

Clinical presentation	Additional data needed for diagnosis
Two or more attacks with objective clinical evidence of at least 2 lesions	None
Two or more attacks with objective clinical evidence of 1 lesion	Dissemination in space on MRI *or* Up to 2 MRI lesions plus +ve CSF OCBs
One attack with objective clinical evidence of 2 or more lesions	Dissemination in time on MRI
One attack with objective clinical evidence of 1 lesion (i.e. CIS)	Dissemination in space on MRI *or* Up to two MRI lesions plus +ve CSF OCBs *AND* Dissemination in time on MRI
Insidious neurological progression	+ve CSF OCBs *AND* Dissemination in space on MRI *or* Abnormal VEPs associated with 4-8 brain lesions, or less than 4 brain lesions and 1 spinal cord lesion on MRI *AND* Dissemination in time on MRI *or* Continued progression for 1 year

CIS: clinically isolated syndrome. Dissemination in time and space on MRI scans are defined in detail in (McDonald et al. 2001).

From (McDonald et al. 2001).

Table 3.3: Expanded disability status score

0.0	Normal examination
1.0	No disability, minimal signs 1 FS
1.5	No disability, minimal signs in more than 1 FS
2.0	Minimal disability in 1 FS
2.5	Minimal disability in 2 FS
3.0	Moderate disability in 1 FS Fully ambulatory with moderate disability in 1 FS or
3.5	mild disability in 3 or 4 FS Fully ambulatory without aid for up 12hrs / day AND able to walk 500m without rest AND
4.0	1 FS grade 4 or combinations more than EDSS 3.5 Up and about much of day AND
4.5	able to walk without aid or rest for more than 300m
5.0	Ambulatory without aid or rest for 200m
5.5	Ambulatory without aid or rest for 100m
6.0	Intermittent or constant unilateral assistance required to walk 100m
6.5	Constant bilateral assistance required to walk about 20m
7.0	Unable to walk beyond 5m even with aid
7.5	Unable to take more than a few steps
8.0	Restricted to bed or chair with effective use of arms
8.5	Restricted to bed some use of arms
9.0	Restricted to bed can communicate and talk
9.5	Totally helpless bed patient
10.0	Death

FS: functional score (these incorporate various elements of neurological examination).

From (Kurtzke 1983)

CHAPTER 4: VALIDITY OF PATIENT DERIVED DISABILITY AND CLINICAL DATA IN MULTIPLE SCLEROSIS

4.1 Introduction

MS is a complex and heterogeneous disease of the CNS in which clinical features including presentation, disease course and rates of accumulation of disability, demonstrate high degrees of individual variation (Compston et al. 2002; Hirst et al. 2008). Since the considerable investment required for long term surveillance commonly exceeds the attention of individual clinicians, descriptive accounts of the disease are commonly derived from cross-sectional studies of population-based or clinic cohorts (Runmarker et al. 1993; Bronnum-Hansen et al. 1994). These data are effective in demonstrating the phenotypic range of disease; however, single assessments of disability are of limited value since the rate of accumulation of disability is variable, long periods of disease stability common and the initial pattern of disease is not necessarily predictive of final outcome (Weinshenker et al. 1989). Where reliable, extensive longitudinal natural history data has been available in large, robust populations, it is considered a rare and valuable resource, contributing to a wide range of epidemiological, genetic and interventional studies. (Weinshenker et al. 1991; Confavreux et al. 2000; Confavreux et al. 2006a). For a detailed examination of biomarkers in MS, it was perceived that an accurate and extensive clinical database was necessary. Although the database outlined in Chapter 3 had been established for 10

years prior to the development of the biobank, it was apparent that a proportion of data was missing, especially from patients not seen frequently in a clinic setting and with a disease course of more than 10 years. This Chapter outlines the development of a simple and cost-effective method to collect accurate historical data from MS patients, thereby increasing the extent and reliability of our prior established clinical resource.

In an effort to produce cost-effective methods to derive reliable disability data in large epidemiological studies and in order to gain information from patients who have difficulty accessing formal clinical settings, attention has turned to the feasibility of patient administered measures. In particular, the use of telephone or paper questionnaires to assess current disability using the EDSS has become a more widely accepted method of data collection, showing good levels of correlation with more formal clinician-led assessments (Bowen et al. 2001; Cheng et al. 2001; Lechner-Scott et al. 2003). However, even with the use of these self-administered questionnaires, collection of sufficient data to develop a detailed, longitudinal profile of disease related disability remains a time-consuming process extending over many years and remains beyond the scope of many studies.

Historical analysis of disability milestones derived from individual patient testimony is commonly presumed to be unreliable, prone to recall bias, and complicated by cognitive deficits in affected patients. Despite this, and in the absence of objective records, patient testimonies detailing current and historical events, including information on disease onset, temporal evolution of disability and relapse frequency,

are widely employed to develop clinical decisions on management and therapeutic interventions. The validity of data collected in this manner has rarely been the subject of detailed analysis since it requires access to an established cohort of patients with reliable disability and clinical milestone data collected over extended time periods

In this study a patient questionnaire was designed (appendix 1) based on the EDDS with incorporated algorithms to determine current EDSS and the year in which patients reached well-recognized disability and clinical milestones. Validity of these patient derived data, collected either by postal questionnaire or structured telephone interview, was examined by employing a well described population-based cohort of patients for which comparable and detailed clinician derived clinical and disability data had been amassed regularly over a period of more than twenty years. Similar comparisons were also made for a range of MS related clinical phenomenology, including year of onset and diagnosis, initial and current disease course, time to secondary progression and relapse frequency.

4.2 Methods

Subjects

Inclusion criteria were definite or probable MS according to Poser criteria or MS or possible MS according to the McDonald criteria (Poser et al. 1983; McDonald et al. 2001), an EDSS of between 6.0 and 9.0, full clinical data sets including multiple assessments in clinics throughout their disease course (allowing accurate assessment of

time of disease onset and time to EDSS > 4, 6 and 8) and previous consent to be contacted by telephone or letter for research purposes. Given that primary endpoint evaluated in this study was time in years to EDSS >4, 6 and 8, patients were selected who had already reached 2 of the 3 specified milestones and patients with EDSS < 6.0 were excluded. Of 661 living MS patients with EDSS 6.0-9.0, 160 were considered to have complete data sets and of these 99 patients were consented to take part in research. To assess the possibility of selection bias, the demographic characteristics of the 562 patients not included in the study (mean age 54.2, 67.2% female, mean disease duration 20.4 years, mean EDSS 6.8 (range 6-9.0), disease course including 16.9% with PPMS, 16.4% with RRMS and 66.7% with SPMS) were examined and found to have a significantly higher age (p=0.001), disease duration (p=0.008) and EDSS (p=0.001) than the assessed population. The characteristics of the 20 non-responders (median age 42.5 years, 75% female, median disease duration 10.5 years, median EDSS 6 (range 6-8.5), disease course included 10% with PPMS, 25% with RRMS and 65% with SPMS) were assessed and were found to be similar to the examined population. To increase the proportion of participants, non-responders to the postal questionnaire were contacted via telephone after 3 weeks; in addition contact via telephone was attempted 5 times before patients were classified as non-responders.

Patients were stratified according to gender and age (≤49, >49) and then randomly allocated one of two groups – postal questionnaire (PQ) or telephone interview (TI). Patients in the TI group also used a supplemental 'life course grid', which identified the timing of important life (i.e. marriage, house move, birth of a child or retirement)

and / or national and world (i.e. the Queen's coronation or a particularly hot summer) events to help improve their recollection of relevant disease-specific events (Parry et al. 1999). The telephone assessor was blinded to the recorded clinician-derived information.

Questionnaire

The questionnaire (appendix 1) initially identified characteristics of the disease presentation, such as year of onset, year of diagnosis and whether initial presenting symptoms had fully resolved ("In what year did you first have symptoms of Multiple Sclerosis?"). Patients were subsequently asked to identify their current disease course from a selection comprising RRMS, SPMS and PPMS or unknown. Although these terms were not expanded with an explanation of disease type, in order to provide additional validity for disease course further questions were provided concerning onset of symptom progression ("Looking back, do you think your symptoms of MS are gradually worsening despite the effect of relapses?"). Questions were also asked regarding initial and recent relapse information and the clinical characteristics of relapses from a selection of pre-determined responses. Current EDSS was determined by asking patients to select from a series of statements describing walking ability as specified by the EDSS with reference distance between well recognized local landmarks; for example, 500 meters equated to the length of one of the well known shopping streets in Cardiff. Patients who could walk at least 500 meters without rest or aid were scored at EDSS equal to or less than 4. Patients were also asked to recall the year of reaching specific disability milestones including EDSS >4 (inability to walk at

least 500 meters without rest or aid), 6 (use of a single walking aid to walk at least 100 meters) and 8 (use of a wheelchair for all activity). The milestones were selected because of their common use as endpoints in previously published epidemiological studies and their onset considered as well defined events easily recognized by both clinicians and patients.

Statistical analysis

Data analysis was performed using Statistical package for Social Sciences version 16 (SPSS inc., Chicago, IL, USA). To assess the accuracy of the questionnaire derived data for the year of reaching specified disability milestones, the time in years was calculated from the clinician derived year of first symptom onset to the specified disability endpoints, second relapse, diagnosis and onset of secondary progression. Time to disability milestones, second relapse, diagnosis and SPMS was analysed after log transformation, as the data were positively skewed, using reliability analysis (two-way random ICC with 95% confidence intervals (CI)). Poor correlation was taken to be <0.7, satisfactory correlation 0.7-0.9 and good correlation >0.9. The difference in the accuracy of reporting variables via telephone or patient questionnaire was examined using Students *t*-test (data was normally distributed). Bland Altman plots (Bland et al. 1986) (absolute difference in years plotted against mean difference) were used to examine for any systematic bias or unequal variation. Gender, age and disease duration were assessed to ascertain any systematic effect on the reliability of self-reported measures by dichotomising the data into two groups (perfect agreement – no difference in years taken to reach an EDSS end-point or not perfect agreement) for reaching EDSS 6 (this being the largest group). The odds ratios with 95% CIs were then calculated for

females vs. males, old vs. young (cut-off 60 years) and long versus short disease duration (cut-off 20 years). For categorical data such as disease course, recovery from initial symptoms and occurrence of relapses in the last year, data was cross-tabulated and a weighted Kappa coefficient was calculated, taking a Kappa >0.7 as evidence of good agreement.

4.3 Results

Demographics

PQs were returned by 35/49 patients (71.4%), and 44/50 (88%) patients were successfully contacted for TI. Mean age of patients in the PQ cohort was 49.4 years (SD 10.4) and 62.9% were females; compared with a mean age 48.9 years (SD 10.0) and 68.2% females in the TI cohort. According to clinician derived data, 14.3% of patients in the PQ cohort had PPMS, 22.9% RRMS and 62.9% SPMS. In the TI cohort 9.1% had PPMS, 18.2% RRMS and 72.7% SPMS. Mean EDSS was 6.5 (SD 0.7) in the PQ cohort and 6.4 (SD 0.8) in the TI cohort with 11.4% in each group of EDSS 8 and above.

Disability

Current EDSS derived from the questionnaire (via post or telephone) showed satisfactory levels of correlation (ICC 0.69 to 0.86) with the clinician derived data (table 4.1) with perfect agreement noted in 75.9% and 88.6% allowing for accepted intra-observer variation of +/- 0.5 EDSS points (Goodkin et al. 1992). The proportion with perfect agreement was more modest (between 25 to 32%) when assessing time to selected disability milestones (EDSS>4, 6 and 8), but widening the time window to

either +/- 1 (between 59 and 75%) or +/- 2 years (between 75 to 100%) substantially improved this (table 4.2). All disability milestones showed good ICC between questionnaire and clinician derived data (table 4.1). The Bland Altman plots (fig 4.1) did not show evidence of any systematic bias but there were occasional outliers that were outside the 95% agreement levels (e.g. EDSS 4). Very occasionally patients reported the year of reaching a milestone before clinician reported disease onset; this may have been an error in patient or clinician reporting of year and is seen as a negative mean difference in the Bland Altman plots. In 26 patients, clinician derived time to EDSS>4 was not available due to non-attendance at clinic during this period of disease. Of the 18 patients with perfect agreement in time in years to EDSS 6, 13 were female, 4 were classed as old (>60 years) and 5 classed as having a long disease duration (>20 years). The odds ratio of reporting exact time in years to EDSS 6 for females versus males was 1.37 (95% CI 0.43-4.39), for old versus young was 1.56 (0.42-5.82) and for patients of long versus short disease duration was 1.01 (0.31-3.29).

Disease onset, relapse, diagnosis

Table 4.1 and figure 4.2 show the mean year of symptom onset and time from clinician reported onset to second relapse and diagnosis with satisfactory levels of correlation between questionnaire and clinician derived data for year of initial symptom onset (ICC 0.86) and time to diagnosis (ICC 0.86), but poor agreement for time to second relapse (ICC 0.55). For all three variables the PQ was more accurate than the TI; however, the forms of data collection were only significantly different when concerning time to diagnosis (p=0.03) and second relapse (p=0.02). When reporting initial symptoms, patients were asked if they obtained complete recovery from their first event. In the PQ

group there was agreement in only 22 of the 35 responding patients (Kappa coefficient 0.25), and in the TI group 29 of 44 patients (Kappa coefficient 0.22). When asked to select from a series of descriptions regarding their first relapse, only 24.1% of patients recorded the exact description as that of the clinical records. However 51.9% reported a similar event and of these 34.1% under-reported and 65.9% over-reported their symptoms.

Disease course

Patients were asked to record current disease course; analysis showed good agreement between the questionnaire and clinician derived data in the PQ group (Kappa 0.89) with only 2 of the 33 responding patients reporting RRMS but recorded to have SPMS; both of these patients reported that their symptoms were worsening despite the effects of relapses, implying progressive disease. In the TI group there was moderate agreement (Kappa 0.56) with 7 of the 42 patients reporting RR disease but recorded as having SPMS (of these only 4 of 7 reported gradually worsening symptoms), 2 reporting PPMS and recorded as having SPMS and 1 person reporting SPMS and recorded as having PPMS. To clarify disease progression, patients were asked if they had progressive symptoms despite the effect of relapses; analysis showed agreement in 82.3% (Kappa 0.39). There was satisfactory correlation between questionnaire and clinician derived data for time in years from disease onset to SPMS (table 4.1 and 4.2, fig 4. 1 and 4.2). The method of data collection appeared to be more accurate in the TI group; however, this was due to chance with no significant difference between methods of data collection ($p=0.64$)

Patients were asked to report any relapses they had in the previous year. There was poor correlation between patients and clinician reported data (Kappa = 0.39) with 46.8% of patients reporting at least one relapse compared to 17.7% with clinician reported events. Patients were also inclined to report more relapses, with 6 patients reporting 2 relapses and 3 reporting 3, compared to only 2 patients recorded as having 2 relapses.

4.4 Discussion

This novel questionnaire, when compared to clinician collected longitudinal data, was accurate in the estimation of current EDSS as well as time from disease onset to well-recognised disability milestones EDSS >4, 6 and 8. However, sample size for analysis of time to EDSS 8 was limited to 8 patients, making this analysis underpowered. The proportion of patients with perfect agreement to milestones was improved with increasing the time-window to 2 years, however a 2 year discrepancy in this end-point would limit the use of such data in some short-term studies although a variability of 1-2 years may be more acceptable in studies collecting data over longer periods in a disease which commonly spans more than 30 years. Furthermore, missing data points in analysis of long term studies is likely to create more bias than using data points with a possible 2 years discrepancy. Precise information was also gained from the questionnaire regarding date of disease onset and diagnosis, and time to SPMS. There was accurate reporting of current disease course; however, the patients interviewed by telephone were more likely to incorrectly report their disease as RRMS when the clinician considered they had become progressive, and patient reporting of disease progression only showed moderate agreement. The reasons for this are not clear but

patient recollection of disease course may have been biased by what the patient was last told in clinic. The questionnaire was less accurate when assessing more detailed information related to features at disease onset and relapses where patients tended to over-report symptoms compared to physician records.

No major differences were observed between data collected via postal questionnaire compared to telephone interview; however, a trend was seen for the postal questionnaire to be more accurate, which was significant when assessing year of diagnosis and second relapse, despite the telephone group using the extra memory aid tool of the life course grid. This is possibly because patients completing the questionnaire engage family members and friends for corroboration and spend more time considering individual questions. The telephone questionnaire took between 10 and 30 minutes to complete whereas the postal questionnaire consumed minimal administrative resources and therefore may be more effective in collecting larger datasets providing return rates can be optimized.

It is important to consider the patient sample with regards to the generalisability of results. Because the primary end-point of the study was to assess time to disability milestones, only patients with higher EDSS scores were entered into the study. This sub-group of MS patients may possibly have a lower recall ability than patients with an EDSS <6.0 and a corresponding lower age and disease duration; therefore our study may under-represent the accuracy of the questionnaire. Patients selected to take part in this study had regular clinic attendance (necessary so that their core data was accurate),

this was reflected by a lower age, disease duration and EDSS than the non-assessed population. They therefore may represent a highly motivated group who has a good understanding of their disease and disability levels compared to patients not under regular specialist care.

This study assumes that clinician-derived data represents the gold standard; however, this may not always hold true especially given that some clinician-derived data is based on patient recollection in clinic. Although patients had all been assessed clinically in the year prior to the study, recent variation in symptoms may have been missed. This is of particular importance when considering current disability and relapses which therefore, may have been under-reported in the clinician derived data. However, this patient cohort all had an EDSS of > 6 with a high proportion having SPMS and long disease duration. The patients could therefore have expected to have a lower than average relapse rate and minimal progression over the course of a single year and the impact on results was unlikely to have been significant. Due to the size of the cohort studied, analysis of the impact of age, gender and disease duration on outcome measures was limited and only applied to EDSS milestone 6. Given the small sample size and wide confidence intervals from this analysis, systematic differences due to bias cannot be excluded. Future work using a larger and more representative data set may help further characterise whether there are any particular sub-groups with differential recall characteristics.

Previous work in this area has focused on collection of data to determine a single current measure of the EDSS either via postal questionnaire (Solari et al. 1993; Verdier-Taillefer et al. 1994; Goodin 1998; Bowen et al. 2001; Cheng et al. 2001) or telephone interview (Lechner-Scott et al. 2003). The reliability of self-reported disability has been shown to be good in the majority of these studies, with 2 studies reporting ICC's of >0.8 (Solari et al. 1993; Bowen et al. 2001); however, it was noted that reliability greatly improves if allowing for variability of +/- 1 EDSS point, which equates to varying levels of rise in disability depending on EDSS (Thompson et al. 1998). In other work Hoogervorst et al analysed the validated self-administered questionnaires, Guy's Neurologic Disability Scale (GNDS) (Hoogervorst et al. 2001b) and Multiple Sclerosis Impact Scale (MSIS-29) (Hoogervorst et al. 2004b), demonstrating good correlation with clinician assessed EDSS. This group then went on to assess self-reported changes in disability (showing poor correlation using the GNDS at 1 year but improved reliability at 2 years) (Hoogervorst et al. 2003; Hoogervorst et al. 2004a) and treatment effects on disability (the GNDS having poor sensitivity to pick up acute treatment related changes) (Hoogervorst et al. 2001a). Self-reported mobility scales such as the Rivermead Mobility Index (Collen et al. 1991) and the MS Walking Scale (Hobart et al. 2003) have also revealed good reliability compared with clinician assessment (Pearson et al. 2004); however, inaccuracy of doctors and patients in estimating walking distances has been observed, indicating that they may not be reliable indicators of health (Sharrack et al. 1997). Self-reporting of current disease course has been assessed in only one study, which revealed moderate agreement for patient classification and physician assessment (Kappa 0.45 (95% CI: 0.32-0.59))

(Bamer et al. 2007). To date all these studies examining self-reported measures are restricted to a contemporary analysis of disease state and do not analyse rates of disability progression.

Patients with MS frequently present to clinical services many years after the onset of symptoms. In these situations it is necessary and common practice to record retrospective patient-derived details relating to symptoms at disease onset. This study would suggest that although patient-derived data are likely to be valid for disease course and disability progression, they may give a less accurate representation of symptoms and course at disease onset and relapse rates. If determined retrospectively these data should be used with caution, particularly if likely to modify disease management options. The data also suggests that patients may over report relapsing disease and therefore caution should be observed in a clinical setting to use objective measures of progression. Presence of relapses in the previous year is also an important element in selecting therapeutic interventions and our study would suggest that patients have a tendency to over report clinically significant events and clinicians should be cautious in using self-reported relapses as evidence for treatment.

This questionnaire has been shown to be an accurate and cost-effective method of determining current and retrospective disability data including time to well-recognized disability milestones from MS patients and could be useful for augmenting phenotypic information in clinical practice as well as for research purposes. This work could be extended by obtaining more data and using covariates to model measurement error so

that modern methods of imputation can be used, thereby maximising the time to event data. Such an approach could be used with other datasets to enhance power and may help to reduce bias if patients in longitudinal studies with missing data differ in their natural history, thereby producing more realistic outcome data.

Table 4.1: Correlation of data collection for disease milestones

Differences are shown between data collected from the questionnaire (via post or telephone) and clinician derived data for current EDSS, year of disease onset and time in years from clinician recorded disease onset to disability milestones.

			Number	P Mean (SD)	CD Mean (SD)	ICC (95% CI)
Current EDSS		All	79	6.5(0.8)	6.4(0.7)	0.79 (0.69-0.86)
		PQ	35	6.6(0.8)	6.5(0.7)	0.89 (0.79-0.94)
		TI	44	6.5(0.8)	6.4(0.7)	0.72 (0.54-0.84)
Time to milestones	EDSS>4	All	53	10.3(8.3)	10.3(9.1)	0.94 (0.89-0.96)
		PQ	23	8.0(7.0)	7.8(7.7)	0.93 (0.84-0.97)
		TI	30	11.8(9.3)	12.2(9.8)	0.93 (0.86-0.97)
	EDSS 6	All	76	13.1(10.3)	14.0(11.0)	0.94 (0.90-0.96)
		PQ	34	12.3(10.7)	12.8(11.3)	0.95 (0.90-0.97)
		TI	42	13.7(10.1)	14.9(10.7)	0.93 (0.88-0.96)
	EDSS 8	All	8	16.6(11.8)	17.4(12.3)	0.99 (0.96-1.00)
		PQ	4	17.0(15.6)	17.8(16.3)	1.00 (0.93-1.00)
		TI	4	16.3(9.1)	17.0(9.5)	0.98 (0.78-1.00)
Year of onset		All	79	1992.6(9.5)	1991.6(11.1)	0.86(0.79-0.91)
		PQ	35	1992.8(10.0)	1992.3(11.6)	0.93(0.86-0.96)
		TI	44	1992.3(9.2)	1991.1(10.9)	0.80(0.66-0.89)
Time to milestones	Second relapse	All	79	2.9(3.7)	4.2(5.9)	0.55(0.37-0.69)
		PQ	35	2.2(3.4)	3.4(4.4)	0.64(0.39-0.80)
		TI	44	3.5(4.0)	4.9(6.8)	0.45(0.18-0.66)
	Diagnosis	All	79	6.2(8.2)	6.2(8.0)	0.86(0.79-0.91)
		PQ	35	5.5(7.7)	5.4(7.5)	0.98(0.97-0.99)
		TI	44	6.9(8.6)	6.8(8.4)	0.76(0.61-0.86)
	SPMS	All	57	10.9(10.5)	9.3(8.7)	0.78(0.65-0.86)
		PQ	26	9.5(9.5)	7.9(7.4)	0.69(0.42-0.85)
		TI	31	12.0(11.2)	10.5(9.7)	0.84(0.69-0.92)

p = data from questionnaire. CD; clinician derived data. SD; standard deviation. ICC ; Intraclass correlation coefficient. CI; confidence interval. PQ; postal questionnaire. TI; telephone interview.

Table 4.2: Reporting of disease milestones

Percent of patients who report the year of disease onset, diagnosis and time from symptom onset to disability and clinical milestones to either the exact year or within +/- 1 or +/-2 years of the clinician-derived data.

	Exact year	+/- 1 year	+/- 2 years
Onset	30.4	57.0	72.2
Diagnosis	53.2	81.0	88.6
EDSS >4	32.1	62.3	81.1
EDSS 6	23.7	59.2	75.0
EDSS 8	25.0	75.0	100.0
SPMS	14.0	47.4	61.4

EDSS; Expanded Disability Scale Status;

SPMS; Secondary Progressive Multiple Sclerosis

Figure 4.1: Plots showing no systematic bias in reporting

Bland-Altman plots showing the absolute (clinician - questionnaire data) and mean (average of clinician and questionnaire data) difference in years between questionnaire and clinician derived data for time to EDSS>4, 6 and 8 and SPSS with 95% absolute levels (indicated by dashed line).

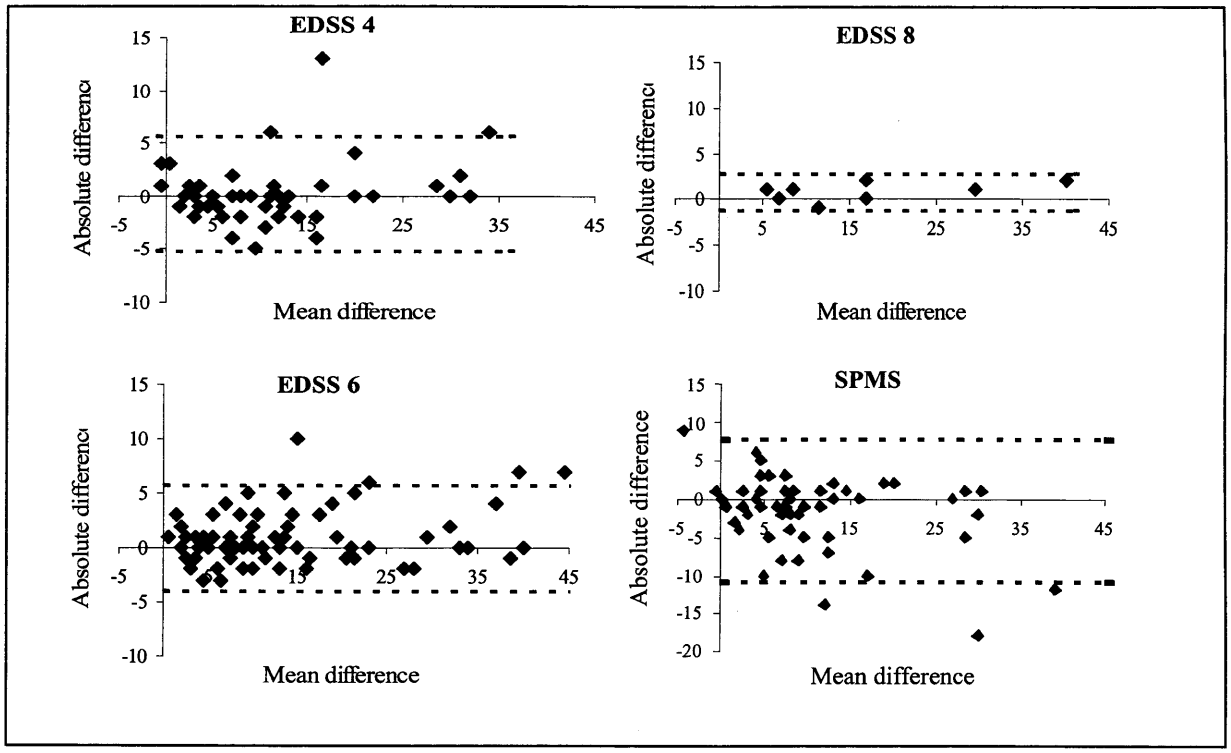
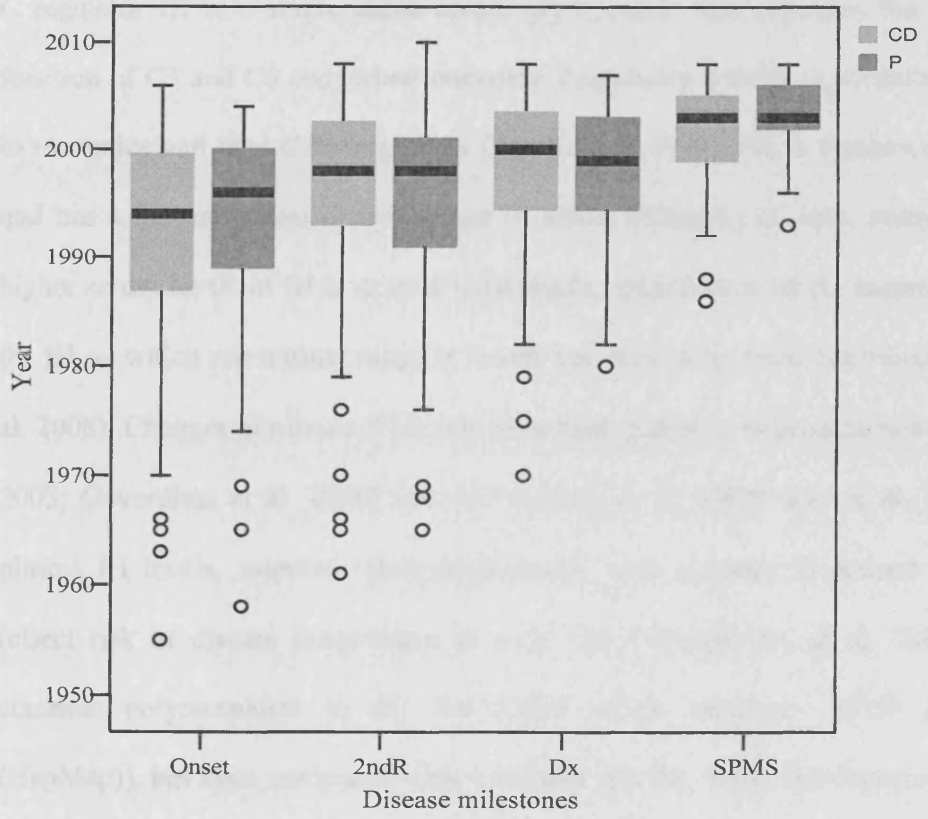


Figure 4.2: Accurate reporting of disease milestones

Box-plots of clinician-derived (CD) and patient reported (P) age of symptom onset (Onset), second relapse (2ndR), diagnosis (Dx) and onset of secondary progressive MS (SPMS). Box plots show the median (bold line) and interquartile range (box). Values more than 1.5 IQR's from the end of the box are labeled as outliers (o).



CHAPTER 5: COMPLEMENT REGULATOR FACTOR H AS A SERUM BIOMARKER OF MULTIPLE SCLEROSIS DISEASE STATE.

5.1 Introduction

C regulator fH is a single chain serum glycoprotein that regulates the formation and function of C3 and C5 convertase enzymes. Regulatory activity is attributed to its ability to recognize and bind C3b fragments (Zipfel et al. 1999). FH is synthesized in the liver and has a normal serum concentration of about 250mg/L; of note, many papers quote higher serum levels of fH in normal individuals; optimization of the assays and standards for fH on which our normal range is based, has previously been described (Hakobyan et al. 2008). Changes in plasma fH levels have been linked to atherosclerosis (Oksjoki et al. 2003; Goverdhan et al. 2006) and AD (Honda et al. 2000; Hye et al. 2006). In AD, plasma fH levels, assessed semiquantitatively, were elevated in disease and shown to reflect risk of disease progression in early AD (Thambisetty et al. 2008). The most common polymorphism in fH, Tyr402His (allele frequency 62:38 in Caucasians (HapMap)), has been associated with increased risk for AMD, homozygosity for the His allele conferring a six-fold increased risk (Edwards et al. 2005; Sjoberg et al. 2007). In AD, one large study found no association with this polymorphism (Hamilton et al. 2007), while another found an association of the His402 allele with disease, but only in those also carrying the ApoE4 risk allele (Zetterberg et al. 2008). FH appears to discriminate self from non-self by recognizing polyanionic structures on self cells, such as sialic acid

and the glycosaminoglycan chains of proteoglycans (e.g. heparin sulphate and dermatan sulphate), thus inhibiting C activation on host surfaces (Kazatchkine et al. 1979; Carreno et al. 1989). The Tyr402His polymorphism located within short consensus repeat 7, a putative binding site for glycosaminoglycans (Skerka et al. 2007), may influence surface binding. FH and its polymorphic variants have not been measured in MS serum or CSF samples to date.

The C system has an established role in the pathogenesis of MS (reviewed in Chapter 2) and in light of recent evidence (as described above) outlining a potential role for fH as a biomarker in other CNS inflammatory conditions and a recent proteomic study showing altered levels of fHL-1 in MS CSF specimens; the hypothesis was tested that serum fH levels would reflect chronic inflammation in MS and therefore help define disease state. Further, it is speculated that differential expression and/or consumption of the fH Tyr402His polymorphic variants would further inform risk of disease progression and other disease characteristics in MS. To achieve this, methods to interrogate fH levels and polymorphisms were developed and optimized using novel monoclonal antibodies and unique enzyme-linked immunosorbant assays (ELISAs) that enable the quantification of total fH and the Tyr402His polymorphic variants in plasma, serum or CSF (Hakobyan et al. 2008). Here, these unique ELISAs were employed to measure fH and the Tyr402His polymorphic variants in subgroups of MS patients with comprehensive clinical phenotypic data.

5.2 Methods

Subjects

Serum samples were prospectively obtained between 2006 and 2008, from 350 patients with MS (McDonald et al. 2001), including 212 patients with RRMS (97 with stable-RRMS (S-RRMS) who had no clinically evident relapse for at least 12 months and 115 with acute-RRMS (A-RRMS) sampled during acute relapse), 85 patients with SPMS and 53 with PPMS. Serial samples were available on 11 patients with clinically stable RRMS and 12 patients who were in transition from RRMS to SPMS. Patients with clinically stable RRMS were sampled over a limited period of 1 year so as to ensure disease stability, whereas transitional patients were sampled over 2 years so that samples were available in both RR and SP disease phases. Parallel information was recorded consisting of disease course, relapse status, disability (measured by the EDSS (Kurtzke 1983)), co-morbidity, inter-current infection and medications. The Multiple Sclerosis Severity Score (MSSS) was calculated from EDSS and disease duration (Roxburgh et al. 2005). The control group comprised 86 non-related age-matched subjects with no personal or family history of neurological disease. A replication study was performed on an additional 105 patients including 70 patients with RRMS (35 of whom were in acute relapse) and 35 with SPMS, and 40 controls.

Demographic details of cases and controls are outlined in table 5.1 and are broadly representative of the population-based characteristics recently reported for this region of the UK (Hirst et al. 2009), no patient reported significant co-morbidity. Mean time to SPMS from disease onset was 10.14 years (SD 9.40), and mean duration of progressive

phase disease in SPMS patients was 8.79 years (SD 6.84). Of the 115 patients in acute relapse (defined according to (Schumacker et al. 1965), 72.2% were treated with steroids, according to the clinical judgment of the assessing neurologist, after blood was sampled. No patients were on steroid treatment at the time of sampling and there were no coincidental infections at the time of relapse. Of the patients, 27.2% were on some form of disease modifying treatment including 45.4% in the S-RRMS group, 23.4% in the A-RRMS group and 25.9% of patients with SPMS. Disease modifying treatments included Rebif (5.3%), Betaferon (5.3%), Avonex (5.0%), Copaxone (0.8%), alemtuzemab (6.1%) and mitoxantrone (4.7%).

CSF was obtained with paired serum samples from 44 patients who had been admitted for investigation of suspected or known MS. Of these, 22 patients were subsequently found to have symptoms not related to demyelinating disease; these samples were used as controls. Subsequent neurological diagnosis within the control group included cervical myelopathy in 5 patients, cerebrovascular disease in 2 patients, trigeminal neuralgia in 2 patients, fibromyalgia, vestibular neuronitis and Sjogrens syndrome. Disease subgroups consisted of 15 patients with S-RRMS, 4 patients with A-RRMS in acute relapse and 3 patients with progressive disease.

Analysis

Assays for quantification of total fH and the fH-His402 polymorphic variant have recently been described (Hakobyan et al. 2008). In brief, microtiter plates (96-well Nunc

MaxiSorp, Life Technologies, Paisley, UK) were coated with affinity-purified rabbit anti-fH IgG diluted in bicarbonate coating buffer (pH 9.6) at 5 μg/well and incubated. After blocking with 1% bovine serum albumin (BSA) in PBS (phosphate buffer solution) (blocking buffer), standards, serum (1:6000 in blocking buffer) or CSF (1:10 in blocking buffer) samples were added in triplicate and incubated. Plates were washed 3 times in PBS/0.1% Tween 20 (washing buffer) before either HRP-labelled affinity-purified rabbit anti-human fH (100μl; 1mg/l) or HRP-labelled MBI-7 anti-fH-His402 (100μl; 1mg/l) was used to measure total fH or fH-His402 respectively. After incubation and washing (3 times with washing buffer with 1 min incubation on the last wash), bound antibody was detected using orthophenylenediamine (OPD; AbD Serotec, Martinsreid, Germany). Development was stopped by the addition of 10% sulphuric acid, and absorbance at 492 nm was measured. All incubation steps were performed motionless for 1 hour at 37°C. Purified fH-His402 and an equimolar mixture of both variants were used as standards for estimation of serum and CSF fH-His402 and total fH respectively. Concentrations of total fH and fH-His402 in serum and CSF were calculated by reference to the appropriate calibration curve prepared from the standards and expressed as mg/L of serum or CSF. Concentration of fH-Tyr402 polymorphic variant was calculated by subtraction of fH-His402 from total fH concentration. The calculated detection limit of the assay was 0.007 mg/l and the working range 0.01 – 0.2 mg/l. The assay performance was assessed by taking multiple measures from independently diluted aliquots of the same plasma samples, either within the same assay or in separate assays. The within-assay precision, measured by the CV%, ranged from 4.1% to 7.0% with an average of 5.5% for total fH measurement and from 7.7% to 12.8% with an average of 11.0% for fH-H402

measurement. Between-assay precision ranged from 4.9% to 10.1% with an average of 8.0% for total fH measurement and from 10.1% to 15.8% with an average of 12.5% for fH-H402 measurement. Serial dilutions of standard, serum and CSF were measured to demonstrate parallelism (fig 5.1). Routine CSF analysis has been outlined in Chapter 3.

Statistical analysis

Data analysis was performed using SPSS version 16 (SPSS inc., Chicago, IL, USA). A pilot study (table 5.2) demonstrated that a sample size of 35 gave a calculated power of 0.997 with 5% precision; pilot data were included in the main study. All data were normally distributed and mean serum fH levels were compared in disease subgroups using one-way ANOVA. Concordance, or *C* statistic, was calculated based on the area under the receiver operating characteristic (ROC) curve, to assess the contribution of fH to disease predictability (a perfect score would be 1.0 or 100% predictability). The effect of demographic and clinical variables (age, disease duration, EDSS, MSSS) on fH levels were determined individually using Pearson's correlation coefficient and then modelled using multivariate regression analysis to assess any dominant effect (data were normally distributed). The effect of gender and treatment with disease modifying drugs was examined in individual subgroups using a Students *t*-test. The effect of disease course on fH was reanalyzed accounting for variables using a univariate linear model. Where sample sizes are limited, medians values and IQRs are presented and data is analysed using non-parametric analysis.

5.3 Results

Serum fH levels reflect disease course in MS

Serum fH levels were significantly elevated in both primary and secondary progressive disease compared to controls and S-RRMS patients ($p<0.001$ for each) (table 5.1, fig 5.2). Within the S-RRMS group, fH levels in remission were higher than controls although this was not significant ($p=0.09$). Relapse was associated with a small but significant increase in fH concentration compared to controls ($p<0.001$) and S-RRMS ($p=0.009$). The *C* statistic based on the area under the ROC curve for SPMS versus S-RRMS was 0.83, 95% CI 0.77-0.89; $p<0.001$ (fig 5.3). Analysis of the data demonstrated that serum fH concentration had value as a surrogate marker of disease course in patients with MS, in particular in distinguishing SPMS from S-RRMS; sensitivity for this distinction was 89.41%, with specificity of 69.47% and a positive predictive value of 72.38% (test cut off value >237mg/L) (table 5.1). When patients in clinical relapse were included, sensitivity and specificity were reduced to 71.18% and 62.40%, respectively, with a positive predictive value of 59.48%. Serum fH concentration was less helpful in distinguishing S-RRMS from patients in relapse, with a sensitivity of 67.37%; specificity 48.00% and positive predictive value 49.61% (cut off value <233 mg/L).

To test whether fH levels fluctuated independent of disease state, concentrations of fH were measured in 33 samples from 11 clinically stable patients (from the S-RRMS group) venesected sequentially over a 12 month period during which time they were deemed by

the assessing neurologist to have clinically stable disease based on lack of reported relapses and lack of change in EDSS. These sequential samples showed little variation in fH concentrations with an average CV% of 6.8% (similar to the documented interassay CV% of 8%), suggesting both reproducibility of the assay and stable serum fH concentration over a protracted time course in patients with clinically stable disease (median fH levels initially were 199.35 mg/L and after 1 year were 193.42 mg/L) (fig 5.4).

An in-house replication study was performed in an additional 105 patients and 40 controls which confirmed a significant difference between controls and SPMS, mean difference 131.58 mg/L (95% CI 100.28-162.88; $p<0.001$); and between S-RRMS and SPMS, mean difference 80.90 mg/L (95% CI 48.57-113.23; $p<0.001$) (table 5.4).

Serum fH levels increase with disease progression in MS patients

To further investigate the dynamic changes in serum fH levels during the transition from relapsing to progressive phase disease, 12 patients were selected in whom 3 serum samples were available over a 2 year period from onset of progression. In this small cohort, fH levels increased over the two year period in 10 of the 12 patients by a median of 59.76 mg/L, with median fH concentrations of 206.64 mg/L (IQR 181.3-220.2) in the 1st year, 232.89 mg/L (IQR 207.4-262.1) in the 2nd year and 266.70 mg/L (IQR 227.4-312.0) in the final year for the whole group (fig 5.4). Non-parametric testing showed that these changes in fH levels over the 2-year period were highly significant ($p=0.007$) and fH levels at the endpoint were significantly higher compared to the S-RRMS group from

the main study (p=0.01). Of note, using the test cut-off for SPMS suggested previously (test cut off value >237mg/L), 9 of the 12 patients had a positive test at year 2. Nine of the 12 patients were on disease modifying treatments throughout the assessed time; this did not affect the outcome.

Serum fH levels do not reflect the nature of the lesion in MS relapse

Relapse clinical characteristics were tested to determine the relation to fH levels. Of the 115 patients in relapse, 29 had a brainstem event, 56 had a spinal or cortical event, 7 had optic neuritis and 23 had a pure sensory event as judged by history, examination and clinical criteria; fH levels were similar in these four relapse groups (mean fH 246.51 mg/L (SD 76.51) brainstem, 247.50 mg/L (SD 65.38) spinal or cortical, 256.61 mg/L (SD 81.06) optic neuritis, 236.94 mg/L (SD 71.90) sensory, p=0.807). In the whole relapse group, mean number of relapses over a four year period was 3.53 (SD 2.16) and the mean interval between relapses was 8.75 months (SD 6.09); neither number of relapses nor relapse interval correlated with fH levels (data not shown). Mean EDSS in the relapse group was 4.55 (SD 1.64) and MSSS was 6.59 (SD 2.31); neither of these parameters correlated with fH levels (data not shown).

Serum fH levels reflect disease activity independent of other patient factors

Correlation of fH concentrations with other phenotypic characteristics in the population was examined and revealed positive correlation with age ($r=0.31$, $p<0.001$), disease duration ($r=0.16$, $p=0.003$), EDSS ($r=0.20$, $p<0.001$) and MSSS ($r=0.14$, $p=0.010$);

however, in a multivariate regression model, only age remained significant. Disease modifying treatment had no effect on fH levels in A-RRMS or SPMS groups; however, in the S-RRMS group, patients on treatment had significantly lower fH levels than those off treatment ($p<0.001$) (table 5.4). The effect of disease course on fH was re-analysed when adjusted for the covariates above, with no significant changes to the results except patients in S-RRMS who, when accounting for treatment, were not different from patients in relapse (table 5.5). No independent effect from gender was observed (mean difference in fH between males and females was 1.75 mg/L). Serum fH concentrations failed to show any additional correlation in the SPMS group with time from disease onset to start of progression ($r=0.08$, $p=0.47$) or duration of progressive disease ($r=0.12$, $p=0.32$). These latter findings demonstrate that serum fH levels predominantly reflect the prevailing disease course at the time of sampling.

CSF fH levels correlate with serum fH and blood-CSF barrier breakdown

It was possible to measure fH in CSF using the assays described, although concentrations were <1% of serum; demographic details of patients and controls are shown in table 5.6. CSF fH levels were higher in MS patients compared to controls, however, these differences did not reach significance (table 5.7). Of note, the CSF:serum fH ratio was significantly higher in MS patients when compared to controls (table 5.7) suggesting that CSF fH levels were increased in MS as a result either of blood-CSF barrier (BCB) leak or intra-thecal synthesis. CSF:serum albumin ratio has been suggested as the most appropriate variable to establish the presence of BCB breakdown (Link et al. 1977; Tibbling et al. 1977; Eeg-Olofsson et al. 1981). In disease cases the CSF:serum fH ratio

was strongly correlated with CSF:serum albumin ratio (Pearson correlation=0.83, $p<0.001$). Patients with BCB leak (demonstrated in 5 cases with a raised CSF:serum albumin ratio) had significantly higher CSF fH levels than patients with a normal CSF:serum albumin ratio (median fH 1.17 mg/L compared to 0.68 mg/L, $p=0.02$). An abnormally high IgG:albumin index has been shown to reflect intra-thecal IgG synthesis (Link et al. 1977; Eeg-Olofsson et al. 1981) and in our patients normal values were exceeded in 17 cases including 4 of the 5 relapse cases. However, CSF fH levels were not significantly raised in these cases (median fH 0.80 mg/L vs. 0.65 mg/L, $p=0.337$) and there was no related evidence of intra-thecal fH synthesis measured by a fH:albumin Index ($p=0.151$) (table 5.7). These data indicate that raised fH CSF concentrations are predominantly due to influx of systemic fH at times of BCB breakdown, rather than local synthesis.

Tyr402His allele frequency is identical in MS patients and controls

The Tyr402His polymorphic status was determined for each sample based on the quantification of total fH and the fH-His402 variant. Out of 350 MS patients, 43 were homozygous for the His allele of the Tyr402His polymorphism, 175 were heterozygous and 132 were homozygous for Tyr allele, giving a His allele frequency of 37.28% which was not significantly different from frequencies observed in the control population (37.21%). Of note, similar analyses in AMD populations readily detect at the protein level the over-representation of the His allele anticipated from genetic studies (Hakobyan et al. 2008). The Tyr402His allelic status also had no effect on fH levels (mean serum fH 248.40 mg/L (SD 68.55) in fH-His402 homozygotes, 250.74 mg/L (SD 73.31) in fH-

Tyr402His heterozygotes and 243.84 mg/L (SD 67.04) in fH-Tyr402 homozygotes), in MS patients or controls. An unexpected finding in post-hoc analysis of the fH-Tyr402His heterozygote group was that higher concentrations of the fH-His402 variant compared to fH-Tyr402 were found in both progressive and relapsing MS but not in controls or MS in remission, with significantly higher levels in the SPMS ($p<0.01$) and PPMS ($p<0.05$) groups (fig 5.5), suggesting either selective synthesis of fH-His402 or consumption of the fH-Tyr402 variant in active disease when fH is up-regulated.

5.4 Discussion

In this chapter I have shown, in a large cohort of comprehensively clinically characterised patients, that raised serum levels of the C regulator fH strongly correlate with disease course independently of other phenotypic parameters such as age, disability or disease duration. Serum fH measurement may therefore be a useful non-invasive and simple clinical test for patient stratification, particularly to distinguish SPMS from RRMS where it has a sensitivity of 89.41% and specificity of 69.47%. FH levels were also raised in acute relapse, but not to a level as would provide a useful disease biomarker. Biochemical outliers to disease subgroups may have been due in part to clinical misclassification, especially in the relapsing and stable RRMS groups where subclinical relapses make disease activity difficult to determine without the use of concomitant MR brain and spinal cord examinations. Clinical inaccuracies for determination of disease progression are more difficult to establish due to the lack of a surrogate marker; it was thought that MR measures of brain atrophy could be useful, but this is not yet established with any degree of certainty (Furby et al. 2010). The amalgamation of multiple potential disease

biological markers is more likely to provide a combined biomarker profile with a higher predictive probability. Other potential markers could be examined within the complement system or from other candidates. In particular when looking at disease progression, it may be worth examining other potential markers of axonal damage as discussed in Chapter 1, such as NFL and Tau proteins.

It may also be that, with further refinements, measurement of fH levels in patients with MS can be used to predict disease course. Clinical details of patients with outlying fH levels were reviewed to identify obvious causes or atypical features. Of 29 patients in the S-RRMS group with fH levels above the 95% CI, 1 year follow-up revealed that 8 had a relapse shortly after initial evaluation, and 4 developed SPMS within a year of evaluation; no other patients in the S-RRMS group underwent relapse or developed SPMS within a year of sampling. Further to this it was demonstrated that patients with S-RRMS have constant serum fH levels, in contrast to patients developing progressive disease in whom serum fH levels increased. These data, though preliminary, suggest that elevated fH levels in clinically perceived S-RRMS may predict risk of relapse and progression and therefore, may also be useful in recognizing or predicting breakthrough disease in patient on disease modifying treatments.

Serum fH is the major fluid-phase regulator of the alternative pathway of C, increasingly recognised as a critical player in many diseases (Oksjoki et al. 2003; Edwards et al. 2005; Pickering et al. 2008; Thambisetty et al. 2008). Changes in fH levels, as well as being a marker for disease course, also imply altered regulation of activation of the alternative

pathway of C throughout the course of disease and in relation to acute inflammatory events. MS relapse is associated with extensive CNS inflammatory infiltration in which both the adaptive and innate immune system are up-regulated causing demyelination and oligodendrocyte damage. Increased levels of fH at times of relapse therefore may be a response to increased C activation. It has been suggested that as patients move through their disease course into the progressive phase, the innate immune system (including macrophages, dendritic cells, mast cells and C) plays a more significant role (Weiner 2008). The alternative pathway is largely responsible for the propagation of inflammation in a range of inflammatory disorders (Brodeur et al. 1991; Oksjoki et al. 2003; Thurman et al. 2006; Scholl et al. 2008); and in animal models of MS (experimental autoimmune encephalomyelitis), the alternative pathway contributes to disease process, while the roles of the classical and terminal pathways are less clear (Chapter 2). These lines of evidence lead us to speculate that increased fH levels in progressive disease reflect chronic activation of the alternative pathway contributing to disease.

Although fH is present in CSF at levels quantifiable in our assays, the low concentrations and low patient numbers made correlation of concentrations to disease parameters non-informative. Due to ethical restrictions in collection of control specimens, collection of CSF from non-neurological controls was limited; as a result, the CSF control patients may have had some other forms of neurological disease, such as vascular pathology, which has also been linked to activation of C. Nevertheless, raised fH CSF concentrations were demonstrated in MS corresponding with breakdown of the BCB (measured by

albumin CSF:serum ratios), thus likely to reflect movement of systemic fH into the CNS at times of disease activity.

Analysis of the frequencies of the fH Tyr402His variants, implicated in other inflammatory diseases, showed no difference in MS patients and controls, indicating that this polymorphism was not linked to disease in the population studied. However, measurement of the individual polymorphic variants in serum from Tyr402His heterozygotes has revealed differences in levels of the fH-Tyr402 and fH-His402 variants, with higher levels of the His402 variant in some subgroups of disease. This apparent allelic imbalance was seen only in those patient groups where total fH levels were also elevated, progressive and relapsing disease, suggesting either that there is selective up-regulation of the His402 variant or selective consumption of the Tyr402 variant in disease. The biological significance of this finding is uncertain.

In summary, work outlined in this chapter has demonstrated that measurement of serum FH, with further refinement, may be a simple, robust, cost-effective and transferable test for distinguishing disease sub-groups in MS which should have important applications in clinical practice. There is now a pressing need for this work to be validated in other cohorts. Elevation of fH levels in serum and CSF might imply that systemic C activation is in part responsible for driving pathology in progressive and relapsing disease. To explore this it will be necessary to measure other C components, activation products and regulators in MS serum or plasma and identify associations with changes in fH. C deposition in MS plaques was described more than 30 years ago (Woyciechowska et al.

1977; Compston et al. 1989) and has recently been confirmed by Lassmann and co-workers who have shown that abundant deposition of antibody and C in a subset of MS patients (Lucchinetti et al. 1996; Lucchinetti et al. 2000). Characterisation of alternative pathway proteins, particularly fH and its isoforms, in MS tissue will clarify the role of the alternative pathway of C in MS and also the relationship of fH to demyelination and axonal damage. Work in other inflammatory disorders has implicated dysregulation of the alternative pathway, linked to genetic polymorphisms in C components and regulators (Scholl et al. 2008; Hecker et al. 2010). A thorough molecular analysis of the C system in MS may, in addition to providing new biomarkers, identify contributions of C dysregulation to specific aspects of MS pathology.

Table 5.1: Demographic details and serum fH concentration of MS disease subgroups compared to control subjects

	No.	Age		Gender	Disease duration		EDSS		FH conc mg/L				FH test	
		Mean	SD	% f	Mean	SD	Mean	SD	Mean	SD	p*	p**	%	p*
Control	86	42.26	16.50	62.9					209.54	61.29	N/A	<0.001	30.23	
S-RRMS	97	39.11	8.95	72.16	9.79	7.16	2.97	1.38	225.29	57.96	0.091	0.009	29.90	1.000
A-RRMS	115	36.27	8.44	80.00	7.97	6.63	4.55	1.64	248.19	68.34	<0.001	N/A	46.96	0.020
SPMS	85	49.99	10.13	62.35	18.74	11.18	6.26	1.27	295.71	51.17	<0.001	<0.001	89.41	<0.001
PPMS	53	53.51	11.34	50.94	10.25	7.38	5.74	1.70	280.58	74.83	<0.001	0.002	60.38	0.001

SD; standard deviation. p = comparison with controls. p** = comparison with A-RRMS. FH test is the % of patients with fH levels above the arbitrary cut-off taken as the upper 95% CI of the S- RRMS group. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive disease course. PPMS; primary progressive disease course. Of note, age, disease duration and EDSS are, as expected, higher in progressive patients; however, when accounted for as covariates, did not significantly alter the differences in fH between disease subgroups.*

Figure 5.1: Parallelism of standards, serum and CSF is demonstrated

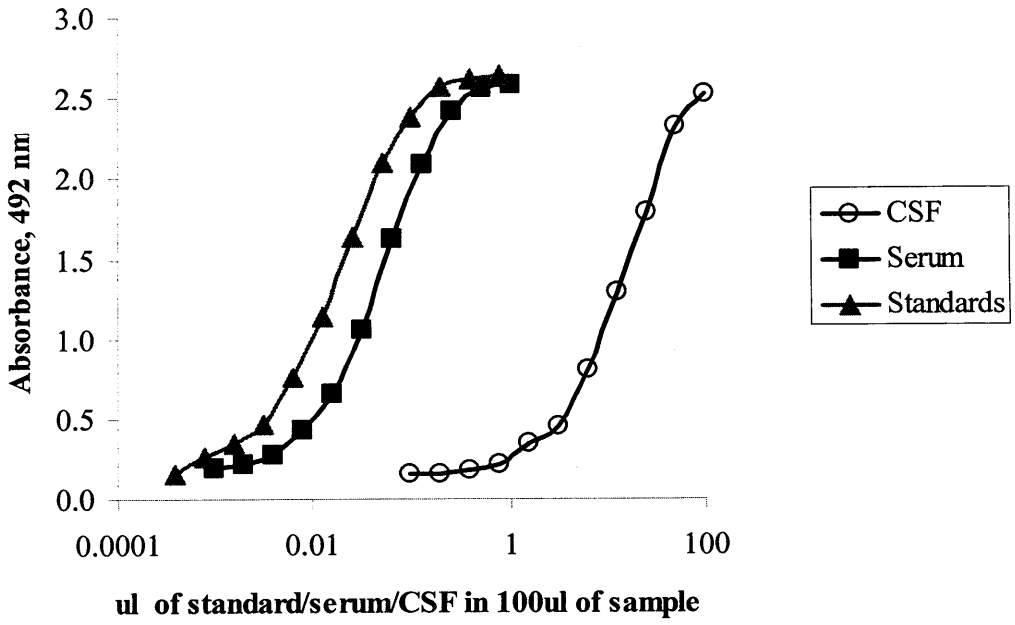


Table 5.2: Pilot data showing demographic details and factor H concentration in MS disease subgroups compared to controls

	Number	Age		Gender	Disease duration		EDSS		FH mg/L		p*	p**
		Mean	SD	% f	Mean	SD	Mean	SD	Mean	SD		
Control	40	37.20	8.86	77.5					220.46	39.81	N/A	0.023
S-RRMS	19	43.00	9.51	73.68	11.53	7.76	3.13	1.71	246.50	43.38	0.105	0.778
A-RRMS	34	36.38	7.52	82.35	6.97	4.81	4.87	1.73	251.15	71.23	0.023	N/A
SPMS	41	51.02	10.55	63.41	19.20	10.24	6.28	1.21	324.39	70.23	<0.001	<0.001
PPMS	20	50.45	11.48	50.00	8.89	4.54	5.33	1.48	270.65	39.40	0.002	0.229

FH; factor H. p = comparison with controls. p** = comparison with "RRMS relapse". SD; standard deviation. f; female. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.*

N.B. Pilot data is included in the main analysis.

Figure 5.2: Serum factor H in MS disease subgroups

Serum fH concentrations are significantly raised in patients with progressive disease compared with control subjects and patients with RRMS ($p<0.001$ for each comparison). RRMS patients in relapse show higher fH levels compared to those in remission ($p=0.016$). *(Data is shown as a dot plot with a horizontal line to denote the mean).*

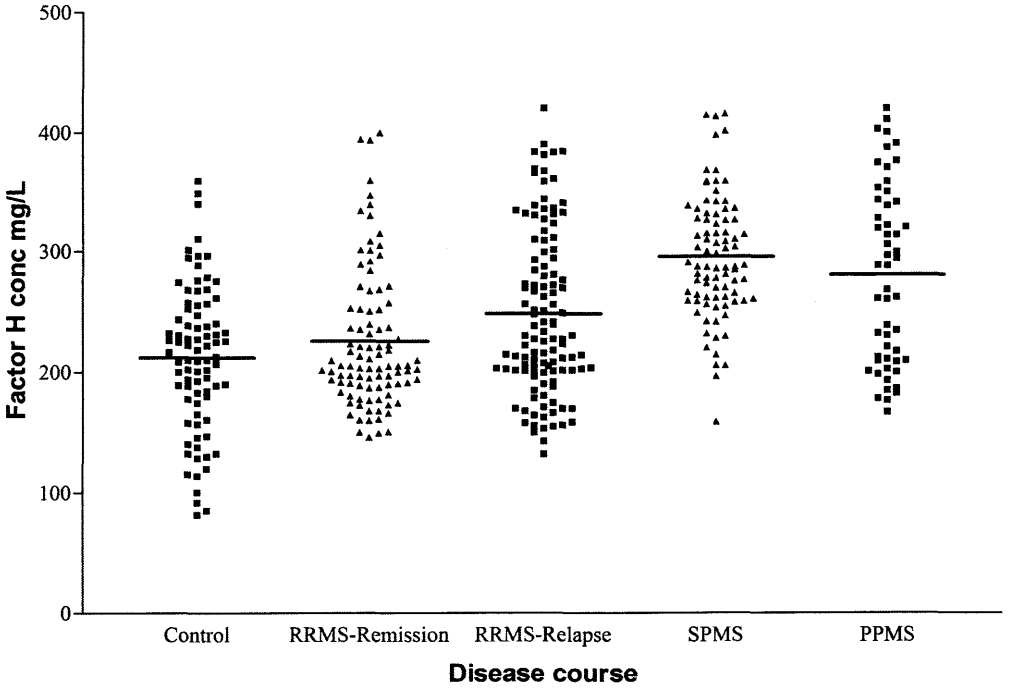


Figure 5.3: Serum factor H receiver operating curve for distinguishing SPMS from RRMS

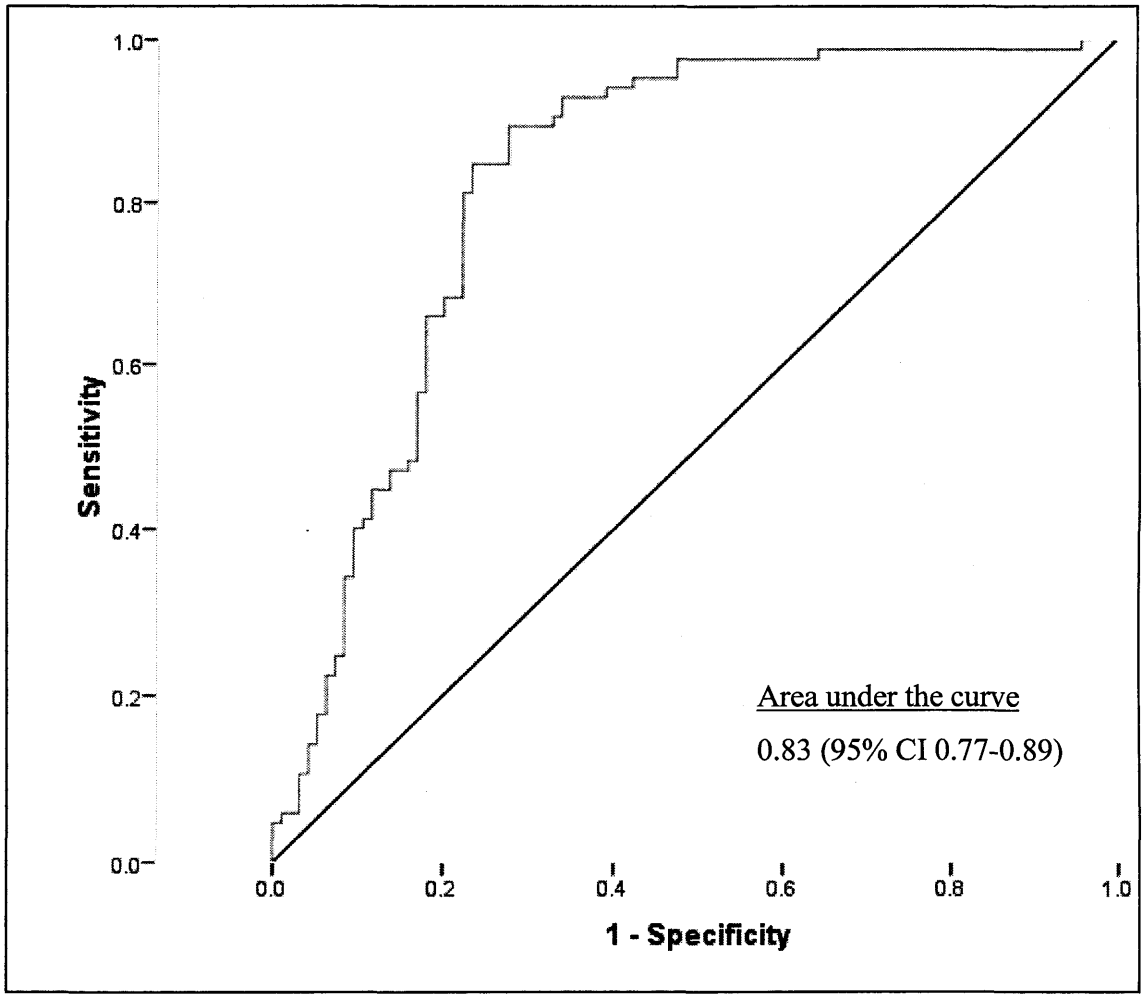
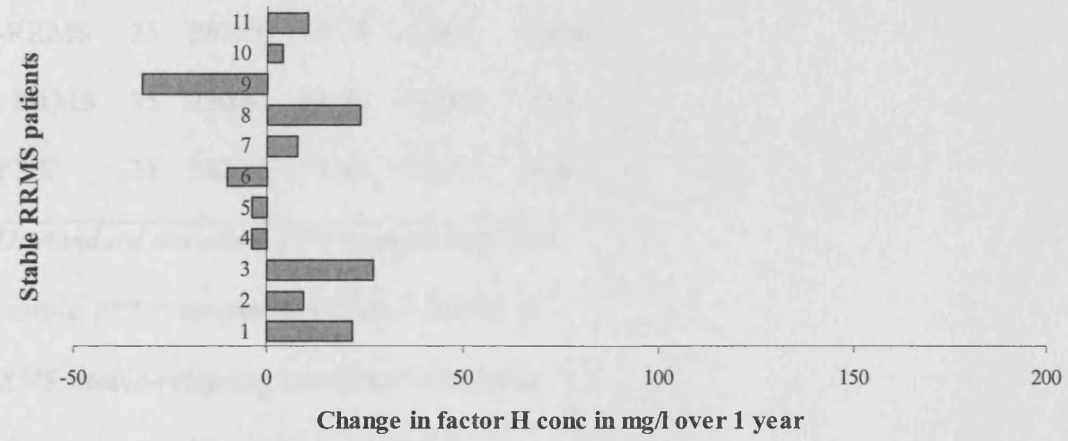


Figure 5.4: Serum factor H over time

FH concentrations increased significantly over a period of 2 years in 10 of the 12 patients who showed a clinical change in their MS disease state from RRMS to SPMS over this period (transitional patients) (p=0.007). However, there was little change in fH concentrations in S-RRMS patients sampled over 1 year with the change in 10 of 12 samples being less than the calculated inter-assay variation.



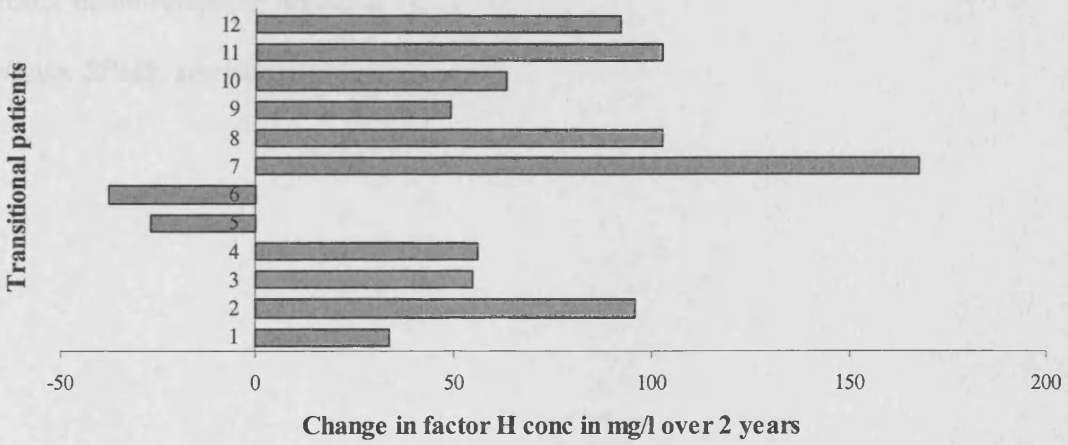


Table 5.3: Replication data showing raised serum factor H in MS patients compared to controls and in patients with SPMS compared to RRMS

		Mean	SD	p*	p**
Control	40	210.88	63.43	N/A	<0.001
S-RRMS	35	261.56	76.78	0.002	0.246
A-RRMS	35	280.62	61.56	<0.001	N/A
SPMS	35	342.46	71.48	<0.001	<0.001

SD; standard deviation. p = comparison with controls. p** = comparison with A-RRMS. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS.*

Table 5.4: Factor H levels in S-RRMS are altered on disease modifying treatment

	DMT	No.	FH mg/L		
			Mean	SD	p
S-RRMS	No	50	246.13	60.28	
	Yes	44	198.86	40.49	<0.001
A-RRMS	No	87	250.60	67.48	
	Yes	28	240.72	71.69	0.509
SPMS	No	61	299.30	54.22	
	Yes	22	282.21	40.55	0.182

DMT; disease modifying treatment. No; number. SD; standard deviation. p = comparison of fH levels on and off DMTs. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS.

Table 5.5: Serum factor H levels adjusted for covariates

Mean serum fH concentrations in mg/L with 95% CI are shown after adjustment for age, disease duration, EDSS, MSSS and treatment with disease modifying drugs; demonstrating that even when accounting for the affect of covariates, fH is predominantly affected by disease course.

	Age		Disease duration		EDSS		MSSS		Treatment	
	Mean difference from controls	p*	Mean difference from S-RRMS	p**	Mean difference from S-RRMS	p**	Mean difference from S-RRMS	p**	Mean difference from S-RRMS	p**
S-RRMS	17.3(-1.2-35.8)	0.066	N/A	N/A	N/A	N/A	N/A	N/A		
A-RRMS	39.6(21.7-57.5)	<0.001	20.9(3.6-38.3)	0.018	26.5(7.9-45.2)	0.005	22.5(3.7-41.2)	0.019	14.5(-2.7-31.8)	0.098
SPMS	82.6(63.3-102.0)	<0.001	71.3(51.2-91.5)	<0.001	81.7(58.0-105.4)	<0.001	71.9(51.7-92.2)	<0.001	64.3(45.6-89.3)	<0.001
PPMS	65.8(43.5-88.3)	<0.001	55.4(33.9-76.8)	<0.001	64.8(40.1-89.5)	<0.001	57.2(33.6-80.8)	<0.001	41.4(18.8-63.1)	0.002

p = p value for mean difference between disease groups and controls after adjustment for covariate. p** = p value for mean difference from S-RRMS group after adjustment for covariate. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.*

Table 5.6: Demographic details and routine CSF results in MS patients and controls

	Number	Age	Gender	Disease duration	EDSS	OCB	Albumin ratio	Ig Index
		Mean(SD)	% f	Mean(SD)	Mean(SD)	% +ve	Mean(SD)	Mean(SD)
Control	22	50.09(14.11)	77.27			0	5.12(2.83)	0.49(0.05)
MS	22	41.68(12.68)	68.18	7.55(9.34)	2.6(2.3)	100	5.53(1.92	1.08(0.47)

EDSS; expanded disability status scale. OCB; oligoclonal bands. Albumin ratio; CSF albumin mg/L / serum albumin g/L ratio. Ig Index; derived from CSF/serum ratios of IgG and albumin concentrations. SD; standard deviation.

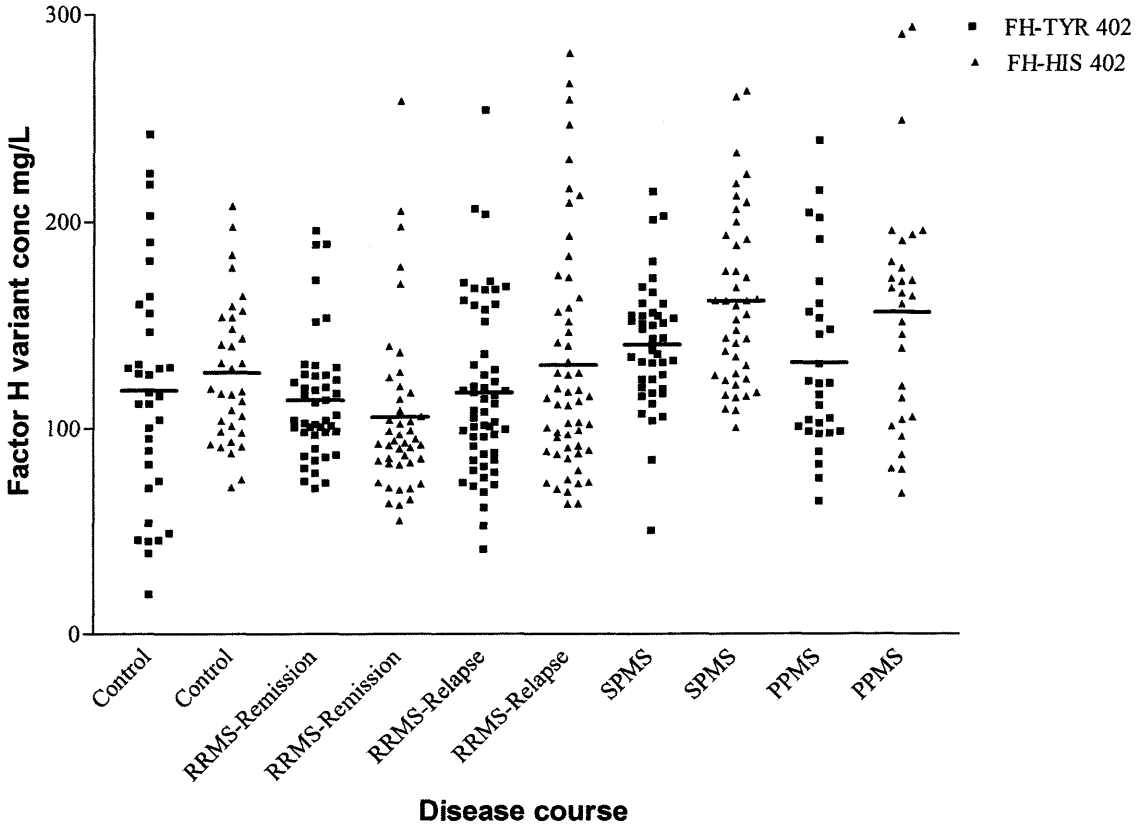
Table 5.7: Factor H CSF and serum concentrations in MS patients and controls

	CSF fH mg/L		Serum fH mg/L		FH CSF/Serum ratio		FH index	
	Mean(SD)	p	Mean(SD)	p	Mean(SD)	p	Mean(SD)	p
Control	0.65(0.32)		288.84(39.97)		2.38(1.24)		0.50(0.23)	
MS	0.78(0.32)	0.209	245.87(39.77)	0.001	3.24(1.43)	0.046	0.60(0.20)	0.151

FH Index; an analogous ratio, calculated by substituting FH for IgG in the Ig Index. SD; standard deviation. p = comparison of MS patients with controls.

Figure 5.5: Factor H Tyr-402His polymorphism in MS

In fH-Tyr402His heterozygotes, significantly higher concentrations of the fH-His402 variant compared to fH-Tyr402 were found in both SPMS (mean fH-His402 166.45 mg/L (SD 51.47) vs. mean fH-Tyr402 144.68 mg/L (SD 53.73); $p<0.01$) and PPMS (156.55 mg/L (SD 57.58) vs. 132.21 mg/L (SD 45.14); $p=0.05$) with higher concentrations also seen in relapsing patients, although this did not reach significance (135.36 mg/L (SD 59.48) vs. 109.13 mg/L (SD 58.54); $p=0.06$). There was no difference in variant concentrations observed in the control (120.35 mg/L (SD 41.53) vs. 117.19 mg/L (SD 61.75)) or RRMS (107.65 mg/L (SD 43.00) vs. 111.44 mg/L (SD 34.15)) groups. *(Data is shown as a dot plot with a horizontal line to denote the mean).*



CHAPTER 6: ELEVATED PLASMA C4A LEVELS IN MULTIPLE SCLEROSIS CORRELATE WITH DISEASE ACTIVITY

6.1 Introduction

C4 is one of the most abundant of the C proteins and is present in both classical and lectin pathways contributing to the formation of C4b2a, C3 convertase. Routinely measured in laboratories, it has been targeted in many studies examining C in both serum and CSF of MS patients. Although one such study has shown increased levels of serum C4 (Jans et al. 1984), this work has not been replicated and results of CSF studies have been conflicting (Kuwert et al. 1965; Link 1972; Jongen et al. 2000). More recent work has utilised proteomic analysis to evaluate biological samples from MS patients. In one such study (Sawai et al. 2010) a global protein/peptide analysis of plasma samples from 31 patients with RRMS obtained during acute relapse, revealed raised levels compared to controls of a peptide derived from the classical pathway component C4, and specifically, present in the C4 activation fragment, C4a (NGFKSHALQLNNRQI). Furthermore in a sub-group of patients on whom 3-6 month convalescent samples were available, levels of this peptide had fallen back to normal. This small study suggested that C4a levels might correlate with certain clinical components of MS disease activity and represent an informative biomarker of disease state. In this chapter I have analysed plasma C4 and C4a levels as well as CSF C4a levels in a large group of comprehensively phenotyped MS patients in order to determine the nature and clinical relevance of these parameters in disease.

6.2 Methods

Subjects

Plasma was collected from 107 patients with MS (McDonald et al. 2001), including 20 with S-RRMS who had experienced no relapse for at least 12 months, 55 with A-RRMS sampled during an acute relapse, 20 patients with SPMS and 12 patients with PPMS. Detailed clinical information was available on all patients including age, age at onset, disease duration and disability assessed by the EDSS (Kurtzke 1983). 9.62% of patients were on disease modifying treatments, including Interferon 1a and 1b, Copaxone, Mitoxantrone and Alemtuzemab. Of 55 A-RRMS patients, 43 subsequently remained relapse free over a 5-7 month period and underwent serial plasma sampling at intervals of between 2-3 months and 5-7 months post-relapse. None of the patients seen in relapse had inter-current infections; 69.64% were treated with oral or intravenous steroids after plasma samples had been collected, the reminder were untreated. Relapses were classified according to detailed anatomical and clinical characteristics; 7 patients had experienced brainstem or cerebellar relapses, 37 pyramidal or long tract motor relapses, 4 optic neuritis and 7 pure long tract sensory relapses. In patients who were serially sampled, mean EDSS at relapse was 4.71 (SD 1.66), this improved by a mean of 1.08 points (SD 1.07) at 2-3 months and by 1.09 points (SD 1.15) at 5-7 months following relapse. The control group comprised 40 non-related subjects with no personal or family history of neurological disease. Demographic details of patients and controls are provided in table 6.1.

CSF was obtained with paired plasma samples from 34 patients who had been admitted for investigation of suspected or known MS. Of these, 11 patients were

subsequently found to have symptoms unrelated to a CNS demyelinating disorder and these samples were used as controls. Subsequent neurological diagnosis within the control group included cervical myelopathy (5), cerebrovascular disease (3), trigeminal neuralgia (1), vestibular neuronitis (1) and lumbar radiculopathy (1). Of patients with MS, 18 had S-RRMS, 2 had A-RRMS and 3 had PPMS.

Analysis

C4a levels were determined using a commercial assay from BD Biosciences (assay kit no. 550947, BD Biosciences, 2350 Qume Drive, San Jose, CA 95131-1807; bdbiosciences.com); all samples were tested in duplicate. C4 concentrations were measured by nephelometry on a Beckman BN11 nephlometer in the University Hospital of Wales Clinical Immunology laboratory using commercial standards. Routine CSF analysis has been outlined in Chapter 3.

Statistical analysis

Data was analysed using SPSS version 16 (SPSS inc., Chicago, IL, USA). All data were normally distributed and are therefore presented as means with SDs and analysed using parametric tests. Disease subgroups were compared using either Students *t*-test (for 2-way analysis) or one-way ANOVA (for multiple comparisons). Concordance, or *C* statistic, was calculated based on the area under the ROC curve, to assess the contribution of fH to disease predictability (a perfect score would be 1.0 or 100% predictability). Correlations were performed using Pearson's correlation coefficient and then modelled using multivariate regression analysis to assess any dominant effect. Subgroup analysis of CSF was limited by number of samples and is therefore presented as median values and analysed using the non-parametric Mann-Whitney test to compare groups.

6.3 Results

Plasma C4 levels weakly correlated with plasma C4a (r=0.27, p=0.001). C4 levels were raised in the MS population overall compared to controls, although this did not reach significance (table 6.2), and levels did not distinguish between clinical subgroups. Plasma C4a levels were significantly elevated in patients compared to controls (p=0.001), but sub-group analysis revealed that C4a levels were significantly elevated only in patients with A-RRMS compared to both controls (p<0.001) and patients with S-RRMS (p=0.004) (table 6.2, fig 6.1). The *C* statistic for A-RRMS versus S-RRMS was 0.69, 95% CI 0.56-0.82; p=0.012 (fig 6.2). Analysis of MS sub-phenotypes revealed weak correlation of C4a with EDSS (r=0.22; p=0.02); however, when accounting for other clinical characteristics including age, age-at-onset and disease duration, using a multivariate regression model, significance was not maintained (Beta coefficient 0.379, p=0.143).

Serial samples were available from 43 patients with A-RRMS who were subsequently relapse-free for 5-7 months. C4a levels were higher in acute relapse compared to convalescent samples in 28 of the 43 patients (fig 6.3). Mean C4a levels in acute relapse were significantly higher than in convalescent samples taken at 2-3 months (p=0.04); however, although relapse C4a levels were higher than those measured at 5-7 months, this did not reach significance (p=0.12) (table 6.3). Univariate analysis accounting for EDSS as a covariate did not alter these findings (data not shown). There was no difference in C4 levels between A-RRMS and convalescent samples (table 6.3).

CSF concentrations of C4a were <1% that of plasma but were quantifiable in the assay. Demographic details for the patient cohort from whom CSF samples were obtained are displayed in table 6.4. CSF C4a levels, C4a CSF:plasma ratio and C4a Index (derived from a ratio of C4a and albumin concentrations in CSF and plasma; C4a CSF ng/mL:plasma mg/L / Albumin CSF mg/L:plasma g/L) were all significantly higher in MS patients than the control population (table 6.5). CSF C4a levels showed moderate correlation with CSF:serum IgG ($r=0.53$, $p=0.01$) but not CSF:serum albumin ($r=0.21$, $p=0.34$). In addition, patients with a raised CSF:serum IgG ratio (indicating intrathecal IgG synthesis (Link et al. 1977; Eeg-Olofsson et al. 1981)) had significantly higher levels of CSF C4a (n=8; median 66.32 ng/mL, IQR 48.72-69.82); $p<0.001$) compared to patients with a normal IgG ratio (n=26; median 27.89 ng/mL, IQR 21.70-32.24). Patients with a raised CSF:serum albumin ratio (indicating leak of the BCB (Link et al. 1977; Eeg-Olofsson et al. 1981)) did not have significantly raised CSF C4a levels (n=4; median 45.63 ng/mL, IQR 42.52-56.86; $p=0.082$) compared to patients with a normal albumin ratio (n=30; median 28.46 ng/mL, IQR 21.90-44.93). This indicates that raised C4a levels in MS patients are most likely a result of intrathecal production of C4a as opposed to leakage from plasma through the BCB. Plasma C4a levels were non-significantly higher in patients with both a raised CSF:serum IgG ratio (median plasma C4a 0.78 mg/L (IQR 0.35-1.19) vs. 0.64 mg/L (IQR 0.37-1.24); $p=0.262$) and a raised CSF:serum albumin ratio (median plasma C4a 1.05 mg/L (IQR 0.62-1.95) vs. 0.64 mg/L (IQR 0.35-1.00); $p=0.082$).

6.4 Discussion

Although C has an established role in the pathogenesis of MS, the extent and nature of activation remains unknown. Previous studies have suggested that the classical and terminal pathways of C may have central roles with the identification of C4 fragments and terminal C complex deposition within MS plaques, one distinguishing a pathological subset of MS patients (Lucchinetti et al. 1996; Storch et al. 1998; Brink et al. 2005). There is also evidence from small studies that C4 may be elevated in MS serum and reduced in CSF compared to controls (Jans et al. 1984), although, to date, this work has not been convincingly replicated (Link 1972; Yam et al. 1980; Jongen et al. 2000). Conversely, evidence from animal models demonstrate that C4-deficient (classical pathway absent) animals, show onset and progression of EAE which is virtually identical to wild-type animals (Morariu et al. 1978; Boos et al. 2005). C4a is a short-lived fragment of C4, generated during activation of the classical or lectin pathways. Although found to be elevated in plasma in several other diseases where activation of the classical pathway occurs (Kasuya et al. 1989; Wild et al. 1990; Abou-Ragheb et al. 1992), it has hitherto escaped detailed examination in MS. A recent peptide biomarker study in MS plasma identified a peptide derived from the C4a sequence (Sawai et al.), motivating us to look more closely at C4a levels in MS patients.

Work in this chapter has demonstrated that MS patients with clinically active disease have raised plasma C4a levels compared to either patients in remission or healthy controls. Furthermore, dynamic changes in C4a post-relapse show C4a levels decrease after the acute inflammatory event. However, these changes were inconsistent within the patient cohort examined and therefore it is unlikely that the observed changes in

C4a levels would be reliable enough to be employed alone as a surrogate marker of inflammation in clinical practice. In particular, convalescent samples at 5-7 months post-relapse did not demonstrate the same degree of reduction in C4a levels as at 2-3 months, for reasons that are unclear. It may be that there is a quiescent period post-relapse during which time further inflammatory events are less likely, before the return of clinical or sub-clinical events in some patients by around 6 months. MR studies would enable further clarification of these issues.

The demonstration of elevation of CSF C4a levels adds to the growing evidence for C activation in the CNS contributing to the pathogenic process in MS. Our results point towards intrathecal activation of the classical pathway of C in CSF; however, since C4a is relatively small (76 amino acids) in comparison with most proteins, it will likely cross the intact BCB to some degree, and changes in barrier permeability may have little effect on C4a transit (Felgenhauer et al. 1976). Indeed, our results show no significant impact of BCB breakdown on CSF C4a levels. Plasma levels of C4a were high during acute relapse, suggesting that there is a significant systemic inflammatory component to the disease. The concept of systemic inflammation in primary central nervous system inflammatory disorders is not novel and is perhaps best illustrated by the presence of serum oligoclonal IgG, indicating systemic immune activation, which is also associated with intrathecal IgG synthesis (Zeman et al. 1996) in MS as well as a number of other disorders. Analyse of CSF C4a in disease subgroups was limited due to the low number of patients.

In summary, plasma and CSF levels of C4a are shown to be elevated in MS, the former restricted to patients in relapse. I speculate that the classical pathway is

activated both in the CNS and systemically in MS patients, particularly at times of relapse highlighting a possible role for anti-C agents in MS, although inter-individual variability implies that different patterns of C activation may represent distinct pathological subgroups. Further work is needed to establish the details of C activation in MS subgroups and disease stages, and the relative contributions of classical and alternative pathways.

Table 6.1: Demographic details of MS disease subgroups and control patients

	No.	Age		Sex	AAO		Disease duration		EDSS	
		Mean	SD	%f	Mean	SD	Mean	SD	Mean	SD
Control	40	36.28	9.84	72.50	N/A	N/A	N/A	N/A	N/A	N/A
Total MS	107	43.80	11.72	67.29	31.39	10.68	11.81	11.51	4.79	2.00
S-RRMS	20	41.85	9.48	55.00	32.25	8.34	8.60	7.41	3.30	1.66
A-RRMS	55	38.44	9.85	76.36	28.45	7.75	9.76	9.07	4.48	1.72
SPMS	20	55.55	8.38	65.00	29.85	13.31	24.70	14.53	6.85	1.40
PPMS	12	52.08	9.75	50.00	46.00	9.82	5.08	4.70	5.29	1.80

AAO; age at onset. EDSS; expanded disability status scale. SD; standard deviation. f; female. S-RRMS; stable relapsing remitting MS with no reported relapse for at least 12 months. A-RRMS; active relapsing remitting MS in acute relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.

Table 6.2: Plasma C4 and C4a levels in MS disease subgroups and control patients

	No.	C4 mg/L			C4a mg/L			
		Mean	SD	p*	Mean	SD	p*	p**
Control	40	247.50	78.57	N/A	1.37	0.75	N/A	<0.001
Total MS	107	282.82	99.16	0.056	1.80	0.96	0.01	N/A
S-RRMS	20	299.33	116.11	0.060	1.39	0.79	0.91	<0.01
A-RRMS	55	278.70	92.41	0.130	2.07	1.07	<0.001	N/A
SPMS	20	292.42	74.96	0.10	1.69	0.70	0.18	0.10
PPMS	12	259.09	139.64	0.72	1.40	0.67	0.89	0.02

SD; standard deviation. p= comparison of C4/C4a levels compared to control patients. p**= comparison of C4a levels compared to acute relapse. S-RRMS; stable relapsing remitting MS with no reported relapse for at least 12 months. A-RRMS; active relapsing remitting MS in acute relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.*

Figure 6.1: C4a levels in MS disease subgroups

C4a levels in MS are raised in patients with relapsing remitting MS in acute relapse (A-RRMS) compared to both controls ($p<0.001$) and stable relapsing remitting (S-RRMS) ($p=0.004$) patients. No elevation of C4a was seen in secondary progressive (SPMS), primary progressive (PPMS) or stable relapsing remitting (S-RRMS) patients when compared to controls. *Box plots demonstrate median levels, with box margins indicating the interquartile range.*

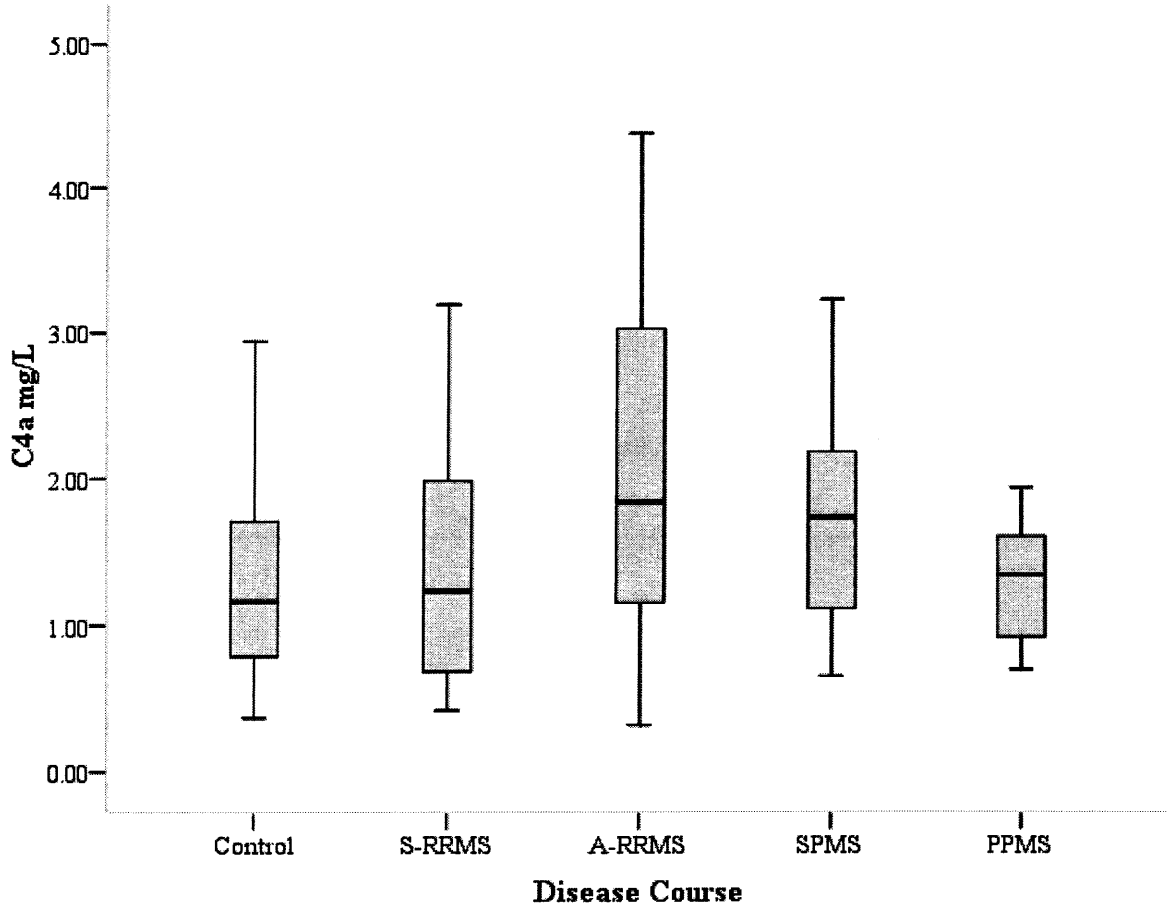


Figure 6.2: Plasma C4a receiver operating curve for distinguishing acute RRMS in relapse from stable RRMS

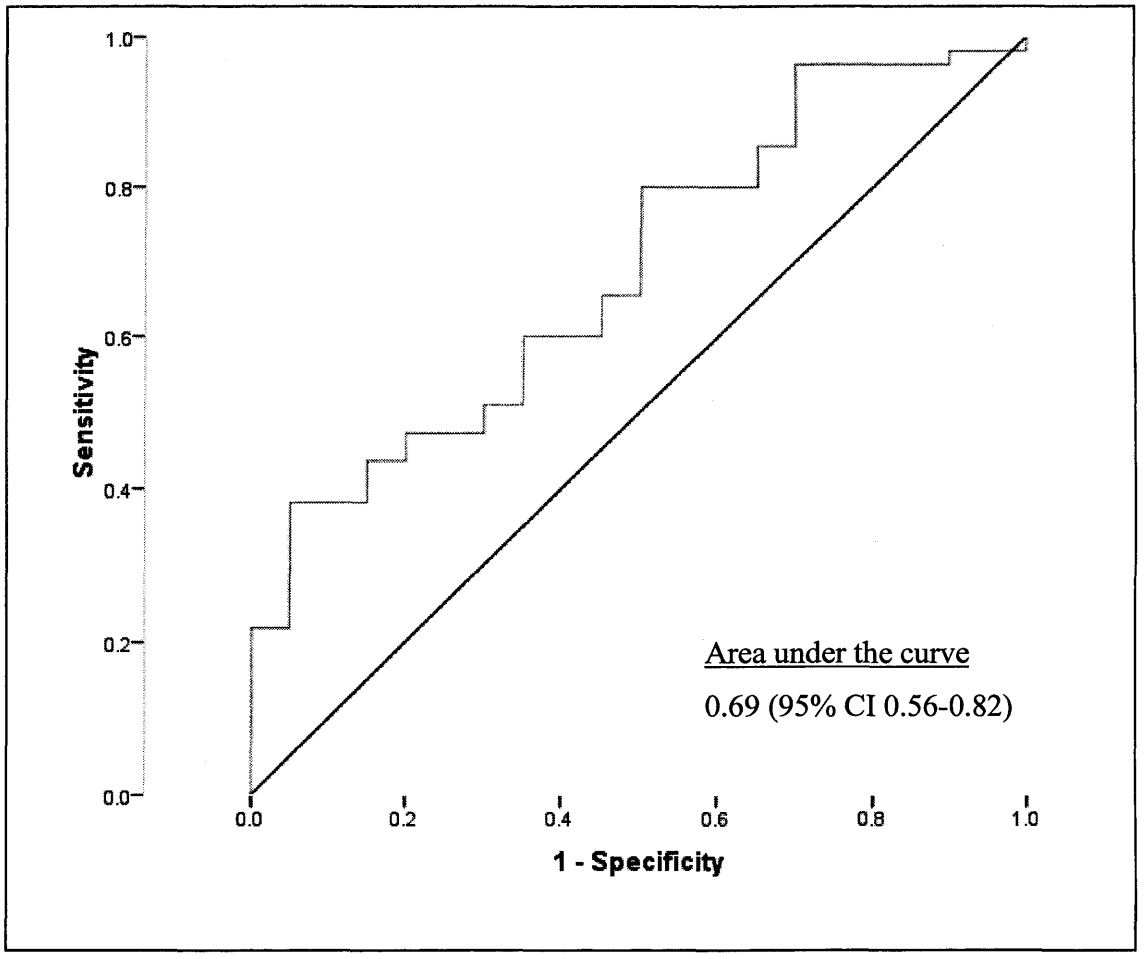


Figure 6.3: Plasma C4a levels post-relapse

Plasma C4a levels are shown in 43 patients with MS in acute relapse (0), from convalescent samples taken 2-3 months or 5-7 months post relapse, showing reduction in plasma C4a in 28 of the 43 patients post relapse. Student's t test demonstrated a significant reduction in mean C4a levels at 2-3 months (p=0.04) but not 5-7 months (p=0.12).

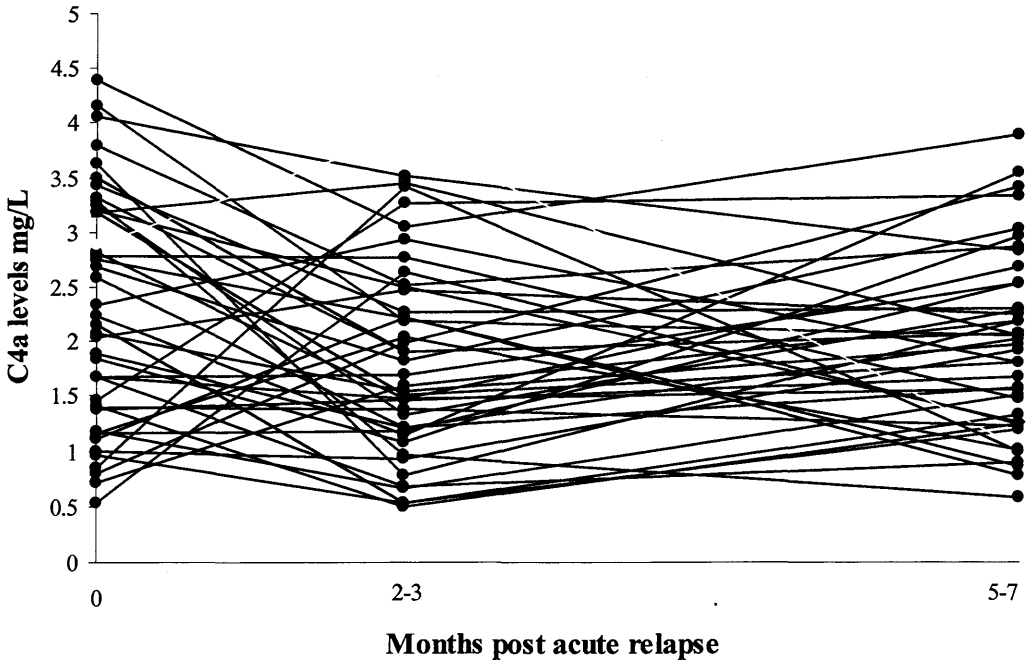


Table 6.3: EDSS, plasma C4 and C4a levels are shown from patients in acute relapse with convalescent samples 2-3 and 5-7 months post relapse

	No.	EDSS		C4 mg/L			C4a mg/L		
		Mean	SD	Mean	SD	p	Mean	SD	p
A-RRMS	43	4.71	1.66	270.53	81.29	N/A	2.27	1.07	N/A
2-3 months	43	3.64	2.08	251.35	77.02	0.27	1.85	0.91	0.04
5-7 months	43	3.61	2.04	260.56	84.07	0.57	1.95	0.81	0.12

EDSS; expanded disability status scale. SD; standard deviation. p= comparison of C4/C4a levels with acute relapse. A-RRMS; active relapsing remitting MS in acute relapse. 2-3 and 5-7 months refer to convalescent samples after acute relapse.

Table 6.4: Demographic details and routine CSF biochemistry is shown from MS patients and controls sampled for CSF

	No.	Age		Sex	DD		EDSS		Alb ratio		Ig Index		OCB
		Mean	SD	%f	Mean	SD	Mean	SD	Mean	SD	Mean	SD	% +ve
Control	11	50.18	18.10	72.73	N/A	N/A	N/A	N/A	5.09	2.64	0.49	0.05	0.00
MS	23	41.30	10.84	78.26	7.09	8.85	2.98	1.89	5.42	2.30	1.05	0.53	95.65

DD; disease duration. EDSS; expanded disability status scale. Alb ratio; Albumin CSF mg/L: Serum g/L ratio. Ig Index; derived from ratios of IgG and albumin concentrations in CSF and serum. OCB; oligoclonal bands. SD; standard deviation. f; female.

Table 6.5: C4a in MS patients and controls sampled for CSF

	No.	CSF C4a ng/ml			Plasma C4a mg/L			C4a ratio			C4a Index		
		Mean	SD	p	Mean	SD	p	Mean	SD	p	Mean	SD	p
Control	11	26.66	10.48	N/A	0.90	0.58	N/A	44.33	36.34	N/A	8.79	6.64	N/A
MS	23	42.41	20.61	<0.01	0.79	0.60	0.63	92.18	84.06	0.04	19.14	18.21	0.03

C4a ratio; C4a CSF ng/ml: plasma mg/L ratio. C4a Index; an analogous ratio calculated by substituting C4a for IgG in the Ig Index (C4a CSF ng/mL:plasma mg/L / Albumin CSF mg/L:plasma g/L). SD; standard deviation.

CHAPTER 7: SYSTEMIC COMPLEMENT PROFILES IN MULTIPLE SCLEROSIS ARE USEFUL AS BIOMARKERS OF DISEASE STATE

7.1 Introduction

So far, work in this thesis has outlined evidence suggesting a contribution from C to pathogenic processes in MS (Chapter 2); however, the extent and precise nature of C activation, and its contribution to disease phenotype and long term outcome, remains unclear. Studies of C levels and activation in MS plasma and CSF to date have been limited and disparities in results may relate either to differences in measurement techniques, low patient numbers leading to underpowered studies, or poor stratification of patients with a complex disease.

Work in Chapters 5 and 6 demonstrate dynamic changes in systemic C levels shown in C protein C4, activation product C4a and regulator fH. This was evident especially in relation to acute relapse, associated with raised C4a and fH; and disease progression, associated with raised fH. Furthermore, serum fH was identified as a potentially useful clinical marker of disease progression with a predictive value based on the *C* statistic of 83%. Local changes in C in CSF were more difficult to analyse given the low levels and limited number of patients. However, it was clear from this work that there are local changes in C production and/or consumption.

Based on evidence from these initial studies, this chapter extends the analysis of the role and extent of activation and regulation of C in a comprehensively phenotyped population of MS patients. Representative elements of various aspects of C activity were strategically chosen to compile a comprehensive set of C proteins, activation products and regulators of classical, alternative and terminal pathways. C profiles were established for different disease courses and evaluated as predictive models of disease state.

7.2 Methods

Subjects

Plasma samples were selected from 194 patients with MS (McDonald et al., 2001), including 40 patients with S-RRMS, with no clinically evident relapse for at least 12 months; 78 with A-RRMS sampled in relapse, 39 with SPMS and 37 with PPMS. Assays were initially performed in a pilot study with 20 patients in each group. Additional analysis of 150 patients was performed for components showing some variation between groups; with further analysis, in relevant components, of 44 patients serially sampled in acute relapse. Detailed clinical information was available on all patients including age, age at onset, disease duration and disability assessed by the EDSS (Kurtzke 1983). Of the 194 patients, 8.76% were on disease modifying treatments, including Interferon 1a (2 patients) and 1b (5 patients), Copaxone (3 patients), Mitoxantrone (4 patients) and Alemtuzemab (4 patients). Of the 78 patients with A-RRMS, 44 remained relapse free over a 5-7 month period and underwent serial plasma sampling at intervals of between 2-3 months and 5-7 months post-relapse. No patients seen in relapse had inter-current infections; 71.79% were treated with oral or intravenous steroids after plasma samples

had been collected, the reminder were untreated. Relapses were classified according to detailed anatomical and clinical characteristics; 13 patients had experienced brainstem or cerebellar relapses, 50 pyrimidal or long tract motor relapses, 5 optic neuritis and 10 pure long tract sensory relapses. In patients who were serially sampled, mean EDSS at relapse was 4.72 (SD 1.64), this improved by a mean of 1.08 points (SD 1.07) at 2-3 months and by 1.09 points (SD 1.15) at 5-7 months following relapse. The control group comprised 35 non-related subjects with no personal or family history of neurological disease. Demographic details of patients and controls are displayed in table 7.1. Patients selected for this study included the RRMS and SPMS patients and controls used in the replication phase of Chapter 5 and the patients from Chapter 6 who were serially sampled post relapse.

CSF was obtained with paired plasma samples from 66 patients who had been admitted for investigation of suspected or known MS. Of these, 5 had a CIS and 24 patients were subsequently found to have symptoms not related to demyelinating disease; these samples were used as controls. Subsequent neurological diagnosis within the control group included cervical myelopathy, cerebrovascular disease, trigeminal neuralgia, fibromyalgia, vestibular neuronitis and Sjogren's syndrome. Of the 37 patients with MS, disease subgroups consisted of 28 patients with S-RRMS, 5 patients with A-RRMS in acute relapse and 4 patients with progressive disease.

Measurement of factor B

A sandwich ELISA for quantification of fB in plasma, serum or CSF samples was developed in-house. Maxisorp (Nunc, Life Technologies, Paisley, UK) plates were coated

with in-house monoclonal mouse anti-human fB (JC1, 100µl, 5mg/l) overnight at 4°C, washed in 0.1% Tween 20 in PBS (washing buffer) and blocked with 200µl 1% BSA/10 mM EDTA (blocking buffer) in PBS. After a single wash in washing buffer, 100µl of standards, plasma (diluted 1:3000 in blocking buffer) or CSF (diluted 1:10 in blocking buffer) samples were added in duplicate and incubated. Wells were washed 3 times in washing buffer and incubated with 100µl of HRP-labelled affinity-purified rabbit anti-human fB (100 µL; 1 mg/L). All incubations were stationary, 1 hour, 37°C, unless stated otherwise. Wells were washed 3 times in washing buffer and bound antibody was visualised with orthophenylenediamine (SIGMAFAST™ OPD; Sigma-Aldrich, Dorset, UK). Development was stopped by the addition of 10% sulphuric acid, and absorbance at 492nm was measured. Purified human fB was used as a standard for estimation of plasma fB. Control standards were included on each plate. Concentration of fB in plasma or CSF was calculated by reference to the appropriate calibration curve prepared from the standards and expressed as mg/L of plasma or CSF. A nonlinear regression model was used to fit standard curves generated by ELISA. The calculated detection limit of the assay was 0.006 mg/L and the working range 0.01-0.2 mg/L. The assay performance was assessed by taking multiple measures from independently diluted aliquots of the same plasma samples, either within the same assay or in separate assays. The within-assay precision, measured by the CV%, ranged from 6% to 8% with an average of 7%. Between-assay precision ranged from 5% to 11% with an average of 9%.

Measurement of C9

Sandwich ELISAs for quantification of C9 in plasma, serum or CSF samples were developed in-house with methods as described above. Coating antibody for C9 detection

was used at 0.1 ug/well (B7, in-house). Plasma samples were diluted 1:2000 in blocking buffer and CSF was diluted 1:10 in blocking buffer. HRP-labelled monoclonal mouse anti-human C9 (MAC68, in-house) was added to wells for detection of C9 (100 μL; 1 mg/L). A pool of control plasma samples was used as a standard and results are expressed as a percentage of the control. The calculated detection limit of the C9 assay was 0.005% with a working range of 0.015-0.25%. The within-assay precision ranged from 0% to 4% with an average of 3% and between-assay precision ranged from 0% to 11% with an average of 6%.

Measurement of C1s

Sandwich ELISAs for quantification of C1s in plasma, serum or CSF samples were developed in-house with methods as described above. Coating antibody for C1s (F33, in-house) was used at 0.5 ug/well. Plasma samples were diluted 1:2000 in blocking buffer and CSF was diluted 1:10 in blocking buffer for both assays. For detection of C1s, goat polyclonal antiserum to human C1s (Quidel; assay no. A302; Quidel Corporation Headquarters, 10165 McKellar Court, San Diego, CA 92121; www.quidel.com) was added to each well, and after 1 hour stationary incubation at 37°C, HRP-labelled polyclonal rabbit anti-goat immunoglobulins (Dako; assay no. P0449; Dako UK Ltd, Cambridge House, St Thomas Place, Ely, Cambridgeshire, CB7 4EX; www.dako.com) was added. A pool of control plasma samples was used as a standard in both assays and results are expressed as a percentage of the control. The calculated detection limit of the C1s assay was 0.003% with a working range of 0.015-0.25%. The within-assay precision ranged from 0% to 5% with an average of 4% and between-assay precision ranged from 0% to 12% with an average of 6%.

Measurement of fH

The assay for the detection of fH has previously been described in Chapter 5.

Measurement of clusterin, TCC, Bb and C4a

Clusterin was quantified using a commercial assay from ALPCO diagnostics (assay no. 44-CLUHU-E05; ALPCO Diagnostics, 26G Keewaydin Drive, Saem, NH 03079; www.alpco.com). TCC was quantified using a commercial assay from Hycult Biotech, working range 8.2 - 2000 mAU/ml (assay no. HK328; Frontstraat 2a, 5405 PB UDEN, The Netherlands; www.hby.nl). Bb was quantified using a commercial assay from Quidel (assay no. A027; Quidel Corporation, 10165 McKellar Court, San Diego, CA 92121; www.quidel.com). C4a was quantified using a commercial assay from BD Biosciences (assay kit no. 550947, BD Biosciences, 2350 Qume Drive, San Jose, CA 95131-1807; bdbiosciences.com) as described in Chapter 6.

Measurement of C3, C4, C1 inhibitor and C-reactive protein

C3, C4 and C1-inh concentrations were measured by nephelometry on a Beckman BN11 nephelometer in the using commercial standards. The assay working range for C3 was 0.02-4.1 g/L, for C4 was 0.01-1.9 g/L and for C1-inh was 0.02-0.6 g/L. High sensitivity C-reactive protein (CRP) was measured by laser nephelometry in the University Hospital of Wales Clinical Immunology laboratory, with a lower detection limit of 0.04 mg/L.

Routine CSF analysis

CSF analysis has been outlined in Chapter 3.

Statistical analysis

Data analysis was performed using SPSS version 16 (SPSS inc., Chicago, IL) statistical package. All data was normally distributed. Quantitative concentrations were compared between disease subgroups using either Students' *t*-test for 2-way analysis, or Anova with Bonferonni correction for multiple comparisons. Odds ratios (ORs) with 95% CIs were calculated individually for each variable and positive variables were then used in a logistic regression model. Objective clinical characteristics such as age, gender and disease duration were added to the model and concordance, or the *C* statistic was calculated based on the area under ROC curve, to assess whether individual components or combined models contributed to predicting binary outcomes (a perfect score would be 1.0 or 100% predictability). Correlations were performed using Pearson's correlation coefficient and then modelled using multivariate regression analysis to assess any dominant effect. Subgroup analysis of CSF was limited by number of samples and is therefore presented as median values and analyzed using the non-parametric Mann-Whitney test to compare groups.

7.3 Results

Plasma complement levels predict MS compared to a control population

Compared with healthy controls, the total MS population analysed showed increased mean levels of plasma C3 (OR 1.00, 95% CI 1.00-1.00, p=0.004) and C4 (OR 1.01, 95%CI 1.01-1.02, p=0.001); reduced levels of plasma C9 (OR 0.98, 95% CI 0.97-0.99, p<0.001); and no difference in mean levels of plasma proteins C1s (OR 1.00, 95% CI

0.99-1.01) and fB (OR 1.00, 95% CI 0.99-1.00, p=0.400) (table 7.2, fig 7.1). There was a significant increase in plasma levels of activation product C4a (OR 1.00, 95%CI 1.00-1.00, p<0.001); however, as shown previously this elevation was seen only in the A-RRMS group. No changes were seen in activation product Bb (OR 1.48, 95% CI 0.22-10.12) and levels of TCC were undetectable in the plasma with a lower assay detection limit of 8.2mAU/ml (table 7.3). C regulators C1-inh (OR 1.02, 95% CI 1.01-1.03, p<0.001) and fH (OR 1.01, 95% CI 1.00-1.02, p<0.001) were significantly raised in MS patients compared to controls with no difference seen in plasma levels of clusterin (OR 1.01, 95% CI 0.99-1.02) (table 7.4, fig 7.1). A logistic regression model examining MS patients and normal healthy controls was constructed and limited to analysis of C9, C3, C4, C1-inh and fH. The *C* statistic based on this model was 0.97 (95% CI 0.94-1.00, p<0.001) and superior to any individual component (fig 7.2).

Dynamic changes in plasma complement levels in acute relapse

Analysis of disease course subgroups demonstrated elevation of mean levels of plasma proteins C9 (p=0.027) and fB (p=0.065) (table 7.2), activation product C4a (p=0.011) (table 7.3) and regulator fH (p=0.557) (table 7.4) in A-RRMS compared to S-RRMS. A logistic regression model was developed to assess A-RRMS compared to S-RRMS; an initial model using fB, C9, C4a and fH along with objective clinical features age, gender and disease duration; revealed there was no contribution to the model from fB (the significance of change if fB was removed from the model = 0.979), gender (the significance of change if gender was removed from the model = 0.214), C4a (the significance of change if C4a was removed from the model = 0.146) or fH (the significance of change if fH was removed from the model = 0.144). The optimal model

used C9 (OR 1.03, 95% CI 1.01-1.04, p=0.003), age (OR 0.90, 95% CI 0.85-0.96, p=0.001) and disease duration (OR 1.07, 95% CI 1.01-1.14, p=0.025). The *C* statistic based on the area under the ROC curve for this model to predict A-RRMS from S-RRMS was 0.75, 95% CI 0.66-0.84 (fig 7.3).

Where higher mean plasma levels were seen in A-RRMS, an extended analysis was conducted with measurement of convalescent samples post-relapse at 2-3 and 5-7 months. There were no differences in mean plasma levels seen between acute relapse and convalescent samples for either C9 or fB (table 7.5). Mean plasma fH levels were significantly higher in A-RRMS compared to both 2-3 (p=0.013) and 5-7 (p=0.007) month samples (table 7.5, fig 7.4); however, levels were only reduced in 26 of 44 subjects at 2 months and 30 of 44 subjects at 6 months suggesting inconsistencies across individuals. Changes in C4a post-relapse have been reported in Chapter 6.

Analysis of phenotypic parameters showed no correlation of any measured components with gender, disease duration, EDSS, MSSS, treatment with disease modifying drugs or time to secondary progressive disease from onset. There was weak correlation of both plasma C9 (r=0.16) and C1s (r=0.12) with age, however, using age as a covariate in our previous analysis did not alter results (data not shown). There was no difference in levels of CRP between the total MS population (mean CRP 4.09, SD 5.23) and the control group (mean 3.16, SD 6.94), or between MS disease subgroups (S-RRMS mean 4.2, SD 5.76; A-RRMS mean 3.04, SD 4.12; SPMS mean 4.86, SD 6.12; PPMS mean 3.94, SD

4.10), demonstrating the absence of another indicator of systemic inflammation at times of C upregulation.

Changes in complement levels in the CSF

It was possible to measure C components fB, C9, C1s, clusterin and TCC in CSF using the assays described (levels of fH and C4a in CSF have been described previously in Chapters 5 and 6); demographic details of patients and controls are shown in table 7.6. Levels of both CSF and plasma fB and C9 were reduced in patients with MS and CIS compared with the control population; however, this only reached significance in plasma C9 levels (table 7.7). C9 CSF/plasma ratio and C9 index were also non-significantly reduced in MS patients compared to controls (table 7.7). Levels of CSF and plasma clusterin, C1s and TCC were increased in patients with MS and CIS compared to controls; this only reached significance in the plasma clusterin CIS cases and given sample size, this finding may be the result of a type 1 error.

In order to examine the degree to which levels of C were due to BCB breakdown or intrathecal synthesis, correlation was assessed with either raised CSF:serum albumin or raised IgG:albumin index respectively (Link et al. 1977; Eeg-Olofsson et al. 1981) (table 7.8). CSF:plasma fB and C9 showed good correlation with both the IgG index and the CSF:serum albumin ratio; in addition, levels of CSF fB and C9 were elevated in patients with a raised IgG index (fB p=0.056, C9 p=0.01) and a raised CSF:serum albumin ratio (fB p=0.173, C9 p=0.010) indicating both systemic and local synthesis of fB and C9. CSF:plasma ratios of C1s and clusterin showed moderate correlation with CSF:serum albumin, and raised CSF levels were seen only in patients with a raised CSF:serum

albumin ratio (C1s, p=0.024, clusterin, p=0.011) indicating that local C1s and clusterin appeared predominantly due to influx at times of BCB breakdown. Levels of CSF TCC showed no correlation with either CSF IgG or albumin.

Examination of disease characteristics showed weak to moderate correlation of CSF fB, C9, C1s and clusterin with age (fB r= 0.23, p=0.068; C9 r=0.39, p=0.002; C1s r=0.23, p=0.079; clusterin r=0.38, p=0.002); however, no correlation was seen with disease duration. CSF C9 showed a weak correlation with disability measured by EDSS (r=0.33, p=0.03).

7.4 Discussion

Although MS is a predominantly T cell mediated disease, it is well established that C plays an important role. I have previously demonstrated both systemic and local up-regulation of C regulator fH (Chapter 5) and fragment C4a (Chapter 6). Here, analysis of local and systemic C in MS patients is extended with the aim of clarifying the contribution of C to disease processes and combined complotype and phenotype models are examined to determine possible combined biomarkers of disease and disease course.

Given the complex and variable immunopathogenesis of MS, the use of combinations of plasma markers to develop informative biomarkers of disease and disease course is likely to be more informative than using isolated measurement. The model developed here for distinguishing MS from controls uses a combination of plasma C components C3, C4,

C9, fH and C1-inh to give a predictive probability of 97%. The use of this model clinically is limited in its current form given that distinguishing MS from differential diagnosis or from CIS was beyond the scope of this study. Additional complotypes to distinguish disease subgroups were not established. No measured component other than fH was specific for disease progression; and although C9, C4a and fH showed differential changes in relapse, when combined, did not produce a predictive model. This may have been because of difficulties in the clinical classification of relapse and progression as discussed in Chapter 5, or it may be that other complement components or inflammatory markers would be more useful in establishing biomarker profiles. The addition of C measurements to objective clinical characteristics, disease duration and age, to distinguish disease state did seem to be of value with a combined model predictive probability for relapse of 75%. This chapter establishes the potential use of complotypes as plasma biomarkers for diagnosis and it is now necessary to evaluate this further with neurological control groups. The use of complotypes for distinguishing disease course needs further evaluation in a prospective cohort of patients with parallel MR data. There is clearly a need for this work to be replicated by independent groups to confirm the use of these models in clinical practice, which would be best achieved in an independent prospective cohort.

Systemic up-regulation of C regulators and systemic and local consumption of C components was demonstrated throughout various stages of the MS disease course, implying continuous activation of C and dynamic effects that are both local and systemic. The previously noted increase in alternative pathway regulator fH predominantly in

progressive disease, contrasts with up-regulation of C1-inh which shows no differential expression between disease subgroups; possibly indicating the need for regulation of the classical pathway of C throughout disease course with the alternative pathway playing a more prominent role as disease progresses. Levels of plasma fH, raised at times of acute relapse, also correlate with clinical disease activity with a significant reduction in plasma fH in convalescent samples post relapse, possibly implying the transient need for systemic regulation of the alternative pathway at times of acute inflammatory events. Although in this study CSF C1-inh was not measured, local expression of fH and increased CSF fH levels corresponding with breakdown of the blood-CSF barrier has previously been demonstrated. I speculate that C regulation is important both locally and systemically in MS.

Reduced levels of both plasma fB and plasma and CSF C9 in the total MS population imply consumption of these components throughout disease with increased systemic production in acute relapse. This corresponds with raised plasma C3 and C4 levels in MS. Chapter 6 demonstrated raised levels of C4a, a fragment of C4, isolated to acute relapse; and raised CSF C4a in the total MS population examined. Together, these results may indicate both local and systemic activation of classical and alternative pathways of C in MS.

Although 2 previous studies have examined plasma C9 in MS patients showing no difference from controls (Morgan et al. 1984; Compston et al. 1986), these studies were limited in terms of patient numbers and may have been underpowered to detect change.

Local reduction of C9 levels shown here is consistent with previous studies (Morgan et al. 1984; Compston et al. 1986); however, given the nature of our CSF control group, there may have been more C activation in the controls than would be seen in normal healthy controls, limiting the difference in C9 observed. Interestingly, CSF C9 in our study did show some degree of correlation with disability, previously only shown in CSF TCC measurements in a single study (Sellebjerg et al. 1998). Further work with extended analysis of CSF C9 and/or TCC in MS disease subgroups and CIS patients may be useful in determining whether they could be clinically informative.

In vitro studies have established that not only can CNS cells produce all C components necessary for C lysis (Gasque et al. 1992; Gasque et al. 1993; Gasque et al. 1995), but also C is a tissue-damaging factor in demyelination and neurodegeneration (Scolding et al. 1989; Wren et al. 1989; Piddlesden et al. 1993b). It is still unclear how this relates to in vivo pathology and whether C components seen in MS white matter plaques are locally produced or are a result of BCB breakdown. This study demonstrates the majority of C components including C9, C1s, fB, fH and clusterin leak into the CSF at times of BCB breakdown; in addition there is suggestion of local biosynthesis of some components including fB, C9 and C4a. Although not conclusive, this evidence suggests both locally and systemically synthesised C contribute to the disease process in MS.

In summary I have developed potential complotype models to distinguish MS from controls and differentiate disease course subgroups. I have shown both local and systemic activation and regulation of C in MS patients, in the absence of changes in CRP, an acute

phase reactant raised in systemic inflammatory responses, indicating C activation is likely due to MS rather than a second disease process. Differential C component concentrations showed correlation with clinical disease activity, disease course or disability and I suggest that both classical and alternative pathways of C play an important role at times of acute inflammatory attack, but also act throughout the whole disease. Systemically produced C is shown to cross the BCB at times of inflammation and given that the vast majority of C synthesis is hepatic, it is possible that systemically targeted anti-C therapies may significantly reduce the extent of local C attack and may be of use in MS patients in addition to more traditional adaptive immune targeted therapies.

Table 7.1: Demographic details of total MS population and controls

	No.	Gender	Age		DD		EDSS		MSSS	
		% f	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	35	60.00	50.57	19.54	N/A	N/A	N/A	N/A	N/A	N/A
Total MS	194	67.53	44.07	12.38	11.74	10.45	4.94	2.07	6.54	2.55
S-RRMS	40	80.00	41.58	9.30	8.00	9.39	2.58	1.38	4.40	2.60
A-RRMS	78	76.92	36.54	9.54	8.68	7.84	4.69	1.51	6.72	2.44
SPMS	39	51.28	53.15	9.56	22.95	10.67	6.95	1.16	7.26	2.00
PPMS	37	51.35	53.05	11.62	10.43	8.02	5.92	1.66	7.71	1.86

DD; disease duration. EDSS; expanded disability status scale. MSSS; MS severity score. f; female. SD; standard deviation. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.

Table 7.2: Comparison of concentrations of complement components between MS disease subgroups and controls

		FB mg/L			C3 mg/L			C1s % of arbitrary standard		C4 mg/L			C9 % of arbitrary standard			
	No.	Mean	SD	p*	Mean	SD	p	Mean	SD	Mean	SD	p	Mean	SD	p	p*
Control	35	194.02	68.28	1	1263.14	268.38	N/A	97.91	44.75	187.43	57.09	N/A	151.11	35.78	N/A	0.001
Total MS	194	184.74	57.55	N/A	1449.13	357.38	0.004	103.42	46.53	237.08	83.39	0.001	113.02	34.09	<0.001	N/A
S-RRMS	40	171.26	34.04	0.065	1450.38	370.23	0.206	100.47	48.34	222.79	66.60	0.581	103.93	20.97	<0.001	0.027
A-RRMS	78	202.29	75.43	N/A	1398.59	348.49	0.549	101.79	47.69	232.23	70.43	0.060	123.74	43.18	0.001	N/A
SPMS	39	169.86	33.59	0.048	1506.26	306.80	0.028	113.16	51.24	258.30	97.83	0.002	104.78	24.09	<0.001	0.044
PPMS	37	177.99	44.54	0.371	1499.58	413.23	0.052	99.79	36.15	249.00	145.97	0.256	108.94	26.84	<0.001	0.282

SD; standard deviation. P values are measured using one-way Anova with Bonferroni correction and are only shown in groups displaying a positive result. P = comparison with control group. P = comparison with A-RRMS group. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.*

Table 7.3: Comparison of concentrations of complement activation products between MS disease subgroups and controls

	Bb mg/L			C4a mg/L				
	No.	Mean	SD	No.	Mean	SD	p	p*
Control	35	1.04	0.21	35	819.65	570.94	N/A	<0.001
Total MS	82	1.07	0.3	194	1487.20	971.28	<0.001	N/A
S-RRMS	20	1.03	0.32	40	1134.22	774.91	1.000	0.011
A-RRMS	20	0.98	0.19	78	1726.57	1100.41	<0.001	N/A
SPMS	22	1.14	0.31	39	1292.25	788.04	0.316	0.190
PPMS	20	1.31	0.42	37	1579.80	646.69	0.154	1.000

SD; standard deviation. P values are measured using one-way Anova with Bonferroni correction and are only shown in groups displaying a positive result. P = comparison with control group. P = comparison with A-RRMS group. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.*

Table 7.4: Comparison of concentrations of complement regulators between MS disease subgroups and controls

	C1 inhibitor g/L					Clusterin mg/L			Factor H mg/L				
	No.	Mean	SD	p	p*	No.	Mean	SD	No.	Mean	SD	p	p*
Control	35	0.29	0.05	N/A	<0.001	35	52.74	23.09	35	212.94	63.08	N/A	0.010
Total MS	150	0.36	0.07	<0.001	N/A	121	55.05	19.55	194	265.55	80.22	<0.001	N/A
S-RRMS	40	0.35	0.06	0.001	1.000	20	55.48	14.90	40	235.82	78.18	1.000	0.557
A-RRMS	34	0.36	0.09	<0.001	N/A	59	55.51	19.76	78	263.89	58.93	0.010	N/A
SPMS	39	0.37	0.06	<0.001	1.000	22	56.99	23.72	39	343.01	70.02	<0.001	<0.001
PPMS	37	0.34	0.05	0.013	1.000	20	51.13	18.91	37	274.11	77.26	0.003	1.000

SD; standard deviation. P values are measured using one-way Anova with Bonferroni correction and are only shown in groups displaying a positive result. P = comparison with control group. P = comparison with A-RRMS group. S-RRMS; stable-relapsing remitting MS with no relapses reported for at least 12 months. A-RRMS; acute-relapsing remitting MS sampled in relapse. SPMS; secondary progressive MS. PPMS; primary progressive MS.*

Figure 7.1: Plasma levels of C3, C4, fH and C1 inhibitor were increased and plasma levels of C9 were decreased in MS patients compared to normal controls

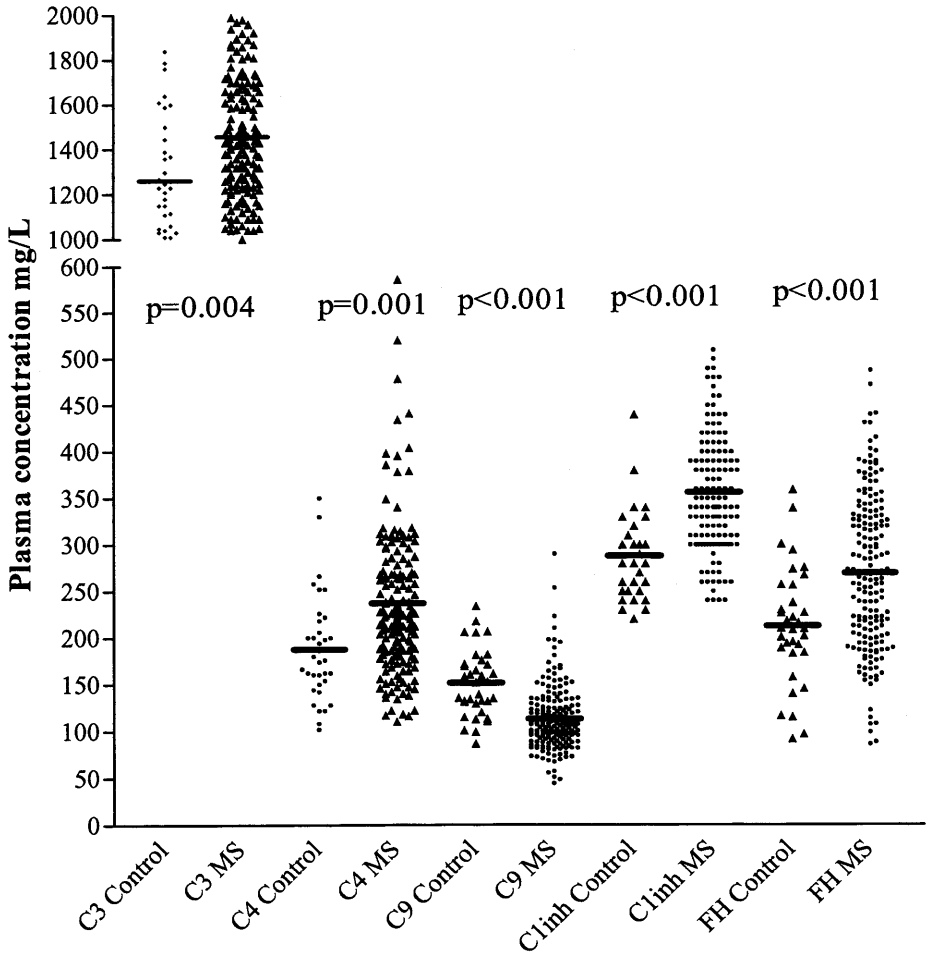


Figure 7.2: Receiver operating characteristic curve to predict the probability of MS compared to control subjects

Combined model *C* statistic 0.97. For the individual components of the model; C3 *C* statistic 0.66, C4 *C* statistic 0.71, C9 *C* statistic 0.15, C1-inh *C* statistic 0.84 and fH *C* statistic 0.79.

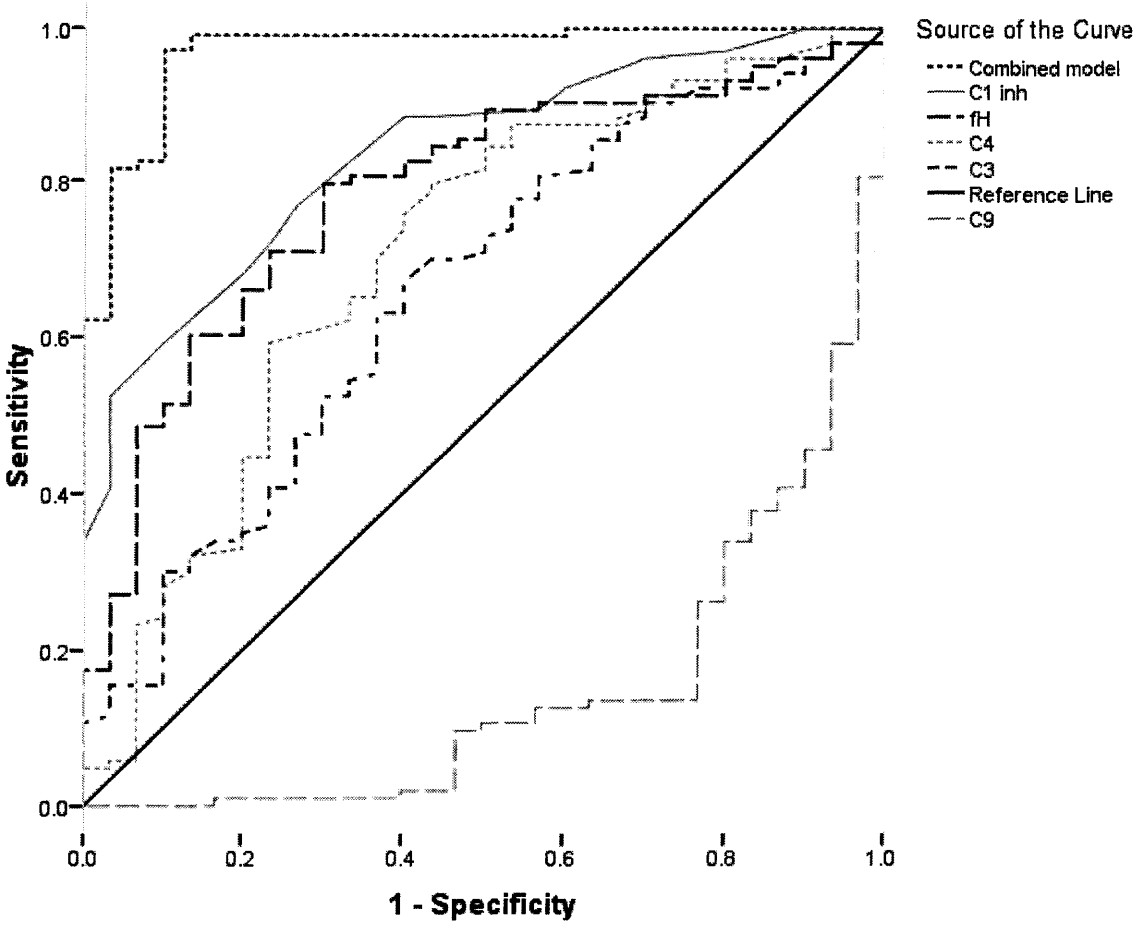


Figure 7.3: Receiver operating characteristic curve to predict the probability of A-RRMS from S-RRMS

Combined model *C* statistic 0.75. For the individual components of the model; C9 *C* statistic 0.63, disease duration *C* statistic 0.56 and age *C* statistic 0.35.

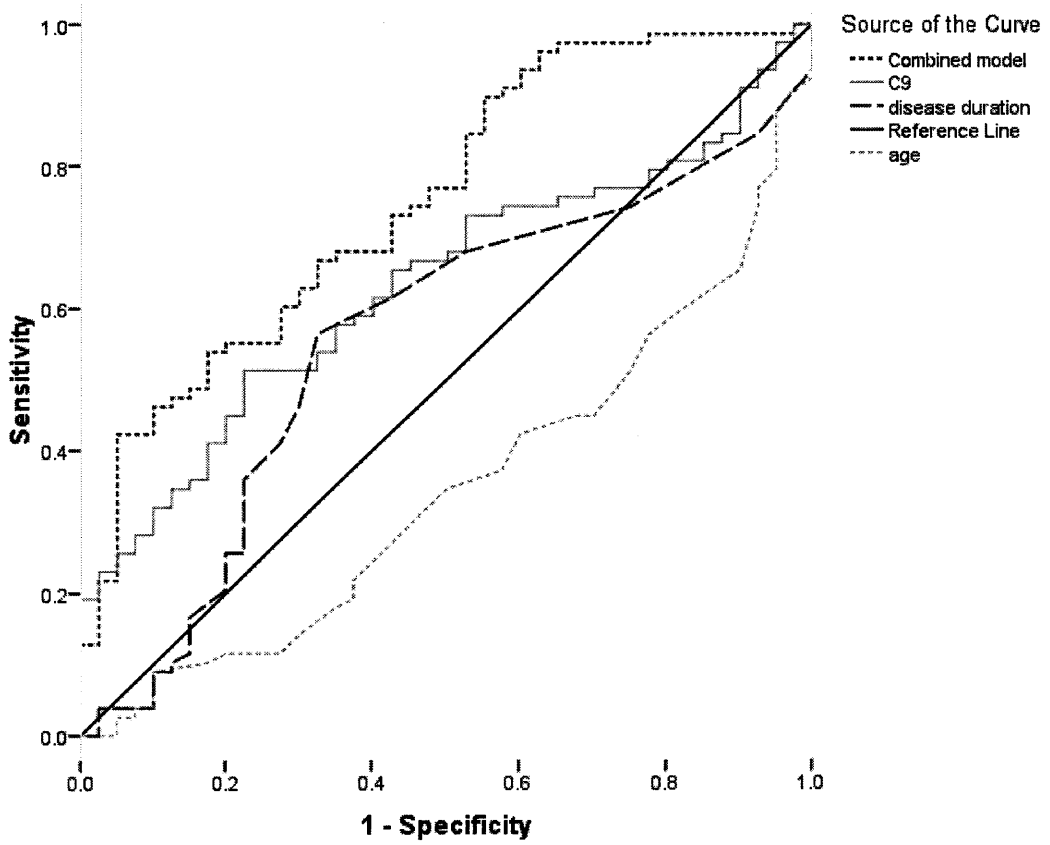


Table 7.5: Mean concentrations of serum factor H, factor B and C9 are shown in 44 patients with serial samples post relapse

Months post relapse		Factor H mg/L			Factor B mg/L			C9 % of arbitrary standard		
	No.	Mean	SD	p	Mean	SD	p	Mean	SD	p
0	44	205.72	63.80	N/A	247.50	86.18	N/A	149.15	48.42	N/A
2-3	44	178.81	42.93	0.013	270.69	70.05	0.158	153.53	45.44	0.678
5-7	44	176.39	39.28	0.007	243.62	71.52	0.813	150.59	49.44	0.891

FH; Factor H. FB; Factor B. SD; standard deviation. p = p value for comparison with acute relapse (month 0).

Figure 7.4: Plasma factor H levels post relapse

A reduction in plasma factor H is seen post relapse in 26 of 44 patients at 2-3 months and 30 of 44 patients at 5-7 months. Mean levels are significantly reduced at both time points (p=0.013 at 2-3 months and p=0.007 at 5-7 months).

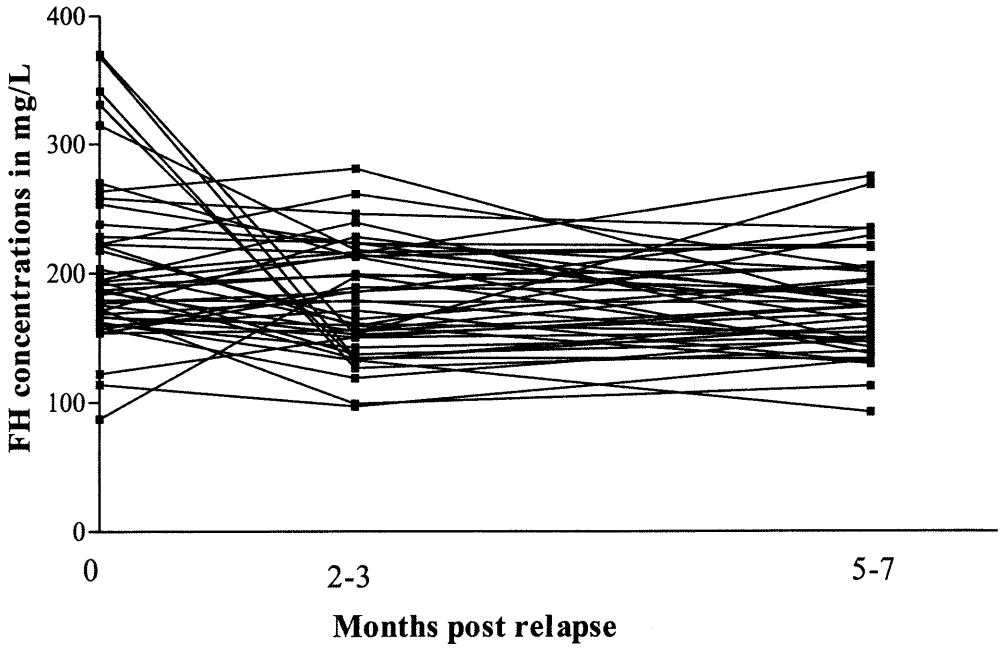


Table 7.6: Demographic details and routine CSF results in controls, patients with MS and CIS

	No.	Age		Gender	Disease duration		EDSS		Albumin ratio		Ig index		OCB
		Mean	SD	%f	Mean	SD	Mean	SD	Mean	SD	Mean	SD	% +ve
Control	24	49.79	13.52	79.17	0.50	0.71			5.24	2.79	0.50	0.05	0.00
MS	37	38.81	10.76	75.68	7.11	8.57	2.55	2.10	5.33	1.93	1.00	0.48	91.89
CIS	5	40.00	9.35	100.00	0.80	0.45	2.00	1.58	6.14	2.31	0.79	0.44	40.00

EDSS; expanded disability status scale. Albumin ratio; CSF albumin mg/L / serum albumin g/L. Ig Index; derived from CSF/plasma ratios of IgG and albumin concentrations. OCB; oligoclonal bands. SD; standard deviation. CIS; clinically isolated syndrome.

Table 7.7: CSF factor B, C9, C1s and clusterin are compared between patients with MS, CIS and controls

		CSF		Plasma		CSF:plasma ratio		Calculated index	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
FB	Control	0.90	0.46	202.81	76.19	4.11	2.08	0.86	0.32
	MS	0.79	0.34	182.40	44.14	4.50	1.96	0.95	0.56
	CIS	0.80	0.30	174.08	62.44	4.80	2.45	0.74	0.15
C9	Control	0.49	0.30	175.30	42.43	3.14	2.17	0.56	0.14
	MS	0.36	0.19	143.98*	38.29	2.59	1.42	0.46	0.17
	CIS	0.34	0.17	121.74*	34.16	4.66	4.41	0.45	0.16
C1s	Control	1.65	0.44	142.19	53.30	13.52	7.16	3.11	1.61
	MS	1.73	0.72	126.49	26.17	13.93	6.85	2.90	1.47
	CIS	2.12	0.95	125.50	35.83	15.56	13.45	2.82	0.99
Clusterin	Control	1.59	0.90	34.67	10.88	54.52	43.97	11.78	9.82
	MS	1.77	0.81	40.68	14.17	54.31	35.72	10.52	7.19
	CIS	1.86	1.04	54.44**	44.69	92.07	63.83	14.39	7.33
TCC	Control	9.96	13.19	N/A	N/A	N/A	N/A	N/A	N/A
	MS	13.23	14.48	N/A	N/A	N/A	N/A	N/A	N/A
	CIS	25.77	42.07	N/A	N/A	N/A	N/A	N/A	N/A

*CSF and plasma values shown in mg/L for factor B (FB) and clusterin, % of arbitrary standard for C9 and mAU/ml for TCC. CSF:plasma Ratio; CSF mg/L / Serum g/L. Calculated index, calculated by substituting the specified C protein for IgG in the Ig Index. SD; standard deviation. CIS; clinically isolated syndrome. * P=0.01, ** P=0.03*

Table 7.8: Correlation of complement components and regulators with raised IgG index and CSF:serum albumin ratios

CSF:plasma C ratio		Correlation		Normal		Raised		
		r	p	Mean C ratio	SD	Mean C ratio	SD	p
FB	IgG Index	0.41	0.001	0.74	0.41	0.92	0.33	0.056
	Albumin ratio	0.38	0.004	0.79	0.39	0.96	0.31	0.173
C9	IgG Index	0.46	<0.001	0.33	0.17	0.48	0.28	0.010
	Albumin ratio	0.65	<0.001	0.35	0.17	0.65	0.34	0.013
C1s	IgG Index	0.11	0.423	1.69	0.59	1.82	0.75	0.468
	Albumin ratio	0.35	0.011	1.67	0.66	2.16	0.57	0.024
Clusterin	IgG Index	0.22	0.110	1.57	0.95	1.86	0.75	0.175
	Albumin ratio	0.36	0.007	1.58	0.83	2.28	0.82	0.011
CSF TCC	IgG Index	0.19	0.210	10.61	13.22	14.95	19.85	0.385
	Albumin ratio	-0.11	0.482	12.96	14.13	10.60	25.81	0.717

Correlations are shown between CSF:plasma C ratio and either the IgG index or CSF:serum albumin ratio (expect for TCC where plasma levels were not detectable and correlation is shown with CSF TCC). Normal and raised refer IgG index or CSF:serum albumin ratio in reference to their normal laboratory values; a raised value indicates either intrathecal IgG synthesis (raised IgG index) or breakdown of the blood-CSF barrier (raised CSF:serum albumin ratio). The p value refers to the difference between mean levels of the CSF:plasma C ratio when the IgG index or CSF:serum albumin ratio is normal or raised. FB; factor B. TCC; terminal complement complex.

CHAPTER 8: AN IMMUNOHISTOCHEMICAL ANALYSIS OF COMPLEMENT IN MULTIPLE SCLEROSIS POST-MORTEM TISSUE.

8.1 INTRODUCTION

Deposition of C components within MS plaques was first demonstrated in 1971 (Lumsden 1971) and several immunohistochemical studies subsequently localised C within white matter lesions suggesting a pathological role for C in demyelination (Woyciechowska et al. 1977; Compston et al. 1989; Gay et al. 1991; Lucchinetti et al. 1996; Bitsch et al. 2000; Prineas et al. 2001; Barnett et al. 2004; Breij et al. 2008). However, labelling in these studies was generally limited to one or several of C proteins C1q, C3 and C4, or activation products C3d, C4d and C9 neoantigen (C9neo). In addition, staining was often reported as either specific to fragmented myelin or diffusely present throughout the plaque with little information provided regarding cellular localisation. A more complete immunohistopathological analysis of the role of C in MS has been described, although limited to one patient, showing diffuse staining throughout selected plaques, with periplaque accentuation, of C proteins C1s, C3, C4, C6, C7, C8 and C9, C activation products C5b and C9neo and C regulators CD59, CD46, S Protein and C1-inh (Storch et al. 1998). Controversially the specificity to MS of C staining of disrupted myelin has been questioned in one study which concluded that demonstrating

deposition of C3d and C9neo provided no direct evidence to support C or antibody mediated injury in MS (Barnett et al. 2009).

It is also unclear whether consistent C immunoreactivity occurs in all cases of MS; one study demonstrated only 53% of cases with staining of C9 neo at sites of active myelin destruction (Lucchinetti et al. 1996). One explanation may be that lesion heterogeneity is present in the initial disease phase and only tissues from more established disease demonstrate consistent C staining, although pathological heterogeneity cannot be excluded (Breij et al. 2008). Furthermore, some observers have reported that C immunoreactivity in MS is exclusive to white matter lesions suggesting that axonal damage within the grey matter may be independent of C (Brink et al. 2005).

In summary, the extent of C activation in MS and its contribution to the various disease components of demyelination and axonal degeneration remains unclear. I speculate that both classical and alternative pathways of C may be important in acute inflammatory demyelinating events and also in chronic progressive phases resulting in chronic inflammation and causing progressive axonal degeneration. In this study I investigated an extensive array of C proteins, activation products and regulators in MS brain and spinal cord, choosing key members of classical, alternative and terminal pathways; to assess the extent of activation and regulation of C in MS and its deposition in relation to MS plaques and axonal damage.

8.2 METHODS

Cases

Human tissue used in this study was provided by the UK Multiple Sclerosis Tissue Bank. Archival, 10% formalin-fixed, paraffin-embedded CNS tissue was obtained at autopsy from 9 MS cases (median age at death 66.0 years, median disease duration at death 26.5 years, median death to tissue preservation time 17.0 hours), 2 non-neurological control cases and 1 Alzheimer's control case (median age at death 91.0 years, median death to tissue preservation time 51.0 hours). One block of tissue was obtained from both brain cerebral cortex and spinal cord for 7 of 9 MS cases and all controls. The remaining two MS cases had blocks available from brain cerebral cortex only. In total, 13 plaques from 8 individuals were examined from MS cases. Clinical background data is summarised in table 8.1.

Histological staining and lesion characterisation

Paraffin wax tissue sections 4 microns in thickness were stained with Luxol fast blue (LFB) to assess presence of myelin and identify areas of demyelinated plaque, peirplaque and normal appearing white matter (NAWM). Areas of NAWM were defined as a region of white matter at least 1cm away from the edge of the demyelinating plaque. Anti-HLA-DR antibody staining was carried out to assess the number of positive microglia within the plaque or surrounding white matter. MS white matter lesions were classified as either active (plaque centre heavily infiltrated with HLA-DR positive microglia and LFB positive macrophages), chronic active (inactive centre surrounded by area of activity with

HLA-DR positive microglia and LFB positive macrophages) or inactive (few or no HLA-DR positive microglia or LFB positive macrophages) (Ferguson et al. 1997; Kornek et al. 2000). Accuracy of lesion classification was confirmed by two independent observers.

Immunohistochemistry

Parraffin wax sections were de-paraffinated using xylol and rehydrated in reducing concentrations of ethanol (100%, 96%, 70% and water). Endogenous peroxidase was blocked by 30-minute incubation in methanol with 0.02% H_2O_2. For antigen retrieval sections were either heated in citraconic anhydride (pH 7.4) or immersed in protein kinase 20 μg/ml (for primary antibody B7 only). Prior to staining, sections were blocked in 10% normal goat serum. Staining was achieved using DAKO autostainer with DAKO EnVision™+ System, Peroxidase (DAKO EnVision™+ System, HRP). Primary antibodies, including antigen target, clone, concentration, dilution and source, are listed in table 8.2.

For double labelling experiments, after an initial 30 min blocking step with 10% normal goat serum, sections were incubated for 1 hour with the first primary antibody followed by 30 mins with the secondary antibody (Dako REAL™ EnVision™/HRP reagent). Sections were stained for visualising using Vector SG peroxidase substrate kit (grey) (Vector Laboratories LTD, www.vectorlabs.com) before undergoing a shorter 10 min blocking step with 10% normal goat serum followed by a 1 hour incubation with the second primary antibody and 30 min incubation with the secondary antibody (as above). The second primary was visualised with Dako REAL™ diaminobenzidine (DAB) +

Chromogen (brown) so that the 2 primary antibodies were visualised on the same slide in a different colour. The sections were dehydrated for reading.

Positive controls used were from renal biopsies of patients with glomerulonephritis sectioned in microarrays (Soverchia et al. 2005). Negative controls were performed using non-immune immunoglobulins of the same isotype as, and diluted to the same concentration as, the primary antibody. Negative controls were also performed with omission of the primary antibody.

Quantitative analysis

All sections were digitally scanned and 5 different areas of plaque, peri-plaque, NAWM and grey matter (GM) were selected manually from each specimen at x40 magnification of the objective lens ($0.037mm^2$/field) for further analysis. For quantification, the immunostained fields were overlaid by a grid and positively stained cells for each of the individual antibodies were manually counted. Values were expressed as the number of positively stained cells per mm^2. In order to verify data, an inter-rater agreement was assessed between two independent investigators over 30 scanned fields from different sections, patients and primary antibodies, with an ICC of 0.99.

Statistical analysis

Data was not normally distributed and therefore was analysed using non-parametric tests and results are displayed with a median and range. Differences between disease

subgroups were analysed using the Kruskal-Wallis test with post-hoc procedures using Mann-Whitney tests. Given the inflation of type I errors due to multiple testing, a p value of <0.016 was taken as significant (0.05 divided by the number of tests).

8.3 RESULTS

For 7 of 9 MS patients, a single plaque from each of brain cerebral cortex white matter and spinal cord was analysed. For the remaining 2 patients, one case had a section from a brain cerebral cortex white matter lesion and a section from cortex NAWM available, and the other case had only a cortex NAWM section available. Lesions were classified as described and in total 2 active, 8 chronic active and 3 inactive lesions were analysed (table 8.1, fig 8.1). Paraffin wax sections from kidney tissue with glomerulonephritis were used as positive controls and showed staining for all antibodies used (fig 8.2). Sections stained for isotype control antibodies were consistently negative in both MS cases and controls (fig 8.2).

Activation and regulation of complement in late stages of MS

All C proteins (C3b, fB and C1q), activation products (C3b, iC3b, C4d and C9neo) and regulators (fH, C1-inh, clusterin, CD59 and CD46) examined were present in MS lesions (table 8.3 and 8.4, fig 8.3). Staining was not consistent in all sections with some patients showing more C immunoreactivity than others; however, staining was seen to some degree in every specimen analysed independent of plaque activity. Within brain sections, significantly more staining was seen in the plaque compared to periplaque areas and areas of NAWM (table 8.5, fig 8.3, fig 8.4). In a few cases marked staining was seen for some

antibodies (predominantly C1q and fH) in the periplaque area. NAWM from MS patients was generally devoid of staining except for C3b staining seen in 1 brain specimen, C1q in 2, clusterin in 4 and C1-inh in 3, with marked staining of C1-inh seen in 1 case with no additional C staining seen (fig 8.5). In addition, myelin staining of fH was seen in NAWM of 1 brain specimen with no plaques present (fig 8.5).

C3b showed the strongest immunoreactivity with similar iC3b staining in 3 verified sections (intraclass correlation coefficient between C3b (stained with C330) and iC3b for quantitative cellular staining = 0.85, 95%CI 0.76-0.91, $p<0.001$) indicating activated C3b present in MS plaques. Marked cellular staining was also seen for C1q and clusterin with weaker cellular staining seen for fB, C4d, C9neo and fH, (fig 8.3). Control specimens were consistently negative for cellular staining with the only positive staining seen in the Alzheimer's tissue which showed positive C staining within amyloid plaques and adjacent vessels.

Complement co-localizes with astrocyes in MS brain plaque

C staining within the plaque and periplaque area was consistently specific to cells morphologically resembling astrocytes (fig 8.3). Double labelling experiments showed no co-localization of C3b staining with HLA +ve microglia; however, positive co-localization with GFAP +ve astrocytes was seen within the plaque and periplaque (fig 8.5). Astrocytes within the grey matter did not stain for C3b (fig 8.5). In some sections, periplaque areas stained more strongly for C1q; however, even in these sections there was little or no co-localization with HLA, and positive co-localization with GFAP.

Complement staining of myelin localises in areas of axonal damage

Myelin staining for C was limited and variable (table 8.3 and 8.4). Within the plaque, staining of fragmented myelin with C3b and clusterin antibodies was only seen in 1 brain section. Myelin staining in areas of NAWM with antibodies to C1q and fH was seen in several MS brain sections from different patients (also seen strongly in the periplaque areas); while myelin staining for clusterin in NAWM was seen in 2 different patient sections. Staining of white matter myelin was more commonly seen in spinal cord sections with 2 of 6 cord sections showing fH staining (fig 8.5), 1 showing C1-inh staining (fig 8.5) and 1 showing C1q staining. Staining of C antibodies was often specific to areas in which axons were positive for APP indicating damage (fig 8.5), with little C staining of myelin seen in sections that were APP negative.

Complement localizes to neurones in brain periplaque grey matter

Within the brain, GM showed relatively little C staining; however, in areas of GM adjacent to the plaque there was frequently increased immunoreactivity of C components C3b and C1q (fig 8.2) and regulators fH, C1-inh and clusterin (fig 8.4) in normal appearing neurones, suggestive of C protein synthesis from neurones. These areas had the appearance of a white matter plaque extending into the cortex with increased HLA +ve microglia and astrocytes within the area.

Complement staining switches from white to grey matter in spinal cord

Less cellular staining of C was seen within plaque and periplaque areas of spinal cord sections; however, there was marked staining of C3b in the anterior horn cells of affected spinal cord sections (table 8.4). In the 5 cord sections with plaques present, a median of 75.8% of neurones were positively stained with C3b (range 40.5%-100%). Neuronal staining for other C antibodies was absent except for clusterin in 1 section (68.8% of neurones) and fH in another different section (47.4% of neurones). The observed neuronal staining may be due to the close proximity of the inflammatory lesion and neurones in spinal cord sections and therefore similar to the periplaque GM staining seen in cortical lesions. However, this does not explain the lack of white matter cellular staining seen in the spinal cord.

8.4 DISCUSSION

There is ongoing debate as to the specific contribution of C to pathology in MS. It is well known that in vitro, neuronal cell lines can express a full lytic C cascade along with an array of C inhibitors, and that neurones and oligodendrocytes are susceptible to C mediated attack (Gasque et al. 2000). In addition, there is strong evidence from the animal model of MS, EAE, for C driven pathology (van Beek et al. 2005). Several studies in human MS brain have shown C deposition in areas of myelin degradation (Storch et al. 1998; Prineas et al. 2001) and serum and CSF show changes in C levels (detailed in Chapter 2). However, it is still not clear from literature to date whether C activation in MS, is a primary event contributing to myelin and axonal damage, or a non-specific feature similar to that seen in other diseases (Barnett et al. 2009).

This study shows extensive cellular staining of C proteins (C3b, fB and C1q), activation products (C3b, iC3b, C4d and C9neo) and regulators (fH, C1-inh, clusterin, CD46 and CD59), indicating activation and deposition of C in areas of disease activity. This demonstrates that activation of C via both classical and alternative pathway persists into the chronic phases of MS, even when there is little other evidence of immune mediated inflammation. Staining seen was specific to astrocytes and neurones in MS patients but not in the normal controls or the Alzheimer's control; in addition the observed pattern of cellular staining has not been described previously in other neurological inflammatory diseases (Barnett et al. 2009) indicating this is an MS specific phenomenon.

A characteristic feature of MS is production of oligoclonal IgG seen in >95% of patients (Andersson et al. 1994). There is some evidence to suggest that oligoclonal IgG in MS is the results of clonal expansion of B cells resident within the CNS (Qin et al. 1998). This, along with deposition of C immunohistochemically, has been cited as evidence for MS being an antibody driven process (Storch et al. 1998). However, in vitro studies have shown C activation due to disrupted myelin and independent of antibody (Vanguri et al. 1982) and even the classical pathway, traditionally considered to be activated primarily by immune complexes, can be activated in other ways such as by apoptotic cells and C-reactive protein bound to ligand. C activation via the classical pathway in MS, therefore, cannot be taken as proof of an antibody driven process. It was not possible from data in this chapter to distinguish whether the classical or alternative pathway predominantly

contributed to C activation and the results provide no evidence to support a pathogenic role for antibodies in MS.

Staining of disrupted myelin in MS lesions, previously cited as evidence of C-mediated pathology (Compston et al. 1989; Storch et al. 1998; Prineas et al. 2001), was limited and variable in this study. Myelin staining of proteins C3b (1 case), fB (1 case) and C1q (1 case both brain and spinal cord sections) and regulators fH (3 cases and 1 control), clusterin (1 case) and C1-inh (3 cases) was seen to some degree in a total of 10/23 sections from 7 of 9 MS patients and 1 of 3 controls. This positive myelin staining was seen in cases with active, chronic active and inactive plaques; in some cases in the centre of the plaque and in other cases in periplaque tissue or in adjacent areas of NAWM; also myelin staining was observed independently of age at death, disease duration and tissue preservation time. Although myelin staining can be a non-specific feature when C is present in brain tissue, in this study most C staining of myelin localized to the same areas of the plaque or periplaque that showed APP positive axons indicating axonal damage, suggesting C-mediated myelin damage.

The median age of patients seen in this study was 66 years (range 42-78) with median disease duration 25 years (range 8-50). The majority of plaques had an inactive centre with or without a periplaque rim of inflammatory activity. However, even in these plaques with little inflammation, evidenced by lack of HLA-DR positive microglia or LFB positive macrophages, in progressive patients with relatively long disease durations; there was evidence of ongoing C activation with synthesis and cellular deposition of C

proteins, activation products and regulators providing evidence of an inflammatory component to pathology in progressive disease phases. This is supported by our previous studies showing increased levels of C proteins and regulators in MS throughout disease course (Chapter 7); in particular, increased levels of fH were specific to progressive disease (Chapter 5) suggesting up-regulation of C in late disease stages.

Traditionally it was considered that axonal degeneration in MS was caused by inflammation, evidenced predominantly by the frequency of transected axons in MS lesions showing correlation with the degree of inflammation (Trapp et al. 1998). However, this relationship has been questioned, most importantly, because of the lack of effect of anti-inflammatory treatments on preventing accruement of disability in progressive disease secondary to axonal loss (Molyneux et al. 2000), and lack of MRI correlation between inflammation and brain atrophy (Filippi et al. 2005). A more recent immunopathological study of 67 MS patients and 28 controls has demonstrated highly significant association between inflammation and axonal injury in all lesions and disease stages (Frischer et al. 2009) and a recent MRI study of 963 patients has shown relentless brain atrophy throughout MS disease course (De Stefano et al. 2010). The findings of this chapter demonstrate ongoing C activity in late disease and would support the concept of ongoing inflammation causing axonal loss and atrophy throughout disease.

The pathological heterogeneity of MS lesions was first described by Lucchinetti et al (Lucchinetti et al. 1996) who suggested 4 distinct patterns of MS lesion with only 50% of patients showing C staining; this has been contested by another study which

demonstrated more consistent C staining throughout their patient sample (Breij et al. 2008). It was postulated that this variation could have been a result of differing disease durations in the 2 populations studied (discussed in Chapter 2); however, measurement of C in the study by Lucchinetti et al was limited to antibody against C9neo, sourced and analysed using the same technique described in this chapter. The staining observed for C9neo in this chapter was not consistent across patients with 2 MS patients showing no C9neo staining, despite staining of other C components; and weak staining in most sections examined. This could explain some, but not all, of the heterogeneity seen in the study by Lucchinetti et al. Despite this, there were clear differences in staining intensity across the MS patients in this study supporting the idea of heterogeneous immunopathological processes in MS patients, possibly arising from the diverse genetics and resulting in the variable phenotypic disease courses. It could be suggested that anti-C or anti-B cell therapies may have a role in combination with more traditional T cell mediated therapies in limiting disease progression in some, but perhaps not all, patients.

In summary, this chapter demonstrates upregulation and activation of C to a varying degree, in all MS patients. Specific staining of astrocytes and neurones in areas of inflammation indicate a pathogenic role for C in disease processes throughout MS disease course. It is now essential to clarify these findings in a larger set of MS patients with more disease subgroups. In particular, this study was limited to patients with long-standing disease and comparison with a group of early relapsing or aggressive MS patients with active plaques would provide insight into changes in complement expression throughout disease. Additional tissue would also allow examination of an

expanded set of inflammatory markers such as Ig and T cell and B cell markers. Colocalization of inflammatory markers along with axonal marker NFL, and neural cell markers such as for oligodendrocytes, to complement staining, would offer a more comprehensive assessment of the pathological implications of complement expression in disease.

Table 8.1: Demographic details, number of lesions and cause of death for MS patients and controls

Case	Disease status	Age at death	Sex	Death to TPT (hrs)	MS Disease duration	MS Disease course	Lesions			Cause of death
							A	CA	I	
MS055	MS	47	f	15	32	SPMS	0	1	1	Pneumonia
MS058	MS	51	f	15	21	SPMS	0	2	0	MS
MS061	MS	56	f	6	>15	PRMS	0	1	1	Adenocarcinoma
MS064	MS	66	m	56	28	SPMS	0	2	0	Pneumonia
MS128	MS	78	f	22	50	SPMS	0	1	1	Pneumonia
MS179	MS	70	f	20	25	SPMS	1	1	0	Pneumonia
MS306	MS	78	m	17	42	SPMS	0	0	0	Infective diarrhoea
MS383	MS	42	m	17	8	PPMS	1	0	0	Pneumonia
MS24	MS	71	f	96	22	PPMS	0	0	0	Pneumonia
C21	Control	91	f	127	N/A	N/A	N/A	N/A	N/A	Pneumonia
C29	Control	71	m	33	N/A	N/A	N/A	N/A	N/A	Abdominal haemorrhage
C23	Control	93	f	51	N/A	N/A	N/A	N/A	N/A	Alzheimer's disease

TPT; tissue preservation time. A; active. CA; Chronic active. I; inactive. f; female. m; male. SPMS; secondary progressive MS, PRMS; progressive relapsing MS, PPMS; primary progressive MS.

Table 8.2: Primary antibodies used for immunohistochemistry

Antibody	Antigen / Target	C pathway	Type	Source	AR Method	Conc (mg/ml)	Dilution
Complement proteins / activation products							
C3-30	C3b	CP &AP	MAb, m	In-house	CA/Heat	4.82	1:1500
Catalogue no. A209	iC3b	CP & AP	MAb, m	Quidel	CA/Heat	1.2	1:500
JC1	Factor B	AP	MAb, m	In-house	CA/Heat	1.0	1:300
SE9G11	C1q	CP	MAb, m	In-house	CA/Heat	1.16	1:60
Catalogue no. A215	C4d neo	CP	MAb, m	Quidel	CA/Heat	1.0	1:50
B7	Neo C9	TP	MAb, m	In-house	PK	0.6	1:20
Complement regulators							
Ox-24	Factor H	AP	MAb, m	In-house	CA/Heat	2.8	1:20
Mbi 6	Factor H-Tyr402	AP	MAb, m	In-house	CA/Heat	5.7	1:300
Mbi 7	Factor H-His402	AP	MAb, m	In-house	CA/Heat	6.9	1:600
C1inh	C1-inh	CP	PAb, r	In-house	CA/Heat	16.93	1:400
Material no. 552886	Clusterin	TP	MAb, m	BD Pharmingen	CA/Heat	5.47	1:300
MEM 43	CD59	TP	MAb, m	In-house	CA/Heat	0.4	1:400
F11	CD46	TP	MAb, m	In-house	CA/Heat	0.9	1:300
Markers							
APP	Axons	N/A	MAb, m	Millipore	CA/Heat	38.7	1:200
GFAP	Astrocytes/ Ependymal cells	N/A	PAb, m	DAKO, Denmark	CA/Heat	3.94	1:500
HLA-DR	Microglia	N/A	MAb, m	DAKO, Denmark	CA/Heat	5.93	1:150

CP; classical pathway. AP; alternative pathway. TP; terminal pathway. MAb; monoclonal antibody. PAb; polyclonal antibody. M; mouse. R; rabbit. AR method; antigen retrieval method. CA/Heat; heated in citraconic anhydride. PK; protein kinase.

Figure 8.1: MS lesions

Case A = MS382; active brain lesion shown with LFB positive macrophages and multiple HLA positive microglia within the plaque. Case B = MS58; brain lesion shown is chronic active with a rim of HLA positive microglia around the plaque and few LFB containing macrophages within the plaque. Case C = MS61, brain lesion shown is inactive with few LFB or HLA positive cells. 1 = LFB stain, plaque x4. 2 = LFB stain, plaque x40. 3 = HLA stain, plaque x40. 4 = HLA stain periplaque x40.

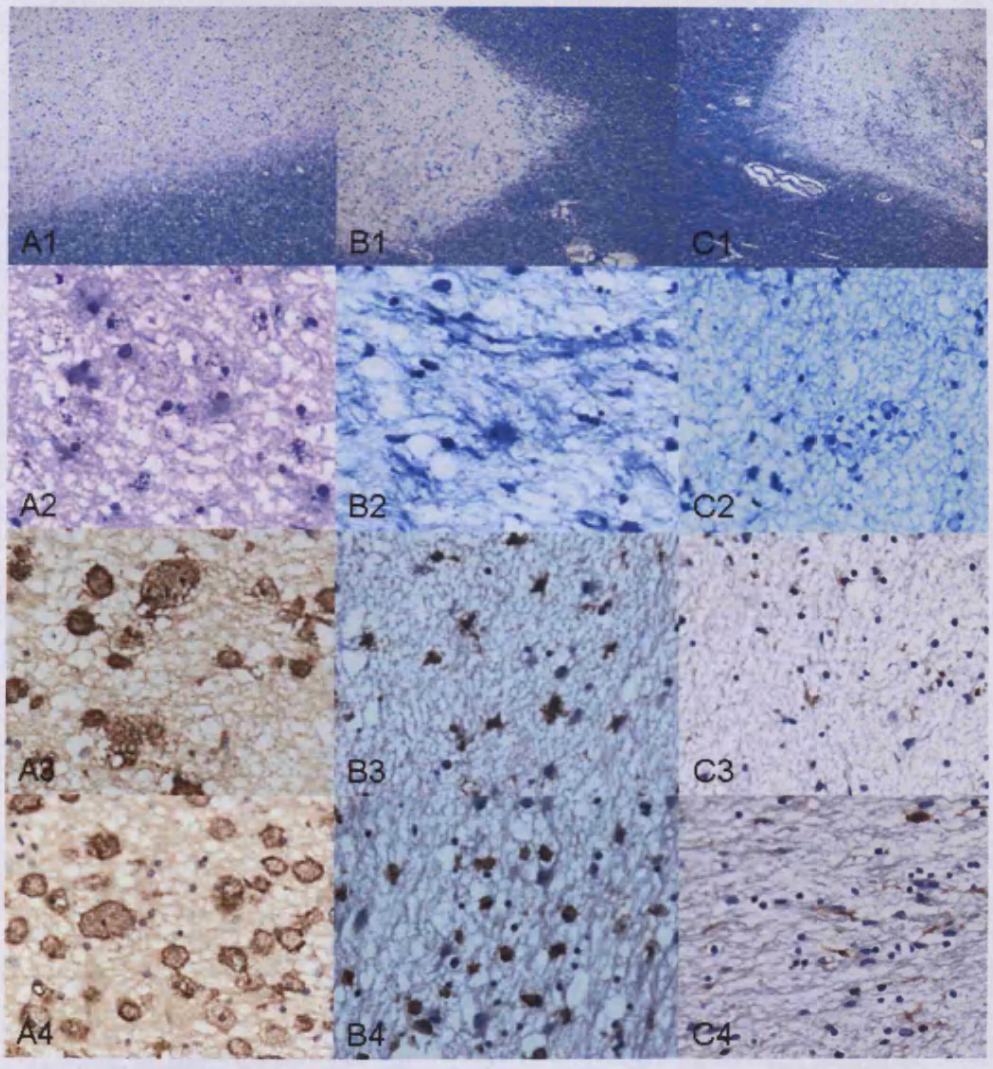


Figure 8.2: Control samples

Tissue microarrays of kidney for positive controls (A-L). A=C330, B=iC3b, C=JC1, D=C1q, E=B7, F=MBI6, G=MBI7, H=C1inh, I=Clusterin, J= CD59, K=CD55, L=CD46. Negative controls in MS tissue (M-P). M= IgG1 in MS55 brain lesion plaque. N = IgG1 in MS 55 brain lesion grey matter. O = IgG2 in MS58 spinal cord plaque. P= IgG2 in MS58 spinal cord grey matter.

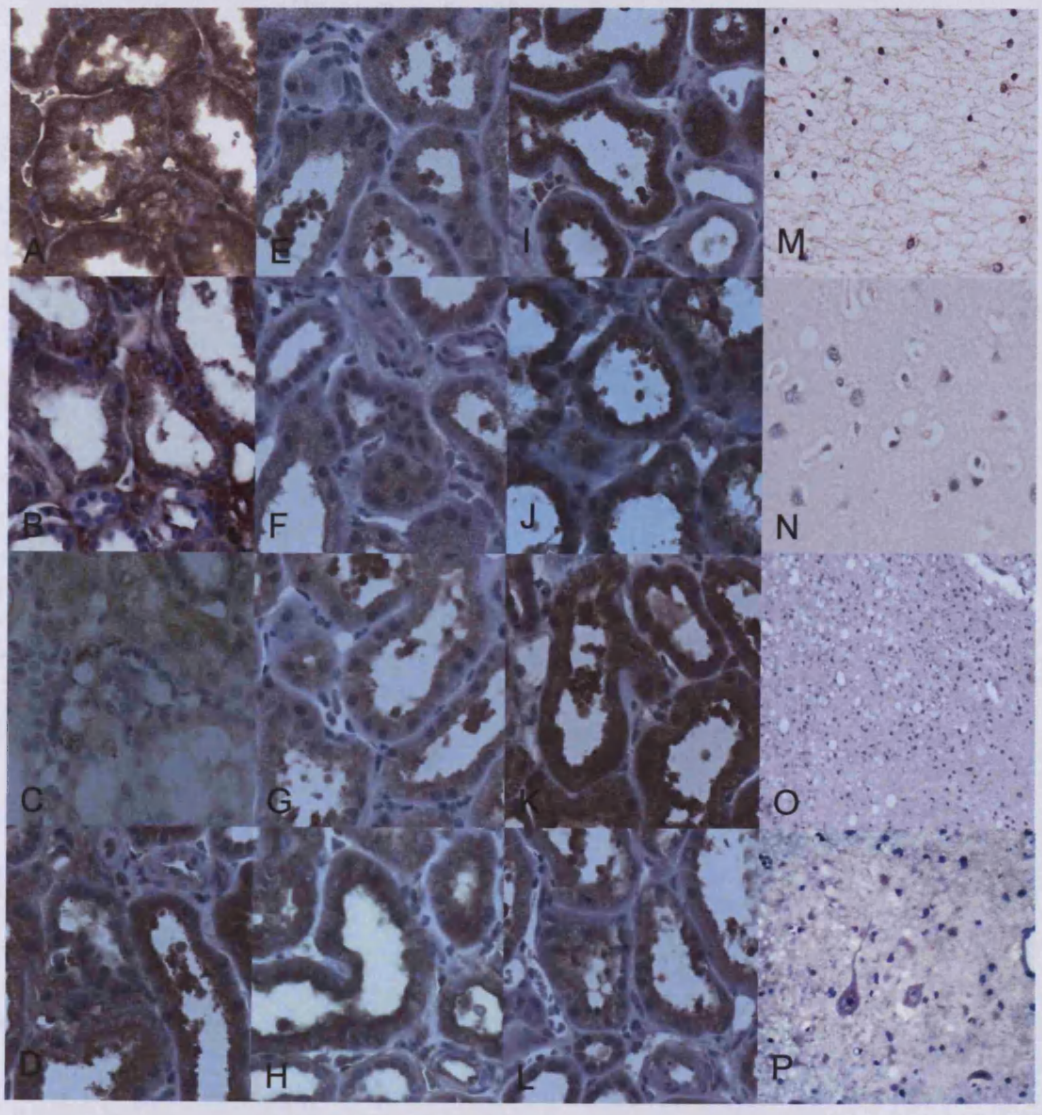


Table 8.3: Cellular and myelin staining of complement in individual brain sections examined

ID	Plaque type	C3b	Factor B	C1q	neo C4d	neo C9	Factor H-Tyr402	Factor H-His402	Clusterin	C1 inh
MS 55	CA	P +++	P +	P +++ G ++	P +	P ++	P +	-	P +++	-
MS 58	CA	P ++	-	P +++ N +	-	P ++	P +	P +	PP++ P+	P ++
MS 61	Inactive	P +++ G ++	-	P ++ G ++	-	-	-	-	P + G ++	-
MS 64	CA	P +++ PM + N + G ++	-	PP+++ P++ G ++	-	P +	P ++ G +++	P ++ G +++	P ++ PM + G +	-
MS 128	Inactive	P ++	P +	P ++ NM+	-	P +	P ++ PPM ++	P + PPM ++	P ++ G +	P ++
MS 179	Active	P +++ G ++	P +	P ++ N +	P +	P +	P ++ G +++	P +	P +++	P ++
MS 383	Active	P +++ G ++	-	PP ++ P+	-	P +	PP +	P +	PP++ P+	P ++ PM + PPM +
MS 383	None	V	NM +	-	-	-	-	-	G++	N ++ NM ++ G ++
MS 306	None	-	-	-	-	-	-	-		N ++ NM ++ G ++
MS 24	None	-	-	-	-	-	NM +++	NM ++	-	-
C 21	None	-	-	-	-	-	-	-	-	-
C 23	None	V	-	V	-	-	AP	AP	V	-
C 29	None	-	-	-	-	-	-	-	-	-

MS; MS case. C; control case.CA; chronic active, P; plaque, PP; periplaque, N; NAW, G; grey matter. P; myelin staining in the plaque, PPM; myelin staining in the periplaque, NM; myelin staining in the NAW. V; staining of vessels. AP; staining of amyloid plaques.+++; strong staining, ++; moderate staining, +; weak staining, -; no staining seen. Apart from myelin staining, this refers to specific cellular staining.

Table 8.4: Cellular and myelin staining of complement in individual spinal cord sections examined

ID	Plaque type	C3b	Factor B	C1q	neo C4d	neo C9	Factor H-Tyr402	Factor H-His402	Clusterin	C1 inh
MS 55	Inactive	P +++ G +++	-	PP+++ P++	-	PP +	PP ++	-	P ++	NM ++
MS 58	CA	P +++ G +++	-	PP ++	-	P +	-	-	P +++	P +
MS 61	CA	P ++ G +++	-	-	-	-	-	-	-	-
MS 64	CA	P ++ G ++	-	P ++	-	-	-	PP + G ++	-	P +
MS 128	CA	G ++	-	NM ++	-	-	-	-	PP + G ++	-
MS 179	CA	G ++	-	-	-	-	NM +++	N ++	-	-
MS 24	None	-	-	-	-	-	NM ++	NM +	-	-
C 21	None	-	-	-	-	-	-	-	-	-
C 23	None	-	-	-	-	-	-	-	-	-
C 29	None	-	-	-	-	-	NM +	-	-	-

MS; MS case. C; control case. SC; spinal cord, CA; chronic active, P; plaque, PP; periplaque, N; NAW, G; grey matter. P; myelin staining in the plaque, PPM; myelin staining in the periplaque, NM; myelin staining in the NAW. V; staining of vessels. AP; staining of amyloid plaques.+++; strong staining, ++; moderate staining, +; weak staining, -; no staining seen. Apart from myelin staining, this refers to specific cellular staining.

Figure 8.3: Complement staining in MS sections (I)

MS64, sections shown are from a chronic active brain lesion. Series A shows marked iC3b staining of cells and myelin predominantly within the plaque (insert in A1 is a iC3b +ve astrocyte x100 magnification). C1q staining shown in series B was more prominent in the periplaque area but also seen in the plaque. C1q staining of neurones was seen in areas of grey matter adjacent to the plaque. Series C shows C9neo staining predominantly in the plaque. A = iC3b, B = C1q, C = C9neo. 1 = Plaque, 2 = Periplaque, 3 = NAW, 4 = GM. Images magnification x40.

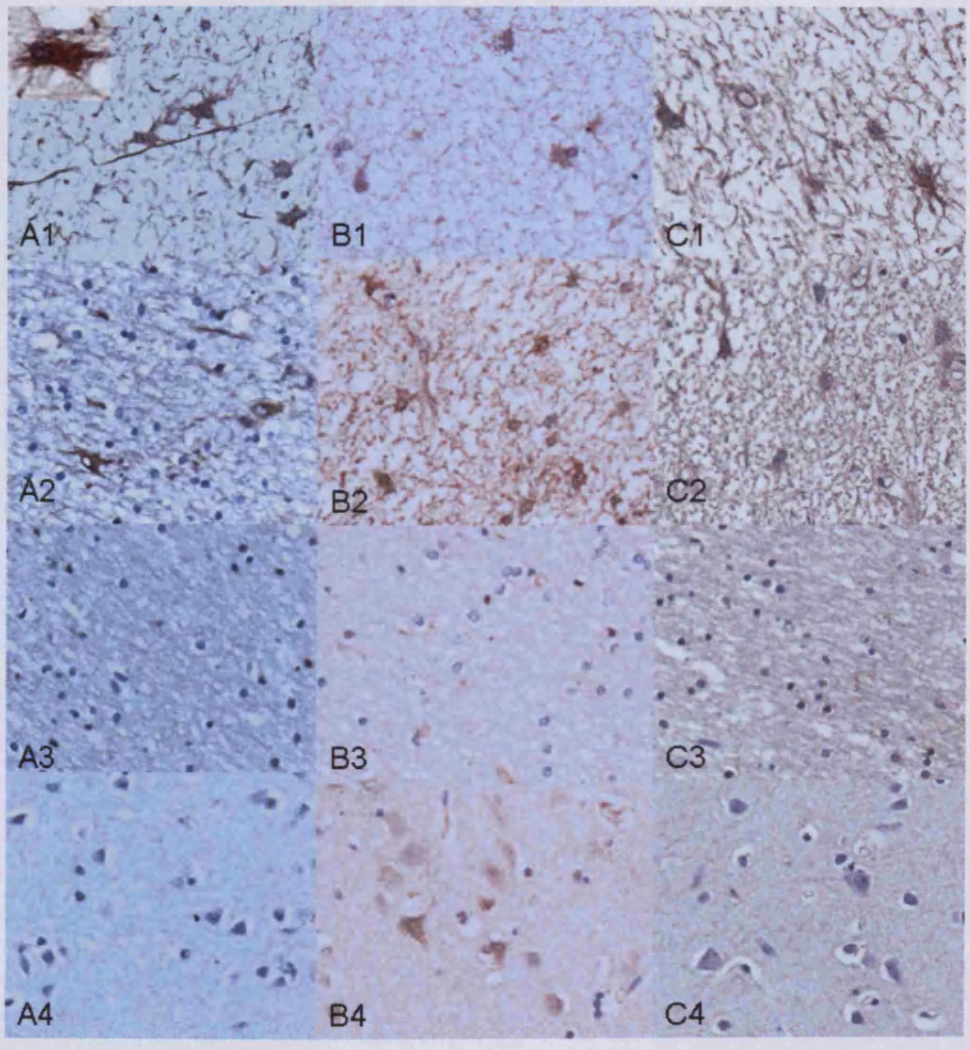


Table 8.5: Cellular immunoreactivity of complement proteins, activation products and regulators

			C3b		Factor B		C1q		C4dneo		C9neo	
		No.	Med (min-max)	p	Med (min-max)	p	Med (min-max)	p	Med (min-max)	p	Med (min-max)	p
Brain	Plaque	7	269 (54-591)		0 (0-296)		134 (27-350)		0 (0-323)		108 (0-376)	
	Periplaque	7	81 (0.0-215)	<0.001	0 (0-81)	0.018	81 (0-296)	0.003	0 (0-81)	0.044	0 (0-242)	<.0001
	NAW	10	0 (0-188)	<0.001	0 (0-0)	<0.001	0 (0-81)	<0.001	0 (0-0)	<0.001	0 (0-0)	<.0001
	GM	10	0 (0-108)	<0.001	0 (0-0)	<0.001	0 (0-269)	<0.001	0 (0-0)	<0.001	0 (0-0)	<0.001
Spinal cord	Plaque	6	81 (0-511)		0 (0-0)		0 (0-430)		0 (0-0)		0 (0-81)	
	Periplaque	6	0 (0-54)	<0.001	0 (0-0)	1	0 (0-403)	0.993	0 (0-0)	1.000	0 (0-27)	0.238

			Factor H-Tyr402		Factor H-His402		C1inhibitor		Clusterin	
		No.	Med (min-max)	p	Med (min-max)	p	Med (min-max)	p	Med (min-max)	p
Brain	Plaque	7	81 (0-350)		108 (0-350)		215 (0-645)		81 (0-269)	
	Periplaque	7	27 (0-108)	<0.001	27 (0-81)	<0.001	81 (0-296)	<0.001	0 (0-215)	0.073
	NAW	10	0 (0-0)	<0.001	0 (0-0)	<0.001	0 (0-81)	<0.001	0 (0-296)	<0.001
	GM	10	0 (0-457)	<0.001	0 (0-108)	<0.001	0 (0-424)	<0.001	0 (0-161)	<0.001
Spinal cord	Plaque	6	0 (0-350)		0 (0-215)		54 (0-538)		0 (0-108)	
	Periplaque	6	0 (0-484)	0.973	0 (0-54)	0.185	0 (0-188)	0.047	0 (0-54)	0.445

Cell counts are expressed as immunoreactive positive cells / mm^2, with the median (med), minimum (min) and maximum (max) values. P value is for comparison to the plaque.

Figure 8.4: Cellular staining in MS brain sections

More positively stained cells are seen in plaque the compared to periplaque, NAW and GM areas (data shown for MS brain sections).

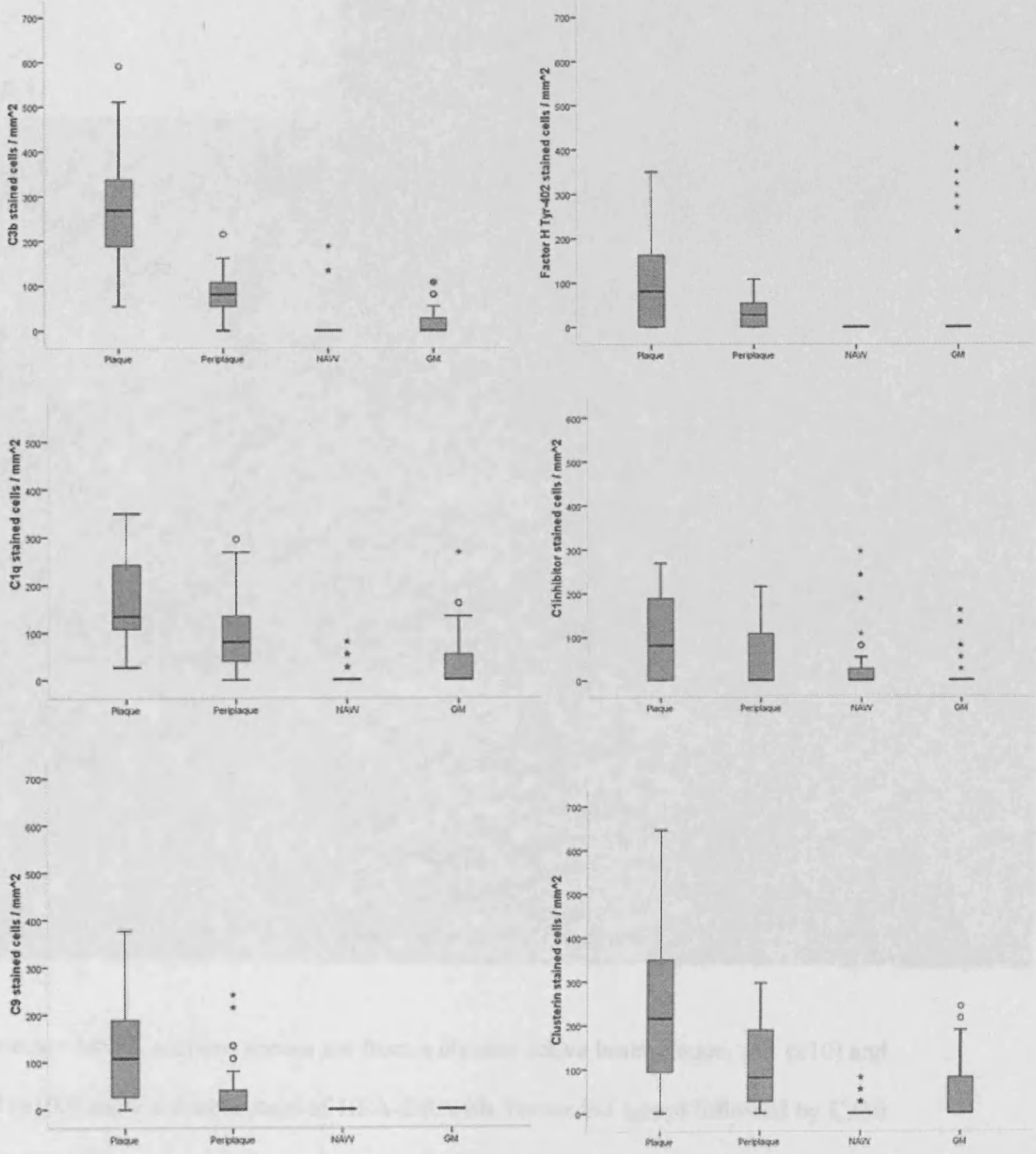
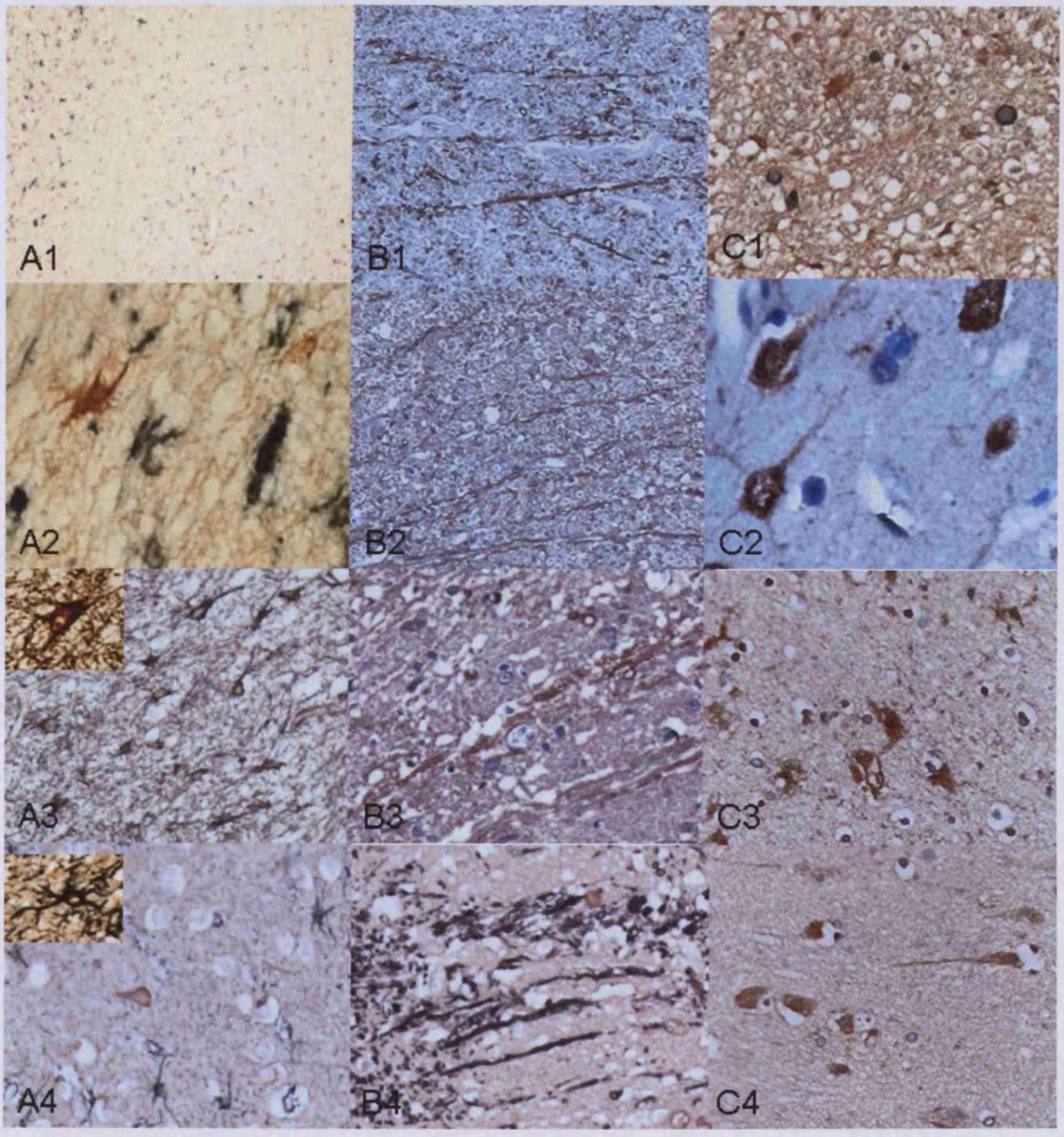


Figure 8.5: Complement staining in MS sections (II)



Case A = MS55, sections shown are from a chronic active brain plaque. A1 (x10) and A2 (x100) show a double stain of HLA-DR with Vector SG (grey) followed by C330 with DAB (brown), taken at the lesion edge, with no co-localization shown between C3b and HLA +ve microglia. A3 (x40) and A4 (x40) show double staining of GFAP

with Vector SG (grey) followed by C330 with DAB (brown). A3 shows co-localization of C3b (brown) with GFAP (grey) +ve astrocytes in the plaque (insert shows an astrocyte x100 magnification with double staining). A4 shows no co-localization of C3b with the GFAP +ve astrocytes in cortex where there are C3b +ve neurones (insert shows an astrocyte stained only grey with GFAP). Case B = MS24, sections shown are from spinal cord with no lesion present. There is positive fH staining of axons shown in both the Tyr402 (B1, x40) and His402 (B2, x40) fH antibodies. B3 (x40) shows positive APP staining of axons and B4 (x40) shows a double stain of Tyr402 fH with Vector SG (grey) co-localizing with APP stained axons with DAB (brown). C1 shows MS55 spinal cord (x40); co-localization is seen of APP (Vector SG, grey), with C1-inh (DAB, brown). C2 shows MS179 brain (x100) with +ve staining of MBI6 in neurones adjacent to the active plaque. C3 and C4 (MS306, x40) show C1-inh cellular staining in the NAWM (C3) and neurones in the GM (C4) of a section with no plaque present and no staining of other C proteins, activation products or regulators.

CHAPTER 9: INFLAMMATORY DISEASE ASSOCIATED COMPLEMENT POLYMORPHISMS DO NOT CONTRIBUTE TO DISEASE PHENOTYPE IN MULTIPLE SCLEROSIS

9.1 Introduction

There is growing evidence in CNS disorders with a significant inflammatory component, such as AD, AMD and vascular disease, for a significant contribution from C. Some of this is thought to be attributed to dysregulation of the C system caused by polymorphisms in C components and regulators. AMD is a neurodegenerative disease with several susceptibility genes within the C pathway including fH, fB, C2 and C3. SNPs in the locus encoding C regulator fH (rs1061170, Tyr402His and rs800292, V62I) have been shown by a number of groups to be significantly associated with increased risk of disease (Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005). Furthermore, it has been suggested that rs1061170 is associated with classical AMD with no occult choroidal neovascularization rather than other disease phenotypes (Wegscheider et al. 2007). Variants within C3 (rs2230199, R102G and rs1047286, P314L) (Despriet et al. 2009), fB (L9H and R32Q) and C2 (E318D and a variant in intron 10) (Gold et al. 2006) have also been shown to confer a significantly reduced risk of disease, and combined haplotype analysis of fB and C2 has shown variation in these two loci predicts clinical outcome in 74% of the affected individuals and 56% of the controls.

In AD, deposition of fH in brain tissue (Honda et al. 2000) and elevated serum fH levels correlated with cognitive decline (Hye et al. 2006; Thambisetty et al. 2008), suggesting to investigators that molecular analysis of the C fH gene may be a productive line of investigation. Association with the Tyr402His polymorphism of fH was shown in AD patients, but was restricted to those also carrying the ApoE4 risk allele (Hamilton et al. 2007; Zetterberg et al. 2008). More recently a GWAS in AD has revealed further SNPs in C regulator clusterin (rs11136000) and C receptor 1 (CR1) (rs6656401) with odds ratios for disease of 0.81 and 1.21 respectively (Lambert et al. 2009).

There is considerable evidence to suggest a role for C in Schizophrenia with activation of classical, alternative and lectin pathway demonstrated in serum (Hakobyan et al. 2005; Boyajyan et al. 2008; Mayilyan et al. 2008). Genetic analysis in Schizophrenia shows variants of the C3 and fB gene associated with disease risk (Rudduck et al. 1985; Fananas et al. 1992).

Cerebral ischemia and reperfusion initiate the inflammatory system resulting in secondary neuronal damage. C cascade activation has been demonstrated following cerebral ischemia (Komotar et al. 2008) and strong activation has been associated with worse outcome (Szeplaki et al. 2009). Also, therapies directed at the C system have been suggested to reduce cerebral damage in the mouse modal (Komotar et al. 2008; Gesuete et al. 2009), and SNPs in C5 (rs17611, I802V) (Greisenegger et al. 2009) and fH (rs1061170, Tyr402His) (Volcik et al. 2008) have been shown to be significantly associated with an increased risk for stroke.

C is known to play an important role in MS and in this thesis altered levels of local and systemic C proteins, activation products and regulators have been demonstrated associated with disease state. Also, work in Chapter 5 suggested that allelic imbalance of the fH Tyr402His polymorphism was seen only in those patient groups where total fH levels were also elevated, suggesting either selective up-regulation of the His402 variant or selective consumption of the Tyr402 variant in disease. Previous work looking for susceptibility genes within complement components and regulators have so far been unsuccessful (reviewed in Chapter 2); however, evidence, outlined above, from other diseases suggests that the impact of genetic variation in C genes may not be solely restricted to disease risk and may have an independent effect on phenotype. No studies to date have examined MS phenotype-genotype correlations in C polymorphisms. The work in this chapter assesses 8 SNPs (described above) in C genes, known to be associated with other neuro-inflammatory diseases, and examines genotype-phenotype correlations in a comprehensively characterised population of MS patients.

9.2 Methods

Subjects

Samples were collected from 887 patients with clinically definite or probable MS on whom multiple clinical assessments (7725 current status datasets) were available for multiple time points throughout their disease course. Additional information was collected from the validated locally relevant historical patient questionnaire (Chapter 4, appendix 1), designed to harvest information on disease onset and diagnosis, time to SPMS, current EDSS and time to EDSS milestones >4, 6 and 8.

Venous blood was collected for serum and DNA, and sputum samples were collected for DNA. Processing of serum samples and extraction of DNA has been described in Chapter 3.

SNP analysis using Taqman

384 well plates with 15ng DNA in 3 μl per well were dried overnight. A mix of 1 x TaqMan Universal PCR Mastermix, and 0.5 x SNP primer (from Applied Biosystems) made to a volume of 4 μl, was added to each well. The plates then underwent PCR using the Perkin Elmer 9700 in cycles as follows: initial denaturing step of 95°c for 10mins, 50 cycles of 95 °c for 10 mins and 50 °c for 1 min. Plates were read using the TaqMan SNP Genotyping assays and samples determined as either homozygous for either allele or heterozygote.

Molecular analysis of Factor H Tyr402His polymorphism

A wet nitrocellulose membrane was marked with 1µl serum and, when dry, was incubated in 5% milk with PBS for 1 hour at room temperature under rotation. The membrane was subsequently probed for a further 1 hour incubation step (at room temperature under rotation) with either HRP-labelled affinity-purified rabbit MBI-6 anti-human fH-Tyr402 (100µl; 1mg/l) or HRP-labelled MBI-7 anti-fH-His402 (100µl; 1mg/l) to detect fH-Tyr402 or fH-His402 respectively (development of antibody MBI-6 (Hakobyan et al. 2008) and MBI-7 (Hakobyan et al. 2010) has been previously described). Membranes were washed 3 times in PBS/0.1% Tween 20 with incubation of 15 minutes for each wash and bound antibody was detected using TMB substrate

and chemiluminesence detection. Films were scored for presence of either MBI-6 and/or MBI-7 and classified as homozygote or heterozygote for variants (fig 9.1).

Statistical Analysis

Association between different subgroups was performed using chi-square test and one-way ANOVA with Bonferonni correction. Association between genotype and disease progression was tested using standard survival analysis methods and hazard ratios were calculated (95% CI and p-values) for median time to EDSS 4, 6 and 8 using an additive model of genetic risk. Because of the low numbers in the minor allele homozygote group for 5 of the 8 SNPs, this group was combined with the heterozygote group for analysis. The analysis was not corrected with Bonferroni method as the tests were not independent; therefore each EDSS milestone was corrected for the number of SNPs analysed and associations were deemed posistive if $p<0.006$ (0.05 divided by the number of tests).

9.3 Results

Genotyping success rate ranged between 91% and 93% for all SNPs analysed on taqman (table 9.1). Minor allele frequency for each SNP was not significantly different from European control group data on the 1958 birth cohort (http://www.b58cgene.sgul.ac.uk/) or HapMap (http://hapmap.ncbi.nlm.nih.gov/) (table 9.1). Demographic characteristics including gender, age, disease duration, disease course and % of patients with EDSS>6.0, were similar in all genotypic subgroups except for a trend towards lower age in rs1065489 minor allele group ($p=0.039$), a trend to lower disease duration in the minor allele groups of both

rs11136000 (p=0.046) and rs1061170 (p=0.071) and a slight overrepresentation of SPMS in the major allele subgroup of rs1061770 (p=0.002) (table 9.2).

The hazard ratios for time to reaching disability milestones EDSS>4, 6 and 8 were reduced in C5 SNP rs17611. An addition, the 2 SNPs in C3 showed a similar trend only observed in time to EDSS>4 and 6 (rs1047286 and rs2230199); this effect may not have been seen for milestone EDSS 8 given the lower number of patients reaching this point (table 9.3). Two other SNPs showed a reduced hazard ratio for time to either EDSS 6 (rs11136000) or 8 (rs1061770); however, this was not born out across the other milestones and therefore is more likely to be due to a type I error (table 9.3). After correction for multiple analysis only one of the associations seen remained significant (EDSS>4 in C3 SNP rs1047286) and it is likely that the other associations seen were due to type I errors. Other disease characteristics (age at onset, age at diagnosis, time from disease onset to SPMS, age at SPMS, disease course at onset and % of patients with PPMS) were not different between disease subgroups except for a slightly higher age at onset in the rs6656401 (CR1) heterozygote group (p=0.041) (table 9.4).

Information on characteristics at disease onset was also analysed in relation to C genotypes. There was no difference seen for disease course at onset (full or partial recovery from relapse or progressive disease from onset) in any genotypic subgroup (table 9.4). There was also no difference seen in the clinical features of first relapse between genotypic subgroups (data not shown). Correlation of the results of initial investigations MRI, OCBs and VEPs were examined in relation to genotypic subgroups (table 9.5). There were a higher proportion of patients with positive MRI

scans in the minor allele group, with a similar trend in the heterozygote group, for the fH SNP, rs1065489. The same trend was also observed in the other two fH SNPs, rs1061770 and rs800292; however, these did not reach significance probably due to the low numbers in the minor allele subgroup. There were no genotype correlations seen with any other parameter.

9.4 Discussion

Detailed analysis was performed of phenotype-genotype correlations in MS selecting 8 SNPs, on a candidate gene approach, in various C genes based on prior evidence for those SNPs conferring increased risk and altered phenotype in other neuroinflammatory diseases. SNPs located in chromosome 6 (genes for fB and C2) were excluded due to their effect being masked by the dominant association of MS with the HLA genes located in this region.

No convincing associations were shown between these candidate SNPs and MS phenotype. Although 3 SNPs showed some degree of association with time to disability milestones, after correcting for multiple comparisons to limit type I errors, only one of these remained significant. Higher age at onset was seen in the CR1 polymorphism heterozygote group (rs6656401); and positive MRI at diagnosis was associated with a SNP in regulator fH (rs1065489) with a similar trend seen in the other 2 fH SNPs (rs1061770 and rs800292). Replication in larger cohorts is needed to test relevance of these findings.

Previous studies looking at genotype-phenotype correlations in MS have been limited. One Scandinavian study has reported correlation of HLA-DRB1*15 with a younger

age at disease onset (corrected p=0.009) (Hensiek et al. 2002); whilst another group examined 808 patients with cross-sectional data and reported HLA-DR2 homozygotes to be associated with a more aggressive disease course ($p<0.05$), although patient numbers were small with only 35 patients in the aggressive disease group (Barcellos et al. 2003). Another group employed cross-sectional data from patient subsets with benign and aggressive MS, reporting HLA-DRB1*01 was under-represented in the patients with aggressive disease (DeLuca et al. 2007). More recently a group have also analyzed the affect of DRB1*15 on disease severity stratified according to MSSS and time to EDSS 6 concluding that DRB1*15 is able to modulate the course of RRMS and hypothesizing that this occurred as a result of early initiation of the progressive phase (Cournu-Rebeix et al. 2008). A further study has examined the effects of functional polymorphisms in the immune mediators CCL5 and CCR5 which although not associated with an absolute risk for MS did seem to be correlated with observed variations in MRI markers and MSSS (van Veen et al. 2007).

Numbers of patients employed in all of these studies varied and since none has been convincingly replicated it is difficult to determine numbers needed to appropriately power such a study especially given that the effect size from epidemiological studies is unclear. Also, the power to detect effect will vary depending on the minor allele frequency of the analysed SNP; however, the positive effects seen in this study were not in those with the highest minor allele frequency. It may be this study was appropriately powered and there is little genetic dysregulation of C from the analysed SNPs contributing to disease phenotype in MS; but the limited positive results seen in this study may suggest that the study was under-powered. If similar numbers are needed as for susceptibility studies, it will be some time before this kind of in-depth

study with longitudinal disability data and comprehensive phenotypic data, can be achieved. An optimal approach would be to use data gained from GWAS with similarly detailed phenotypic data as has been employed in this study; but such an analysis was beyond the scope of this thesis. In addition, there is an increasing precedence in GWAS for additional SNP associations to become evident only on pathway analysis. The use of these approaches may reveal phenotype-genotype associations in C genes.

Figure 9.1: Molecular analysis of Factor H Tyr402His polymorphism

The upper photographed membrane shows staining of 96 patient samples with the MBI-6 antibody (fH Tyr402), and the lower shows the same 96 patients stained with the MBI-7 antibody (fH His402). Arrow 1 denotes a His402 homozygote, arrow 2 a Tyr402 homozygote and arrow 3 a heterozygote patient.

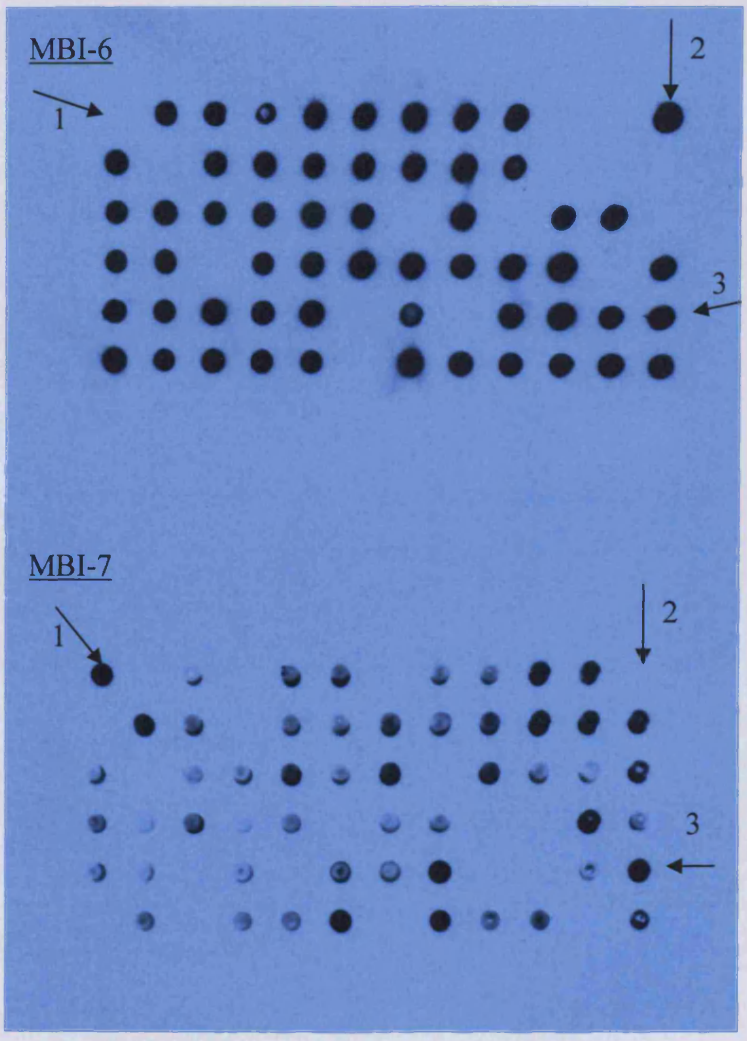


Table 9.1: Genotyping details

	rs1047286	rs2230199	rs17611	rs1065489	rs800292	rs1061170	rs11136000	rs6656401
No.	887	887	887	887	887	819	887	887
Gene	C3	C3	C5	Factor H	Factor H	Factor H	Clusterin	CR1
Chromosome	19	19	9	1	1	1	8	1
Genotyping success rate	91.2	91.5	91.4	92.8	91.4	100.0	91.5	92.3
Minor allele f	18.3	18.9	40.1	16.6	20.5	38.5	37.9	14.7
Control minor allele f	23.9	17.5	42.0	17.0	24.0	38.0	40.0	18.0

Table 9.2: Demographic characteristics of the cohort are not different in genotypic subgroups

		No.	Sex	Age			Disease duration			RRMS	SPMS	PPMS	EDSS>6.0
			% f	Mean	SD	p	Mean	SD	p	%	%	%	%
rs1047286	C C	514	69.46	45.82	11.88	N/A	14.07	10.62	N/A	50	40.47	9.53	29.77
	C T	266	69.92	46.24	12.59	1.000	15.22	11.48	0.513	50.75	39.85	9.4	29.7
	T T	29	68.97	45.59	14.31	1.000	15.34	14.54	1.000	62.07	31.03	6.9	27.59
rs2230199	C C	507	68.84	46.18	12.03	N/A	14.27	10.86	N/A	49.7	41.22	9.07	30.18
	C G	275	70.91	45.77	12.7	1.000	15.28	11.56	0.686	51.27	39.64	9.09	28.36
	G G	30	66.67	45.67	11.43	1.000	14.8	12.57	1.000	60	33.33	6.67	26.67
rs17611	G G	250	70.00	46.57	12.3	N/A	14.72	11.61	N/A	50	41.6	8.4	31.2
	A G	411	70.32	46.33	12.54	1.000	14.98	11.28	1.000	49.39	39.17	11.44	28.71
	A A	150	64.67	44.64	10.92	0.377	13.32	10	0.684	53.33	41.33	5.33	29.33
rs1065489	G G	559	68.16	45.31	12.26	N/A	14.09	10.93	N/A	51.7	38.46	9.84	29.34
	G T	233	71.67	47.67	12.08	0.039	15.36	11.4	0.433	46.78	44.64	8.58	30.04
	T T	31	74.19	44.13	10.97	1.000	15.9	12.19	1.000	58.06	32.26	9.68	25.81
rs800292	C C	494	70.24	46.24	12.66	N/A	14.53	11.57	N/A	49.8	40.08	10.12	30.77
	C T	271	70.11	45.46	11.47	1.000	14.12	10.14	1.000	52.03	38.75	9.23	25.83
	T T	46	58.7	45.22	11.45	1.000	15.87	12.07	1.000	47.83	47.83	4.35	39.13
rs1061170	Y Y	307	68.73	46.33	11.77	N/A	15.9	11.93	N/A	*46.25	*46.58	*7.17	34.53
	H Y	394	67.77	45.44	11.96	0.991	13.42	10.49	0.011	56.09	34.01	9.9	26.65
	H H	118	72.88	44.22	12.95	0.319	13.17	10.94	0.071	51.69	36.44	11.86	27.12
rs11136000	C C	272	66.18	47.24	12.4	N/A	15.2	11.29	N/A	47.06	41.91	11.03	28.31
	C T	408	70.83	45.73	12.29	0.338	14.71	11.38	1.000	53.68	37.99	8.33	30.88
	T T	132	72.73	44.42	10.94	0.087	12.35	9.49	0.046	49.24	41.67	9.09	25.76
rs6656401	G G	588	68.88	45.89	12.11	N/A	14.81	11.19	N/A	49.32	41.33	9.35	29.93
	A G	202	70.30	46.84	12.4	1.000	13.92	11.11	0.973	52.97	38.12	8.91	27.23
	A A	29	72.41	44.14	12.82	1.000	13.48	9.02	1.000	55.17	31.03	13.79	31.03

*f; female. SD; standard deviation. p = comparison with major allele group with Bonferroni correction. *rs1061170, analysis of disease subgroups, chi = 20.92, p=0.002. Positive results highlighted in grey.*

Table 9.3: Hazard ratios for reaching time to disability milestones are shown for each SNP

SNPs	Time to EDSS>4			Time to EDSS 6			Time to EDSS 8		
	HR	95% CI	p	HR	95% CI	p	HR	95% CI	p
rs1047286	0.73	0.59-0.90	0.003	0.70	0.54-0.91	0.007	0.84	0.52-1.39	0.511
rs2230199	0.77	0.63-0.94	0.012	0.72	0.56-0.93	0.012	0.84	0.51-1.36	0.476
rs17611	0.73	0.57-0.95	0.019	0.66	0.48-0.91	0.011	0.51	0.28-0.92	0.025
rs1065489	0.92	0.74-1.13	0.404	0.97	0.75-1.26	0.843	0.58	0.33-1.03	0.065
rs800292	0.97	0.80-1.19	0.791	0.87	0.68-1.12	0.275	0.71	0.43-1.16	0.178
rs1061770	0.94	0.70-1.27	0.704	0.89	0.62-1.28	0.540	0.50	0.27-0.94	0.031
rs11136000	0.94	0.70-1.27	0.530	0.76	0.58-0.99	0.045	1.03	0.63-1.70	0.902
rs6656401	1.01	0.81-1.27	0.898	0.86	0.64-1.15	0.304	0.96	0.55-1.69	0.890

HR; hazard ratio. CI; confidence interval. In each case the minor homozygote group was combined with the heterozygote group for comparison. Positive p values (<0.006) are highlighted in grey.

Table 9.4: Phenotypic disease characteristics are shown in genotypic subgroups

		PPMS	Age at onset			Age at diagnosis			Time to SPMS			Age at SPMS			Disease course at onset		
		%	Mean	SD	p	Mean	SD	p	Mean	SD	p	Mean	SD	p	RO	SO	POR
rs1047286	C C	9.53	31.12	10.08	N/A	35.40	10.32	N/A	10.16	8.71	N/A	41.52	10.14	N/A	62.06	28.02	9.53
	C T	9.40	30.49	9.74	1.000	35.63	10.50	1.000	12.13	12.93	0.424	43.53	9.59	0.359	62.41	27.44	9.40
	T T	6.90	29.76	10.12	1.000	34.76	12.00	1.000	11.43	14.95	1.000	37.14	14.71	0.781	68.97	24.14	6.90
rs2230199	C C	9.07	31.30	10.06	N/A	35.51	10.29	N/A	10.26	8.68	N/A	41.84	10.11	N/A	62.33	28.21	9.07
	C G	9.09	29.96	9.69	0.213	35.42	10.86	1.000	12.15	13.18	0.468	42.75	9.73	1.000	62.91	27.27	9.09
	G G	6.67	30.23	9.25	1.000	33.77	7.84	1.000	12.33	12.52	1.000	40.44	13.33	1.000	66.67	26.67	6.67
rs17611	G G	8.40	31.15	9.76	N/A	35.89	10.42	N/A	11.69	12.69	N/A	42.72	10.15	N/A	66.00	24.80	8.40
	A G	11.44	30.84	10.07	1.000	35.50	10.73	1.000	11.33	9.96	1.000	42.66	10.42	1.000	59.61	28.71	11.44
	A A	5.33	30.72	9.84	1.000	34.95	9.45	1.000	8.66	7.96	0.280	40.21	9.93	0.454	64.67	29.33	5.33
rs1065489	G G	9.84	30.63	9.93	N/A	35.07	10.33	N/A	10.33	10.32	N/A	40.77	9.50	N/A	60.82	28.80	9.84
	G T	8.58	31.72	10.01	0.481	36.07	10.57	0.658	11.74	10.44	0.872	44.38	10.98	1.000	64.81	26.18	8.58
	T T	9.68	27.61	9.59	0.302	36.23	10.73	1.000	14.00	13.90	0.915	43.11	11.68	1.000	74.19	16.13	9.68
rs800292	C C	10.12	31.11	10.42	N/A	35.59	10.86	N/A	10.89	11.16	N/A	42.27	10.14	N/A	63.97	25.30	10.12
	C T	9.23	30.76	9.36	1.000	35.47	9.71	1.000	10.55	9.43	1.000	41.82	9.83	1.000	61.62	28.78	9.23
	T T	4.35	28.85	8.69	0.429	34.04	9.56	1.000	11.50	10.12	1.000	40.76	11.62	1.000	56.52	39.13	4.35
rs1061170	Y Y	7.17	29.82	9.55	N/A	35.07	9.75	N/A	12.31	12.22	N/A	41.17	10.22	N/A	68.08	24.43	7.17
	H Y	9.90	31.44	9.55	0.088	35.59	10.37	1.000	10.22	9.24	0.382	42.78	8.79	0.596	62.44	26.90	9.90
	H H	11.86	30.50	10.79	1.000	34.26	10.89	1.000	8.85	8.24	0.225	39.95	10.05	1.000	58.47	29.66	11.86
rs11136000	C C	11.03	31.44	10.28	N/A	36.15	10.45	N/A	11.58	11.32	N/A	42.76	10.06	N/A	63.97	24.63	11.03
	C T	8.33	30.43	9.32	0.579	35.01	10.15	0.476	10.98	10.35	1.000	42.68	9.71	1.000	62.01	28.92	8.33
	T T	9.09	31.51	10.59	1.000	35.81	10.63	1.000	9.26	9.14	0.631	39.60	11.35	0.228	62.12	28.79	9.09
rs6656401	G G	9.35	30.45	9.81	N/A	35.46	10.52	N/A	11.23	11.10	N/A	42.00	10.12	N/A	62.59	27.38	9.35
	A G	8.91	32.45	9.96	0.041	35.69	10.16	1.000	9.97	8.84	1.000	42.64	10.16	1.000	64.36	26.73	8.91
	A A	13.79	30.17	10.92	1.000	34.79	10.19	1.000	11.00	3.21	1.000	39.88	14.94	1.000	58.62	27.59	13.79

RO; full recovery. SO; partial recovery. POR; progressive disease from onset. p = comparison with major allele group with Bonferroni correction. Positive results highlighted in grey.

Table 9.5: Genotypic correlations with initial investigations

		MRI findings				OCB				VEP findings			
		No.	Neg %	Pos %	p	No.	Neg %	Pos %	p	No.	Neg %	Pos %	p
rs1047286	C C	441	6.6	93.4		333	9.3	90.7		226	40.8	59.2	
	C T	244	4.3	95.7		220	5.3	94.7		187	9.1	90.9	
	T T	29	0.2	99.8	0.405	28	0.8	99.2	0.321	28	1.4	98.6	0.131
rs2230199	C C	433	6.0	94.0		334	8.9	91.1		236	38.1	61.9	
	C G	255	4.5	95.5		225	6.0	94.0		189	10.7	89.3	
	G G	30	0.4	99.6	0.580	29	0.6	99.4	0.585	29	1.0	99.0	0.867
rs17611	G G	231	3.7	96.3		204	3.9	96.1		183	10.8	89.2	
	A G	364	5.3	94.7		301	7.7	92.3		229	24.4	75.6	
	A A	145	2.2	97.8	0.537	136	2.5	97.5	0.975	124	5.0	95.0	0.942
rs1065489	G G	469	7.0	93.0		342	10.8	89.2		212	47.8	52.2	
	G T	218	3.4	96.6		198	4.7	95.3		177	8.5	91.5	
	T T	31	1.1	98.9	0.024	31	0.6	99.4	0.557	30	1.6	98.4	0.274
rs800292	C C	419	7.1	92.9		340	9.0	91.0		234	37.5	62.5	
	C T	254	3.4	96.6		215	5.5	94.5		188	9.8	90.2	
	T T	45	0.8	99.2	0.648	45	0.6	99.4	0.271	43	1.4	98.6	0.155
rs1061770	Y Y	243	6.1	93.9		204	6.8	93.2		173	14.2	85.8	
	Y H	365	5.1	94.9		301	6.6	93.4		222	24.9	75.1	
	H H	126	1.0	99.0	0.079	121	1.6	98.4	0.240	114	5.7	94.3	0.394
rs11136000	C C	282	2.9	97.1		253	4.8	95.2		220	11.8	88.2	
	C T	350	6.4	93.6		285	8.1	91.9		216	23.4	76.6	
	T T	114	2.0	98.0	0.397	107	1.7	98.3	0.769	101	5.3	94.7	0.822
rs6656401	G G	496	8.5	91.5		355	12.5	87.5		202	53.3	46.7	
	A G	189	2.8	97.2		177	4.1	95.9		160	7.9	92.1	
	A A	29	0.4	99.6	0.997	28	0.2	99.8	0.599	28	1.4	98.6	0.171

MRI; magnetic resonance imaging. OCB; oligoclonal bands. VEP; visual evoked potential. Neg; negative (in the MRI group this refers to any scan not fulfilling Barkof diagnostic criteria). Pos; positive (in the MRI group this refers to any scan fulfilling diagnostic criteria. p= comparison of disease subgroups by Chi square. Positive results highlighted in grey.

CHAPTER 10: CONCLUDING REMARKS AND DIRECTIONS FOR FURTHER RESEARCH

10.1 Development of a biological bank

Central to this thesis was the establishment of a well-maintained and functional biobank with rigorously processed serum, plasma, CSF and DNA samples linked to comprehensive clinical information. This biobank was established with the aim of being readily available for a wide range of projects extending over many years. The samples were therefore processed in a manner designed to make them fit for analysis of even unstable products. Given the large amount of samples being taken from clinics, it was not possible to foresee and account for all problems. There would be benefit when analysing some potential markers for samples to be immediately placed on ice, separated and frozen within 30mins. In addition the extraction of cells, not planned for by this biobank, may be beneficial for some work. However, for the purposes of many projects our protocol is sufficient and multiple aliquots from each sample makes measurement of different factors in the same individual easier. One of the main problems encountered was gaining samples from sufficient patients in various disease subgroups so as to make biomarker studies feasible. At the end of the project, serum and plasma samples had been collected from over 900 patients and DNA from over 1100 patients. The collection of CSF was limited to about 30 patients and 30 controls which meant CSF studies in the project were limited. Collection of longitudinal samples from individuals is valuable for biomarker studies especially when looking at prognostic markers. The biobank has been set up to collect samples yearly from patients attending clinic and more frequently if there is a change in their

disease state, so that in-depth studies looking at prognosis, although beyond the scope of this thesis, can be achieved in future projects.

The extent of clinical information linked to the sample biobank was a strength of this project; however, given the nature of MS and the disease duration there were gaps in the data which I attempted to fill in with a historical postal questionnaire. This was successfully used but further validation of some components such as the reporting of relapses, is needed in patients with relapsing remitting disease.

10.2 Systemic complement biomarkers for multiple sclerosis

This work firmly establishes C regulator fH as a biomarker of MS disease course with a test sensitivity and specificity to distinguish secondary progressive from relapsing remitting disease sufficient to make this a practical and useful clinical marker. Although replicated in-house, there is a need for this work to be validated and replicated by other groups so as to firmly establish the reproducibility of this work. Despite its usefulness as a biomarker of disease course, the data available on its use in predicting disease outcome is limited. To establish the ability of a biomarker to predict disease outcome, it would be necessary to have samples, together with corresponding clinical data, collected over many years. In Chapter 5 I present preliminary data with serial samples collected from 12 patients over a 2 year period in which the patients change disease course from relapsing to progressive; however, the collection of more samples over a protracted period was beyond the scope of this thesis. Another important element in establishing its usefulness as a biomarker, was to look at the stability of fH levels in clinically stable disease. This was performed in 11

patients over 1 year; extending this work would make findings more robust and should be possible in future projects when the biobank is more established.

It seems more likely that a combination of different markers will produce a sensitive and specific model for particular elements of disease. Chapter 7 shows combined phenotype-C component models which increase the predictive value of a particular test compared to using the individual component alone. The models described in this thesis allow the distinction of MS from healthy controls and relapse from remission. These models now need testing in prospective cohorts of patients. Firstly, to be useful as a clinical marker of MS, the model would have to be tested in patients with other neurological disease within the MS differential such, as vasculitis; as well as in patients with a CIS. The optimal method of examining this in more detail would be to use a prospective cohort of patients with CIS allowing correlation of the objective model with time to conversion to MS; although again this would require collection of samples over a longer period than this thesis allowed. The optimal way to validate a model to distinguish relapse would be in a prospective group of patients attending an acute rapid access MS service who believe they have a clinical relapse. The objective model could then be correlated with clinical features and disease activity on MR scans.

Further ways to improve the models generated would be the use of objective phenotypic variables such as age, gender and disease duration. This was discussed in Chapter 7 and left out of the initial analysis so as to not cause confusion in the initial steps of generating an objective biomarker. Correlation with other inflammatory markers or axonal injury markers would also provide additional information which

may add to the models generated. In addition, the use of accepted markers such as MRI would enable correlation of a potential biomarker with more accepted and objective disease measures.

10.3 The contribution of complement to the pathophysiology of multiple sclerosis

The extent and nature of C activation and its involvement in the acute and chronic phases of disease is currently controversial. Although animal model studies have given us some indication of the role of C in acute MS, they have hitherto provided little information on any contribution to axonal degeneration seen throughout disease but more predominantly in progressive phase. The development of a novel mouse model for chronic EAE may lead to more informative research regarding axonal damage and remyelination in demyelinating diseases. Pathological studies in human brain have mostly been limited to the examination of relatively few components of the C system and provide only a limited snap-shot view of the pathological process depending on the patients' disease course at death. This thesis examines an array of C components, fragments and regulators both systemically in serum/plasma and locally in CSF and immunohistochemically in brain and spinal cord sections, in an attempt to clarify some of these issues.

Work in this thesis demonstrates extensive systemic and local activation and regulation of the C system even in late stages of MS indicating a contribution from C to pathogenesis throughout the disease course, and supporting the hypothesis of inflammatory driven axonal degeneration causing progressive MS. Furthermore, in

Chapter 8, immunohistochemical analysis shows C deposition in long-standing MS in and around plaques which would otherwise be classified as inactive; also C deposition on damaged axons co-localized with APP. Further work examining brain tissue from patients with early, active disease; comparing C deposition to patients with long-standing, chronic disease, would provide additional information on changes in the immune system over disease course. In addition the establishment of a brain bank from local patients with prior collected serum, plasma, CSF and DNA during their disease would be of great benefit when examining the contribution of systemic and local C to pathology.

CSF studies were limited in this thesis due to a relative lack of specimens; further, the control group available included neurological controls with a degree of inflammation possibly contributing to the condition; for example, cerebrovascular disease. Further CSF studies with a larger non-inflammatory neurological control group in addition to larger MS and CIS groups would be of benefit not only to confirm the source of C in MS brain whether it be largely local or systemic in origin, and therefore how amenable it will be to systemically administered therapies. This work suggests both the classical and alternative pathways contribute to disease, although further investigation of other alternative pathway components in progressive disease may help further elucidate the differential expression of fH seen in these patients.

Most treatments for MS have so far focused predominantly on controlling the T cell mediated immune response which initiates and drives relapsing disease. The majority of anti-inflammatory therapies have failed to have any significant effect in delaying disease progression when used in either the relapsing remitting or progressive disease

phase, leading to speculation amongst investigators as to the contribution of inflammation to axonal degeneration. New evidence outlined in this thesis supports the role for inflammation in chronic disease and suggests therapies targeting innate immune responses in combination with more traditional therapies, may have greater success in preventing or halting axonal degeneration.

PUBLICATIONS ARISING FROM THIS WORK

- **Ingram G**, Hakobyan S, Hirst C, Harris CL, Pickersgill TP, Cossburn MD, Loveless S, Robertson NP, Morgan BP. Complement Regulator Factor H as a Serum Biomarker of Multiple Sclerosis Disease State. Brain. 2010 Jun;133(Pt 6):1602-11. Epub 2010 Apr 25.

- **Ingram G,** Hakobyan S, Roberston NP, Morgan BP. Plasma C4a levels in multiple sclerosis correlate with disease activity. J Neuroimmunol. 2010 Jun;223(1-2):124-7. Epub 2010 Apr 20.

- **Ingram G,** Colley E, Ben-Shlomo Y, Cossburn MD, Hirst CL, Pickersgill TP, Robertson NP. Validity of patient derived disability and clinical data in Multiple Sclerosis. Multiple Sclerosis. Mult Scler. 2010 Apr;16(4):472-9. Epub 2010 Feb 11.

- **Ingram G**, Hakobyan S, Robertson NP, Morgan BP. Complement in multiple sclerosis: its role in disease and potential as a biomarker. Clin Exp Immunol. 2009 Feb;155(2):128-39. Epub 2008 Nov 24 (Review).

PUBLICATIONS ARISING FROM RELATED WORK

- **Ingram G**, Robertson NP. The association of Anti-EBNA-1 IgG and multiple sclerosis clinical disease activity – response. Eur J Neurol. 2010

- **Ingram G,** Hirst CL, Robertson NP. Correspondence: What is the risk of permanent disability from a multiple sclerosis relapse? Neurology. 2010 Aug 31;75(9):837

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Appendix 1: EDSS questionnaire

ID	Name

Question 1. When were you diagnosed with Multiple sclerosis?

Month…..……..Year……...

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

Question 2. When did you first have symptoms of MS?

Month……Year……...

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

Question 3. What were your very first symptoms? *please tick yes or no*

Arm or hand weakness	Yes ☐ No ☐	Dizziness / vertigo	Yes ☐ No ☐
Leg weakness or heaviness	Yes ☐ No ☐	Unsteadiness / loss of balance	Yes ☐ No ☐
Facial weakness / drooping	Yes ☐ No ☐	Double vision	Yes ☐ No ☐
Pins and needles	Yes ☐ No ☐	Blurring of loss of vision	Yes ☐ No ☐
Loss of sensation in body	Yes ☐ No ☐	Bladder or bowel problems	Yes ☐ No ☐
Loss of sensation in face	Yes ☐ No ☐	Speech or swallowing problems	Yes ☐ No ☐

Question 4. Did these initial symptoms completely go away at any time in the following year?

☐ Yes – **Please go to Question 6** ☐ No – **Please go to Question 5**

Question 5. Did these initial symptoms gradually deteriorate in the following year? Yes ☐ No ☐

Question 6. How long was it before your next relapse? .....months

(episode of symptoms lasting at least a day but not at a time when you had an infection e.g. urine infection / fever) ...../....years

please specify to the half year

Question 7. What type of Multiple Sclerosis do you understand you have?
(please tick the box that best describes your MS)

Relapsing remitting ☐	Secondary Progressive ☐
Primary Progressive ☐	Don't know ☐

Question 8. Many patients with MS feel that their condition is stable in-between relapses; however, some patients feel that their condition is gradually worsening. Looking back do you think your symptoms of MS are gradually worsening despite the effect of relapses? Yes ☐ No ☐

If yes, when do you think this started to happen? Month.....

Year.....

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

Question 9. Have you had any relapses in the past year?
(episodes of symptoms lasting at least a day but not at a time when you had an infection e.g. urine infection / fever)

☐ Yes - **Please fill in chart below** ☐ No - **Please go to Question 10**

How many relapses have you had in the last year? ……..

Relapse 1

Month…….. Year……

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

Did these new symptoms go away at any time during the following year? Yes ☐ No ☐

Arm or hand weakness	Yes ☐ No ☐	Dizziness / vertigo	Yes ☐ No ☐
Leg weakness or heaviness	Yes ☐ No ☐	Unsteadiness / loss of balance	Yes ☐ No ☐
Facial weakness / drooping	Yes ☐ No ☐	Double vision	Yes ☐ No ☐
Pins and needles	Yes ☐ No ☐	Blurring of loss of vision	Yes ☐ No ☐
Loss of sensation in body	Yes ☐ No ☐	Bladder or bowel problems	Yes ☐ No ☐
Loss of sensation in face	Yes ☐ No ☐	Speech or swallowing problems	Yes ☐ No ☐

If you have had more than 1 relapse this year, there is an additional chart attached at the back of the questionnaire. Please contact us if you need extra sheets.

Question 10. Are you able to walk independently - without the use of a walking stick or frame or person to hold or FES machine or other support? *(please tick)*

☐ Yes - **Please go to Question 11**

☐ No – **Please go to Question 12**

Question 11. At your BEST, how far can you walk without a rest or help from a stick or frame or person or FES or other walking aid? *(please tick)*

☐ Unrestricted *(I can walk for 2-3 hours with no problem)*

☐ Clearly more than 500 metres / 550 yards

☐ About 500 metres / 550 yards
(about the length of Queens Street in Cardiff)

Go to Question 18 at the end

☐ About 300 metres / 330 yards
(the length of 3 football pitches)

☐ About 200 metres / 220 yards
(half of Queens street in Cardiff or 2 football pitches)

☐ About 100 metres / 110 yards
(the length of one football pitch or the outpatients corridor in the UHW hospital)

☐ Less than 100 metres / 110 yards

Go to Question 12

Question 12. Do you remember when, to the nearest month and year, you became *unable* to walk for 500 metres / 550 yards without a rest or help from a stick or frame or person?

Month.....

Year.....

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

If you can walk without the use of a walking aid such as a stick, you have finished the questionnaire, please proceed to Question 18 at the end.

If you need help to walk or use a wheelchair, please continue to Question 13.

Question 13. Please tick the box below that best describes your walking ability.

- ☐ I can walk more than, or about, 100 metres / 110 yards (*the length of a football pitch)* with 1 stick or person for support
- ☐ I use an FES machine to walk (on **one** leg / on **both** legs - *please ring*)
- ☐ I need support on both sides to walk, such as 2 sticks or a walker / frame
- ☐ I can only walk about 10 metres (just about across a room)
- ☐ I can only walk a few steps even with help and normally use a wheelchair
- ☐ I use a wheelchair **all** of the time and **can't take even a few steps**

Question 14. Do you remember when you first started to need constant help from either a stick or person and could only walk about 100 metres / 110 yards *(the length of a football pitch)*?

Month.....
Year.....

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

If you are still mobile please go to Question 18 at the end
If you use a wheelchair all of the time, please go to Question 15

Question 15. Do you remember when you first started to need to use your wheelchair to get around and you were no longer able to take any steps more than to transfer?

Month.....
Year.....

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

Question 16. If you are bed or chair bound, do you have trouble using your hands for writing and eating, etc.

Yes ☐
No ☐

Question 17. Are you totally confined to bed and need help with *all* daily tasks?

Yes ☐
No ☐

Many thanks for filling in this questionnaire which will be very helpful.
Please now return it using the prepaid envelope.
If you have any questions, or struggle to fill in any parts of the questionnaire, please phone Dr Gillian Ingram on 02920 743454. If you leave a message with your name and number I will get back to you as soon as possible.

Question 18.

Could you tell us who filled in this questionnaire?

☐ **You alone**
☐ **You and a carer**
☐ **Carer alone**

Who is your carer *(please ring)* **husband / wife / partner / sibling / parent / child**

Name.....Signature..... Date.....

Other comments.....

.....

.....

.....

.....

.....

.....

.....

.....

.....

Appendix 1

Relapse2

Month..... Year.....

If you can't remember the month please can you ring the season?

Spring Summer Autumn Winter

Did these new symptoms go away at any time during the following year? Yes ☐ No ☐

Arm or hand weakness	Yes ☐ No ☐	Dizziness / vertigo	Yes ☐ No ☐
Leg weakness or heaviness	Yes ☐ No ☐	Unsteadiness / loss of balance	Yes ☐ No ☐
Facial weakness / drooping	Yes ☐ No ☐	Double vision	Yes ☐ No ☐
Pins and needles	Yes ☐ No ☐	Blurring of loss of vision	Yes ☐ No ☐
Loss of sensation in body	Yes ☐ No ☐	Bladder or bowel problems	Yes ☐ No ☐
Loss of sensation in face	Yes ☐ No ☐	Speech or swallowing problems	Yes ☐ No ☐

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