



SHL ITEM BARCODE



19 1707209 4

REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree *MD*Year *2005*Name of Author *Chen, E-T*

COPYRIGHT

This is a thesis accepted for a Higher Degree of the University of London. It is an unpublished typescript and the copyright is held by the author. All persons consulting the thesis must read and abide by the Copyright Declaration below.

COPYRIGHT DECLARATION

I recognise that the copyright of the above-described thesis rests with the author and that no quotation from it or information derived from it may be published without the prior written consent of the author.

LOANS

Theses may not be lent to individuals, but the Senate House Library may lend a copy to approved libraries within the United Kingdom, for consultation solely on the premises of those libraries. Application should be made to: Inter-Library Loans, Senate House Library, Senate House, Malet Street, London WC1E 7HU.

REPRODUCTION

University of London theses may not be reproduced without explicit written permission from the Senate House Library. Enquiries should be addressed to the Theses Section of the Library. Regulations concerning reproduction vary according to the date of acceptance of the thesis and are listed below as guidelines.

- A. Before 1962. Permission granted only upon the prior written consent of the author. (The Senate House Library will provide addresses where possible).
- B. 1962 - 1974. In many cases the author has agreed to permit copying upon completion of a Copyright Declaration.
- C. 1975 - 1988. Most theses may be copied upon completion of a Copyright Declaration.
- D. 1989 onwards. Most theses may be copied.

This thesis comes within category D.

☐

This copy has been deposited in the Library of _____

☒

This copy has been deposited in the Senate House Library, Senate House, Malet Street, London WC1E 7HU.

**NEURODEGENERATIVE MARKERS - INSIGHTS
INTO MULTIPLE SCLEROSIS**

Dr EE TUAN LIM

Department of Neuroinflammation

Institute of Neurology

Queen Square

University College London

London, UK

Thesis submitted for the examination for

DOCTOR OF MEDICINE (M.D.)

UNIVERSITY OF LONDON

2005



UMI Number: U592305

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U592305

Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



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

Abstract

INTRODUCTION:

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) characterised pathologically by inflammation, demyelination and variable degrees of axonal loss and gliosis. Remyelination, axonal and synaptic plasticity have been identified as mechanisms underlying functional recovery. MS typically follows a chronic course, in the sense that new episodes or steady progression are the rule. Currently radiological surrogates of inflammation, demyelination and axonal loss are available, but these correlate only modestly with the development of cumulative disability. In this thesis, potential brain specific protein (BSP) markers for these pathological processes are identified and compared to existing magnetic resonance (MR) markers.

OBJECTIVES:

Levels of the nervous system proteins S100B (astrocytic activation), glial fibrillary acidic protein (GFAP; astrogliosis), neurofilaments (axonal marker) and ferritin (microglial activation) and other inflammatory markers (i.e. anti-myelin antibodies) were measured. These values were correlated with both histopathological measurements in post-mortem specimens and MR imaging measures in patients with MS. Histology and immunochemistry of tissue sections characterised normal and pathological CNS and localized the extent of demyelinating plaques.

METHODS:

Subjects/Specimens

Sera/plasma and cerebrospinal fluid (CSF) from various cohorts of patients with MS or other neurological diseases and blood samples from healthy subjects were stored at -80°C . CSF of subjects with demyelinating diseases obtained from the Stockholm Optic Neuritis study and the Copenhagen intravenous vs. oral steroid trial; sera/plasma from subjects who participated in the primary progressive (PP) MS phase II trial of IFN β -1a and clinically isolated syndrome (CIS) natural history study, Institute of Neurology (ION), United Kingdom, were analyzed for various BSP. Prospective collection of fresh post mortem brain specimen (within 24 hours of death) from MS brain bank at Charing Cross was used for in ex-vivo studies. Cerebrospinal fluid and bloods of individuals with other neurological diseases (OND) from ION served as controls. Full patient consent and ethical approval were obtained.

Laboratory techniques:

Existing in-house ELISAs allowed ferritin (microglial activation; Keir et al., 1993), S100B for astrocytic activation (Green et al.; 1997), neurofilament heavy chain phosphoforms and GFAP to be quantified. Anti-myelin antibodies were measured by western blotting by collaborators in Innsbruck. Tissues from post-mortem specimens were homogenized, and the supernatant measured for the BSP described above. Levels of the BSP proteins in the supernatants were compared

with quantitative histopathological variables that have been established to assess the various pathological processes highlighted above.

ANALYSIS TECHNIQUES

The body fluid markers measured were correlated with MRI and clinical findings; Kurtzke's Expanded Disability Status Scale (EDSS). Post mortem specimens studied were correlated with histopathology.

OUTCOMES

In MS disease mechanisms are complex, and as yet no surrogate marker adequately account for disability. In this thesis, various fluid markers were combined with clinical and neuroradiological findings, in order to better predict disease progression. This is an important aim in that it may allow us to target therapies to patients with more severe disease and a protein marker may prove useful as a surrogate outcome measure in MS treatment trials.

List of Abbreviations

| | |
|-------|--|
| AIDS | Acquired immune deficiency syndrome |
| BSA | Bovine serum albumin |
| BSP | Brain specific proteins |
| CDMS | Clinically definite (MS) |
| CJD | Creutzfeldt-Jacob Disease |
| CI | Confidence interval |
| CIS | Clinically isolated syndrome |
| CNS | Central nervous system |
| CREAE | Chronic relapsing experimental allergic encephalomyelitis |
| CSF | Cerebrospinal fluid |
| EAE | Experimental autoimmune encephalomyelitis |
| EDSS | Expanded disability status scale |
| ELISA | Enzyme-linked immunosorbent assay |
| GFAP | Glial fibrillary acidic protein |
| Ig | Immunoglobulin |
| IF | Intermediate filament |
| IFN | Interferon |
| IFN30 | Interferon- β -1a 30 μ g intramuscularly weekly |
| IFN60 | Interferon- β -1a 60 μ g intramuscularly weekly |

| | |
|----------------------|--|
| Im | Intramuscularly |
| MBP | Myelin basic protein |
| MOG | Myelin oligodendrocyte glycoprotein |
| MRI | Magnetic resonance imaging |
| MS | Multiple sclerosis |
| NAA | N-acetyl aspartate |
| NAWN/BT | Normal appearing white matter / brain tissue |
| NF | Neurofilament |
| NFL | Neurofilament light chain |
| NFM | Neurofilament medium chain |
| NfH | Neurofilament heavy chain |
| NfH ^{SMI34} | Extensively phosphorylated neurofilament heavy chain |
| NfH ^{SMI35} | Phosphorylated neurofilament heavy chain |
| NIND | Non inflammatory neurological diseases |
| OCB | Oligoclonal band |
| ON | Optic neuritis |
| OIND | Other inflammatory neurological diseases |
| OND | Other neurological diseases |
| PPMS | Primary progressive (MS) |
| R | Spearman rank correlation coefficient |
| RRMS | Relapsing remitting (MS) |
| SD | Standard deviation |

Tables

| | | |
|------------------|--|-----|
| Table 1.1 | Recent studies looking at subsequent risk of development of multiple sclerosis in patients presenting with CIS | 34 |
| Table 1.2 | New MS Diagnostic Criteria | 35 |
| Table 1.3 | The Poser criteria for MS | 36 |
| Table 1.4 | Kurtzke's Expanded Disability Status Scale (EDSS) | 45 |
| Table 3.1 | MRI outcome measures in MS | 58 |
| Table 3.2 | Characteristics of the brain specific proteins | 60 |
| Table 3.3 | Neurofilament studies of MS | 68 |
| Table 3.4 | Nomenclature and characteristics of the 9 types of NfH phosphoforms. | 69 |
| Table 3.5 | S100B studies of MS | 74 |
| Table 3.6 | GFAP studies of MS | 78 |
| Table 3.7 | Ferritin studies of MS | 82 |
| Table 3.8 | Anti-MOG antibody studies in MS | 87 |
| Table 4.1 | Characteristics of subjects with other neurological disease | 101 |
| Table 4.2 | Clinical characteristics of subjects | 102 |
| Table 4.3 | CSF characteristics expressed as median (interquartile range) | 103 |
| Table 5.1 | Results of ordered logistic regression in | 118 |

| | | |
|------------------|---|-----|
| | multiple sclerosis trial group examining association between EDSS change and neurofilament levels. | |
| Table 5.2 | Spearman rank correlation coefficients were used to examine associations between CSF neurofilament heavy chain levels and baseline T1 weighted MRI gadolinium area (enhancing lesion volume). | 119 |
| Table 6.1 | Spearman rank correlation coefficients were used to examine associations between brain specific proteins and quantitative histology. | 138 |
| Table 6.2 | Brain specific proteins to total protein ratios in multiple sclerosis brain tissues. | 141 |
| Table 6.3 | Non parametric Spearman associations between different brain specific proteins to total protein ratios in NAGM, NAWM, lesions in WM and in GM. | 142 |
| Table 7.1 | Age and serial serum S100B levels expressed as median (interquartile range). | 152 |
| Table 7.2 | Estimated mean change in 24-month serum S100B associated with unit increase in mean value of T1 and T2 lesion load, ventricular and spinal cord volume, adjusted for baseline | 156 |

values of both serum S100B and of MRI parameters.

| | | |
|------------------|--|-----|
| Table 8.1 | Characteristics of the 47 patients and their disease, according to the antibody status. | 164 |
| Table 8.2 | Conversion to clinically definite multiple sclerosis according to McDonald and Poser criteria. | 165 |
| Table 8.3 | Conversion to clinically definite multiple sclerosis according to McDonald and Poser criteria and the cumulative total that converted to MS compared to those that did not according to each criteria. | 166 |

Figures

| | | |
|-------------------|---|-----|
| Figure 1.1 | Treatment for optic neuritis in adults | 29 |
| Figure 1.2 | Acute right optic neuritis showing high signal lesion in the optic nerve on fast spin echo | 37 |
| Figure 1.3 | Abnormal T2 weighted MRI image of patient with multiple sclerosis | 38 |
| Figure 1.4 | Isoelectric focusing to reveal oligoclonal IgG production in CSF | 39 |
| Figure 3.1 | Schematic diagram of neurofilament monomers | 66 |
| Figure 4.1 | Schematic diagram of the degree of phosphorylation healthy axons vs. damaged axons. | 95 |
| Figure 4.2 | Box-and-whiskers plot of levels of extensively phosphorylated neurofilaments in subjects with ON and those with OND was significantly different | 104 |
| Figure 4.3 | Box-and-whiskers plot levels of the ratio NfH ^{SMI34-SMI35} in subjects with ON and that with OND was significantly different | 105 |
| Figure 5.1 | Number of subjects who received treatment | 108 |
| Figure 5.2 | The association between neurofilament and MBP at baseline in the placebo group. | 120 |
| Figure 5.3 | The association between neurofilament measured after treatment and baseline MBP in the placebo. | 121 |
| Figure 6.1 | Brain slice indicating ventral and dorsal sides with | 135 |

arrow pointing to plaques which were sampled on either side.

| | | |
|-------------------|---|-----|
| Figure 6.2 | MRI T2-weighted image was done 5mm beneath the surface where samples were excised. | 136 |
| Figure 6.3 | Scatterplot of GFAP to total protein ratio versus axonal count in NAWM and lesions. | 139 |
| Figure 6.4 | Scatterplot of GFAP to total protein ratio versus axonal density in NAWM and lesions. | 140 |
| Figure 7.1 | Fifty subjects with PPMS were randomised in a phase II trial of Interferon β -1a and were assessed 3 monthly over a 2-year study period. | 151 |
| Figure 7.2 | Three-monthly serum S100B levels trajectory of subject with PPMS who were given placebo weekly for 2 years. | 153 |
| Figure 7.3 | Three-monthly serum S100B levels trajectory of each patient with PPMS who received IFN β -1a 30 μ g intramuscularly weekly for 2 years. | 154 |
| Figure 7.4 | Three-monthly serum S100B levels trajectory of each patient with PPMS who received IFN β -1a 60 μ g intramuscularly weekly for 2 years. | 155 |
| Figure 8.1 | Hazard function of the risk of CDMS according to antibody status | 167 |

Description of Thesis

The thesis is divided into four parts.

Part 1 gives an introduction to optic neuritis and multiple sclerosis, and describes the immunology that we understand to date. The roles of surrogate markers and the brain specific proteins investigated in this thesis are elaborated.

Part 2 comprises of three studies; the aim of the first study is to evaluate the levels of BSP markers in demyelinating diseases. The second study sets out to confirm the findings from the first study and to determine whether one or more protein markers may be useful surrogate marker(s) i.e. predict clinical outcome. Lastly, comparison is made between the immunological markers studied with histopathology in post-mortem multiple sclerosis brain.

Part 3 answers two important questions, which as yet, have not been answered. Firstly, whether serum S100B is raised in patients with PPMS. Of note, levels of serum S100B has been studied in patients with relapsing-remitting and secondary progressive multiple sclerosis. Secondly, to confirm the exciting and promising findings by Berger *et al.* (2003) as to whether anti-myelin antibodies can predict conversion to clinically definite (CD) MS.

The final part consists of a summary of the principle findings and the conclusions of the thesis.

Acknowledgements

I would like to thank my parents for giving me so much and expecting so little.

This thesis is dedicated to them.

Thank you to Ee Chui, Siew Sing and Ee Theng for putting up with a sister like me.

I have been very lucky to have Dr Gavin Giovannoni and Dr Geoff Keir as my supervisors. They have been so patient, approachable, encouraging and supportive.

Thanks to Donna who taught me how to use a pipette on day 1! Thank you to Prof Ed Thompson, Jan, Rosemary, Miles, Axel, Andrew, Russell, Nick, Pat, Stuart, Paul, Davide, Lotte, Liz and June.

I would also like to thank all the research fellows whom I share my office with, and some of whom I collaborated my research with. They are Klaus, Catherine, Waqar, Stefania, Anand, Michela, Jaume, Mara, Lluís, Marek, Bertrand, Krishani, Jo, Mary, Leonora, Gary, Becky and John.

Thank you to my other collaborators – Professor Miller, Professor AlanThompson, Siobhan, Dr Sellebjerg, Dr Söderström and Dr Berger. A special thanks to Dan Altmann for his help in statistics.

This thesis is possible because of patients and doctors who kindly participated in research. Also, thank you to the UK Multiple Sclerosis Tissue Bank at the Charing Cross Hospital, London.

Finally I would like to thank Gerard for making my first 3 years in London a very happy one. This thesis is also for you.

Publications arising from this thesis

ET Lim, D Grant, M Pashenkov, G Keir, EJ Thompson, M Soderstrom, G Giovannoni. Cerebrospinal fluid levels of brain specific proteins in optic neuritis. *Mult Scler.* 2004 Jun;10(3):261-5.

ET Lim, A Petzold, SM Leary, DR Altmann, G Keir, EJ Thompson, DH Miller, AJ Thompson, G Giovannoni. Serum S100B in primary progressive multiple sclerosis patients treated with interferon-beta-1a. *J Negat Results Biomed.* 2004 Oct 13;3(1):4.

ET Lim, T Berger, M Reindl, CM Dalton, K Fernando, G Keir, EJ Thompson, DH Miller, G Giovannoni. Do anti-myelin antibodies allow earlier diagnosis of multiple sclerosis? *Mult Scler.* (In press.)

ET Lim, F Sellebjerg, CV Jensen, DR Altmann, D Grant, G Keir, EJ Thompson, G Giovannoni. Acute axonal damage predicts clinical outcome in patients with multiple sclerosis. *Mult Scler.* (In press.)

ET Lim, G Giovannoni . Immunopathogenesis and immunotherapeutic approaches in multiple sclerosis. *Expert Review of Neurotherapeutics.* (In press.)

Contents

| | |
|---|-----------|
| Abstract | 2 |
| List of Abbreviation | 5 |
| List of Tables | 7 |
| List of Figures | 10 |
| Description of thesis | 12 |
| Acknowledgements | 13 |
| Publications arising from thesis | 15 |
| Contents | 16 |
| | |
| PART 1 INTRODUCTION AND BACKGROUND | 23 |
| Chapter 1 Optic Neuritis, Clinically Isolated Syndrome and Multiple Sclerosis | 24 |
| 1.1 Optic Neuritis | 24 |
| 1.1.1 Introduction | 24 |
| 1.1.2 Aetiology | 24 |
| 1.1.3 Differential Diagnosis | 24 |
| 1.1.4 Clinical Manifestation | 25 |
| 1.1.5 Clinical Findings | 26 |
| 1.1.6 Prognosis | 27 |
| 1.1.7 Treatment | 27 |
| 1.2 Clinically Isolated Syndrome | 30 |

| | | |
|------------------|--|-----------|
| 1.3 | Multiple Sclerosis | 30 |
| 1.3.1 | Natural History | 30 |
| 1.3.2 | Aetiology | 31 |
| 1.3.3 | Diagnosis | 32 |
| 1.3.4 | Epidemiology | 40 |
| 1.3.5 | Genetics | 41 |
| 1.3.6 | Genes or Germs | 42 |
| 1.3.7 | Clinical Manifestation | 42 |
| 1.3.8 | Investigations | 46 |
| 1.3.8.1 | MRI | 46 |
| 1.3.8.2 | CSF Analysis | 47 |
| 1.3.8.3 | Neurophysiological Studies | 47 |
| 1.3.9 | Treatment | 47 |
| 1.3.9.1 | Disease Modifying Therapy | 47 |
| 1.3.9.2 | Symptomatic Therapy | 49 |
| Chapter 2 | Immunology in Clinically Isolated Syndrome and Multiple Sclerosis | 51 |
| 2.1 | Historical Perspective | 51 |
| 2.2 | Immunology | 51 |
| 2.2.1 | Inflammation | 52 |
| 2.2.2 | Demyelination | 53 |
| 2.2.3 | Axonal Damage | 53 |
| 2.2.4 | Remyelination | 54 |

| | | |
|------------------|--|-----------|
| 2.3 | Histopathology | 55 |
| Chapter 3 | Biological Markers in Multiple Sclerosis | 57 |
| 3.1 | MRI | 58 |
| 3.2 | CSF Oligoclonal Bands | 59 |
| 3.3 | Brain Specific Proteins | 59 |
| 3.3.1 | Neurofilaments | 61 |
| 3.3.1.1 | ELISA method to detect neurofilament heavy chain phosphoforms | 67 |
| 3.3.2 | S100B | 71 |
| 3.3.2.1 | ELISA method to detect S100B | 73 |
| 3.3.3 | Glial fibrillary acidic protein | 75 |
| 3.3.3.1 | ELISA method to detect GFAP | 76 |
| 3.3.4 | Ferritin | 79 |
| 3.3.4.1 | ELISA method to detect ferritin | 80 |
| 3.3.5 | Anti-myelin antibodies | 82 |
| 3.3.5.1 | Anti-myelin basic protein | 83 |
| 3.3.5.2 | Anti-myelin oligodendrocyte glycoprotein | 83 |
| 3.4 | Summary | 85 |
| | | |
| PART 2 | BRAIN SPECIFIC PROTEINS IN PATIENTS WITH DEMYELINATING DISEASES AND IN POST MOSTEM BRAINS WITH MULTIPLE SCLEROSIS | 88 |

| | | |
|------------------|---|------------|
| Chapter 4 | CSF Levels of Brain Specific Proteins in Optic Neuritis | 89 |
| 4.1 | Introduction | 89 |
| 4.2 | Aims | 90 |
| 4.3 | Methods | 91 |
| 4.3.1 | Subjects | 91 |
| 4.3.2 | Follow up | 92 |
| 4.3.3 | CSF analysis | 93 |
| 4.3.4 | Oligoclonal bands, total protein, S100B and Ferritin | 93 |
| 4.3.5 | Neurofilament heavy chains | 93 |
| 4.3.6 | Statistical Analysis | 94 |
| 4.4 | Results | 95 |
| 4.4.1 | ON (total) vs. OND | 95 |
| 4.4.2 | ON (CDMS) vs. Non-MS-type ON | 96 |
| 4.4.3 | MS-type ON vs. Non-MS-type ON | 96 |
| 4.5 | Discussion | 96 |
| Chapter 5 | Cerebrospinal fluid levels of neurofilament heavy chain in acute optic neuritis and multiple sclerosis patients treated with oral high-dose methylprednisolone | 106 |
| 5.1 | Introduction | 106 |
| 5.2 | Aims | 107 |
| 5.3 | Methods | 108 |
| 5.3.1 | Patients and examination | 108 |
| 5.3.2 | MRI | 109 |

| | | |
|---|--|-----|
| 5.3.3 | CSF tests | 109 |
| 5.3.4 | Statistical analyses | 110 |
| 5.4 | Results | 112 |
| 5.4.1 | CSF neurofilament heavy chain levels and clinical disability | 112 |
| 5.4.1.1 | MS attack trial group | 112 |
| 5.4.1.2 | ON trial group | 112 |
| 5.4.2 | Effect of treatment on clinical outcome | 113 |
| 5.4.3 | Effect of treatment on CSF neurofilament levels | 113 |
| 5.4.4 | CSF neurofilament heavy chain levels and MRI measures | 113 |
| 5.4.5 | CSF neurofilament heavy chain levels and MBP | 114 |
| 5.5 | Discussion | 114 |
| Chapter 6 A comparison of the brain specific proteins and histopathology in post mortem multiple sclerosis brain | | 122 |
| 6.1 | Introduction | 122 |
| 6.2 | Materials and methods | 123 |
| 6.2.1 | Protein extraction | 124 |
| 6.2.2 | Brain specific protein analyses | 124 |
| 6.2.3 | Histology studies | 125 |
| 6.2.4 | Statistical Analyses | 128 |
| 6.3 | Results | 128 |
| 6.3.1 | Brain specific protein vs. histopathology | 128 |

| | | |
|---|--|-----|
| 6.3.2 | Brain specific proteins in normal appearing brain tissue vs. lesions | 129 |
| 6.3.3 | Relationships between BSPs (ELISA) | 130 |
| 6.4 | Discussion | 131 |
| PART 3 SPECIFIC MARKERS IN PROGRESSIVE DISEASE AND CLINICALLY ISOLATED SYNDROMES | | 143 |
| Chapter 7 Serum S100B in primary progressive multiple sclerosis patients treated with interferon-beta-1a | | 144 |
| 7.1 | Introduction | 144 |
| 7.2 | Aims | 145 |
| 7.3 | Methods | 145 |
| 7.3.1 | Patients and examination | 145 |
| 7.3.2 | MR imaging and analyses | 146 |
| 7.3.3 | Serum S100B levels | 147 |
| 7.3.4 | Statistical analyses | 147 |
| 7.4 | Results | 148 |
| 7.4.1 | Serum S100B between subjects with PPMS and controls | 148 |
| 7.4.2 | Serum S100B levels change over time | 148 |
| 7.4.3 | Serum S100B versus clinical and MRI parameters | 148 |
| 7.5 | Discussion | 149 |

| | | |
|-------------------|---|------------|
| Chapter 8 | Do anti-myelin antibodies allow earlier diagnosis of multiple sclerosis? | 157 |
| 8.1 | Introduction | 157 |
| 8.2 | Aims | 158 |
| 8.3 | Methods | 158 |
| 8.3.1 | Patients | 159 |
| 8.3.2 | Antimyelin antibody analysis | 159 |
| 8.3.3 | Statistical Analysis | 159 |
| 8.4 | Results | 159 |
| 8.5 | Discussion | 161 |
| PART 4 | SUMMARY AND CONCLUSIONS | 168 |
| Chapter 9 | Summary and conclusions | 169 |
| REFERENCES | | 172 |

PART 1

INTRODUCTION AND BACKGROUND

CHAPTER 1

Optic Neuritis, Clinically Isolated Syndrome and Multiple Sclerosis

1.1 Optic Neuritis

1.1.1 Introduction

Optic neuritis (ON) is a relatively common condition - the annual incidence is about 5:100 000 person-years (Rodriguez *et al.*, 1995), and it typically involves the young adult population. It typically affects Caucasians and females more than males. It may develop at any age though its first onset is rare in children and the elderly. The usual age at presentation is between 20 and 40 years. In the vast majority of cases ON is self-limiting with good functional recovery.

1.1.2 Aetiology

The aetiology of ON is unknown. It is considered a complex disorder with genetic susceptibility that interacts with an environmental trigger. Although it is claimed to be immune mediated, with evidence of autoimmunity there is evidence to support infection as an important trigger.

1.1.3 Differential Diagnosis

In the past, ON was thought only to arise in isolation but more recent long-term studies have revealed, that in at least 60% of cases it is an early manifestation of MS. Other conditions that it can be associated with ON are Lyme disease,

systemic lupus erythematosus, Behcet's disease, syphilis, sarcoidosis, toxocariasis, toxoplasmosis and histoplasmosis. Isolated ON over the age of 50 years becomes increasingly difficult to distinguish from ischaemic optic neuropathy (Rizzo and Lessell, 1991).

1.1.4 Clinical Manifestation

Typically, it manifests as an acute loss of vision or impaired vision, which often becomes apparent on waking from sleep. The defect is progressive over a few days to 2 weeks. Unilateral involvement is common and the less common bilateral disease is seen typically in children following a viral infection (Kriss *et al.*, 1988; Morales *et al.*, 2000). Pain on movement of the affected eye is reported in 92% of cases (Foroozan *et al.*, 2002). Mild tenderness around the globe either just before or at onset may be present and this can occasionally be distressingly severe and accompanied by generalized headache. Pain is also significantly more common in retrobulbar neuritis, where there is no abnormality of the optic disc (Lepore, 1991). Painless ON is less likely to be associated with the subsequent development of MS.

Ninety percent of patients with ON complain of visual loss which varies from minor blurring to blindness in the affected eye. Photophobia and bright dots (phosphenes) on eye movement might be present. In 1889, Uthoff described transient blurring of vision following exercise or elevated body temperature. This phenomenon is not uncommon in early MS and often noted during the recovery

phase rather than at onset. The mechanism underlying Uhthoff's is a temperature dependent intermittent conduction block as a result of a reduced safety factor of conduction in the affected nerve. It is a good indicator that the underlying pathology is demyelinating.

Progressive deterioration of visual acuity after 2 weeks is uncharacteristic of ON and should raise doubts about the diagnosis. In addition, improvement usually begins within the first month of onset.

1.1.5 Clinical Findings

On examination, reduced visual fields, contrast sensitivity and colour perception are typically present in the affected eye. The visual field defect is characteristically a central or paracentral scotoma. A relative afferent pupillary defect or Marcus Gunn phenomenon or an afferent pupillary defect is often present and this is due to a lesion in the afferent limb of the pupillary light reflex.

The term retrobulbar ON is diagnosed when the optic disc appears normal on ophthalmoscopic examination because the site of injury is posterior to the optic nerve head. This seems to be the case in about two-thirds of affected eyes (Optic Neuritis Study Group 1991). If the optic disc is swollen, this is referred to as papillitis and indicates that lesion is in the anterior part of the optic nerve. Optic atrophy can occur at a later stage independent of the site of lesion.

1.1.6 Prognosis

Partial to complete remission of sight is almost an invariable feature and occurs in greater than 90% of cases after the initial attack. The initial phase of recovery is rapid, averaging from 3-5 weeks. Improvement in vision is seen up to a year after the acute episode. Of note remyelination can continue for up to 2 years but it is not usually associated with functional improvement of vision.

Although most patients recover well from ON, vision after ON can vary greatly during the day and from one day to the next, and might deteriorate in situations associated with an increase in body temperature such as a hot bath, hot meal and exercise (Uthoff's phenomenon). Pulfrich's phenomenon, which is misperception of the trajectory of moving objects, that is an object moving in 2D appears to move in 3D, is occasionally noted. Persistent subtle impairment of colour vision, contrast sensitivity, depth perception and cortical evoked potential latency changes are nearly always demonstrable.

1.1.7 Treatment

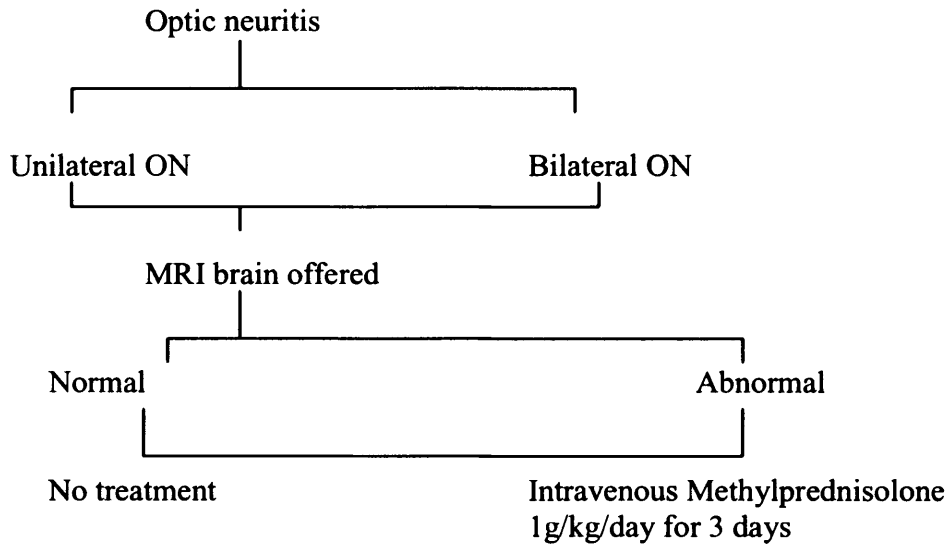
The Optic Neuritis Treatment Trial (ONTT) has documented that ON can recur in 20% of affected eyes, 17% of fellow eyes and in 30% for either eye (Foroozan *et al.*, 2002). The prognosis merely confirms that ON is a common condition and can often recur, and that there should be a high index of suspicion for MS in view of the fact that at least 60% of cases would subsequently develop the condition.

The North American Optic Neuritis Treatment Trial comprised 457 patients randomized to treatment with high-dose intravenous methylprednisolone, oral prednisolone or oral placebo (Beck *et al.*, 1992). It was suggested that high-dose intravenous methylprednisolone increased the rate of recovery but not the ultimate visual outcome. The efficacy of treatment with oral prednisolone did not differ from placebo.

Post-hoc subgroup analyses of the ONTT also suggested that patients who were treated with oral prednisolone alone had a higher rate of recurrent ON, whereas patients treated with methylprednisolone were less prone to develop MS the first 2 years after treatment (Beck *et al.*, 1992; 1993; 1995). These findings have been questioned because assessment of the risks of recurrent ON and of developing MS were not primary aims of the trial (Silberberg, 1993; Goodin, 1993).

In the UK, the Association of British Neurologists' guidelines recommend disease-modifying drugs only in established MS, which is clinically active, i.e. patients should have had at least two significant relapses in the preceding two years. Repeat imaging of patients with CIS could establish earlier diagnosis of multiple sclerosis by using the new McDonald criteria (McDonald *et al.*, 2001). In some patients, this early diagnosis will enable a more informed discussion of diagnosis and prognosis and possibly treatment in the future.

Figure 1.1 Recommended treatment for optic neuritis in adults based on the results of the ON treatment trial (Hickman *et al.*, 2002).



Qualification: The three-year and five-year data from the ONTT however has negated this result (Beck *et al.*, 1992; 1993; 1995).

1.2 Clinically Isolated Syndrome

A clinically isolated syndrome (CIS) can be defined as a single focal or multifocal event of acute onset in the CNS suggestive of demyelination. The presenting attack may be in the optic nerve, brain stem or spinal cord. Although some CIS are self-limited, others are the first clinical manifestation of MS.

1.3 Multiple Sclerosis

An episode of ON increases the risk of developing CDMS, which by definition requires at least two demyelinating events, separated by time and space. MS is the commonest cause of neurological disability in the UK in young adults with a prevalence of 1 per 1000 and shares similar epidemiological profiles with ON. It is a disease of the CNS beginning most often in late adolescence and early adult life and usually expressing itself by discrete and recurrent attacks of optic nerve, spinal cord, brainstem, cerebellar and cerebral dysfunction.

1.3.1 Natural History

At its onset, MS can be clinically categorized as either relapsing-remitting (RR) MS, observed in 85-90% of patients, or PPMS. Relapses or 'attacks' typically present subacutely, with symptoms developing over hours to several days, persisting for several days or weeks, and then gradually abating.

The outcome of patients with RRMS is variable. About 50% of all MS patients who are untreated will require the use of walking aid within 10 years after clinical onset (Weinsehnker, 1994). Clinical predictors of a more rapid deterioration are:

1. Increased attack frequency and poor recovery in the first years of clinical disease
2. Progressive motor onset as opposed to remitting sensory symptoms or ON
3. Older age/male rather than younger age/female
4. Polysymptomatic presentation
5. Multiple MRI lesions, particularly those that gadolinium-enhance¹ on the first MRI scan.

About 40% of patients with RRMS develop a progressive neurodegenerative secondary disorder related to the chronic CNS inflammation, known as secondary progressive (SP) MS. There are fewer gadolinium-enhanced lesions and a decrease in brain parenchymal volume with SPMS (Khoury *et al.*, 1994). Furthermore responsiveness to immunotherapy at this stage decreases with time.

The primary progressive form of MS is characterized from the onset by the absence of acute attacks and instead a gradual clinical decline. This disease is associated with a lack of response to any form of immunotherapy (Hohol *et al.*, 1999).

1.3.2 Aetiology

The cause of MS, like ON, is unknown. In the 19th century, Charcot described the disease as *sclerose en plaques*. The plaques are damaged oligodendrocyte myelin

¹ Gadolinium-enhancement is consistent with acute flux of activated, autoreactive T cells into the CNS causing a blood brain barrier breakdown which may be associated with clinical events.

complexes. Typically, multiple plaques are seen on conventional MRI and biopsy samples, and clinically what determines whether or not a lesion causes symptoms is the size and location of the lesion. For example brainstem lesions are more likely to cause symptoms than lesions in the periventricular white matter of the cerebral hemispheres. In Chapter 2, the current consensus that MS is an immune-mediated condition is discussed further.

1.3.3 Diagnosis

The criteria for diagnosing MS have been standardised. Generally there should be at least two neurological events. However, the application of serial MRI findings allows one to make the diagnosis after one clinical event, for example after an episode of ON, provided one still satisfies criteria for dissemination of disease in time and space (Table 1.2). The 2 types of diagnostic criteria incorporated in this thesis are those designed by the Poser committee (Poser *et al.*, 1983) and McDonald (McDonald *et al.*, 2001) and international panels.

The Poser criteria, published in 1983 were standardised for epidemiological studies and therapeutic trials have been widely incorporated in clinical practice. These criteria were designed to incorporate CSF analysis² and results of evoked potentials (Poser *et al.*, 1983) (Table 1.3).

² Cerebrospinal fluid examination is of prognostic value. About 95% of patients with CDMS have locally synthesized oligoclonal IgG resulting from clonal production of IgG by activated B-lymphocytes and plasma cells within the central nervous system (Figure 1.4).

The recently proposed McDonald criterion is a modification of these criteria and incorporates MRI findings (McDonald *et al.*, 2001). MRI changes have become increasingly important as a prognostic tool in patients presenting with clinically isolated syndromes such as ON. MRI studies may show swelling and high signal lesion in the involved optic nerve (Figure 1.2). Approximately 50% of patients with 3 or more lesions in the cerebral white matter on MRI will go onto develop CDMS (Figure 1.3). In contrast 16% of those with ON and normal MRI scan will develop CDMS.

Table 1.1 Recent studies looking at subsequent risk of development of multiple sclerosis in patients presenting with CIS

| Study | Results | Comments |
|-----------------------------------|---|---|
| Dalton <i>et al.</i> 2002 | 21% patients had MS at 3 months, 48% at 1 year, 58% at 3 years | Using new McDonald criteria to patients with clinically isolated syndrome |
| Tintore <i>et al.</i> 2003 | 37% had MS at 1 year, 80% who fulfilled new McDonald criteria had second clinical attack in mean follow-up 49 months. | Studied a total of 139 patients with clinically isolated syndrome, and used new McDonald criteria |
| Brex <i>et al.</i> 2002 | 88% with abnormal MRI at onset had MS after a mean of 14.1 years. | The increase in MRI lesion load over 5 years correlated with long term disability |
| Frederiksen <i>et al.</i> 1992 | 29% had MS at mean follow-up of 27 months. | All the patients who developed MS had lesions on MRI at onset |

Table 1.2 New MS Diagnostic Criteria

| <i>CLINICAL ATTACKS</i> | <i>OBJECTIVE LESIONS</i> | <i>ADDITIONAL REQUIREMENTS TO MAKE DIAGNOSIS</i> |
|--|-------------------------------------|--|
| <i>2 or more</i> | 2 or more | None; clinical evidence will suffice (additional evidence desirable but must be consistent with MS) |
| <i>2 or more</i> | 1 | Dissemination in <u>space</u> by MRI or positive CSF and 2 or more MRI lesions consistent with MS or further clinical attack involving different site |
| <i>1</i> | 2 or more | Dissemination in <u>time</u> by MRI or second clinical attack |
| <i>1 monosymptomatic</i> | 1 | Dissemination in <u>space</u> by MRI or positive CSF and 2 or more MRI lesions consistent with MS AND Dissemination in <u>time</u> by MRI or second clinical attack Positive CSF AND Dissemination in <u>space</u> by MRI evidence of 9 or more T2 brain lesions or 2 or more cord lesions or 4-8 brain and 1 cord lesion |
| <i>0 progression from onset</i> | <i>1</i> | or positive VEP with 4-8 MRI lesions or positive VEP with less than 4 brain lesions plus 1 cord lesion AND Dissemination in <u>time</u> by MRI or continued progression for 1 year |

Table 1.3 The Poser criteria for MS

| Category | Attacks | Clinical Evidence | | Paraclinical Evidence | CSF |
|--|---------|----------------------|-----|--------------------------|-----|
| A Clinically Definite (CD) | | | | | |
| CD MS A1 | 2 | 2 | | | |
| CD MS A2 | 2 | 1 | and | 1 | |
| B Laboratory-supported Definite (LSD) | | | | | |
| LSD MS B1 | 2 | 1 | or | 1 | OCB |
| LSD MS B2 | 1 | 2 | | | OCB |
| LSD MS B3 | 1 | 1 | and | 1 | OCB |
| C Clinically Probable (CP) | | | | | |
| CP MS C1 | 2 | 1 | | | |
| CP MS C2 | 1 | 2 | | | |
| CP MS C3 | 1 | 1 | and | 1 | |
| D Laboratory-supported Probable (LSP) | | | | | |
| LPMS D1 | 2 | | | | OCB |

OCB = local synthesis oligoclonal band

Figure 1.2 Acute right optic neuritis showing high signal lesion in the optic nerve on fast spin echo (Courtesy of Dr Simon Hickmann, NMR Unit, IoN).

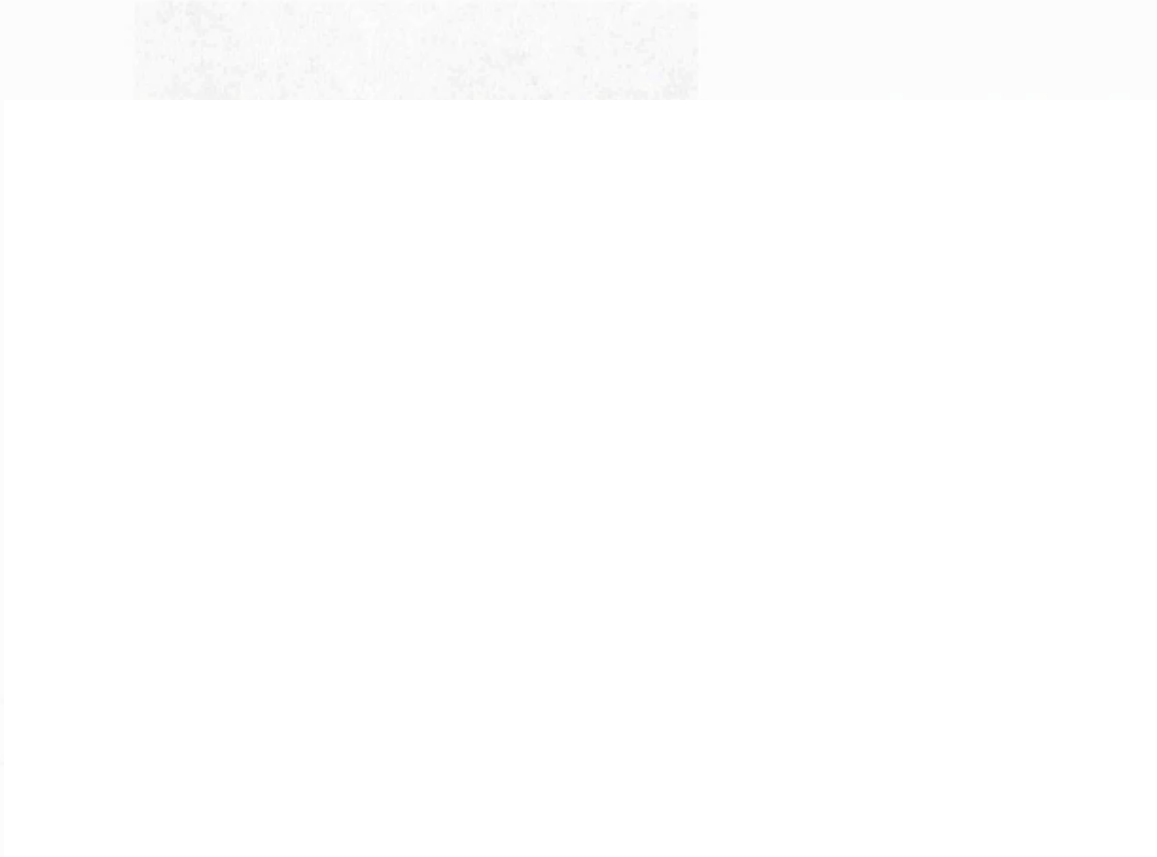


Figure 1.3 Abnormal T2 weighted MRI image of patient with multiple sclerosis

Arrows indicate plaques (Courtesy of Dr Gerard Davies, NMR Unit, IoN).



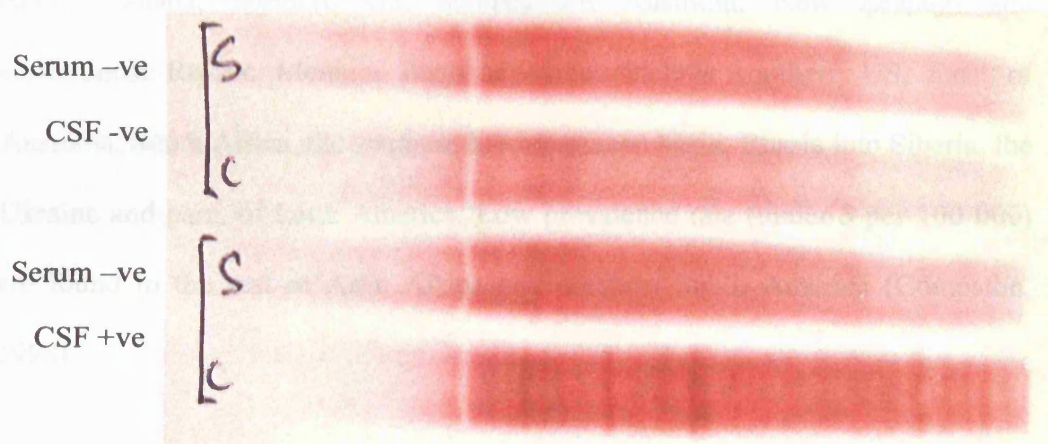
Denial -ve

CSP -ve

Parane -ve

CSP +ve

Figure 1.4 Isoelectric focusing to reveal oligoclonal IgG production in CSF. The upper two lanes are paired serum and CSF samples from a patient without MS. They show a normal or polyclonal pattern. In the lower pair there are numerous IgG bands visible in the CSF lane that are not present in the serum sample. This type of distribution indicates the intrathecal synthesis of oligoclonal IgG bands that is observed to occur in greater than 95% of patients with multiple sclerosis.



1.3.4 Epidemiology

MS is thought to originate in Europe where it remains most frequent. Kurtzke (1993b) suggested that MS originated in Scandinavia in the 18th century and that the disease spread across the Baltic States and Northern Europe including the British Isles over the next century. At a later stage, the disease was thought to spread to North America, Australasia, South Africa and Italy.

The hypothesis that certain racial groups are susceptible to MS is based on the epidemiology of MS. Geographically MS describes 3 frequency zones. High frequency areas (prevalence 30+ per 100 000) now comprise most of Europe, Israel, Canada, northern US, southeastern Australia, New Zealand and easternmost Russia. Medium frequency areas include southern US, most of Australia, South Africa, the southern Mediterranean basin, Russia into Siberia, the Ukraine and parts of Latin America. Low prevalence rate (under 5 per 100 000) are found in the rest of Asia, Africa and northern South America (Compston, 1998).

Migrants from high to low risk areas retain the MS risk of their birthplace if at least age 15 years at migration. Those migrating from low to high-risk areas increase their risk beyond that of their native country. Swingler and Compston (1986) correlated the distribution of MS in the UK with regional variations in population gene frequency and concluded that a frequency gradient exists which correlates with a genetic cline marked by HLA-DR2 susceptibility allele.

1.3.5 Genetics

Familial incidence is low but several times higher than chance expectancy. The risk of the disease in the siblings of affected individuals is 3-5%. The importance of genetic factors in accounting for this increased risk is confirmed by the results of twin and adoption studies (Mumford *et al.*, 1992; Robertson *et al.*, 1997).

Increased susceptibility to MS is genetically linked with the immune molecule, MHC class II (Sawcer *et al.*, 1996; Haines *et al.*, 1996; Ebers *et al.*, 1996). The Genetic Analysis of Multiple Sclerosis in Europeans (GAMES) (Sawcer and Compston, 2003) suggests that there is a polygenic mode of inheritance. The histocompatibility (HLA) antigens found more frequently in the MS population are HLA-DR2, -DR3, -B7 and -A3. The genes that confer susceptibility to MS are the MHC encoded on chromosomes 6q21 and 17q22 (Sawcer and Compston 2003; Sawcer *et al.*, 1996).

Other candidates have been proposed, including genes encoding the immunoglobulin heavy chain, T cell receptor beta chain and APOC2, but none has been confirmed (Sawcer *et al.*, 1996). Evidence for linkage and association to the myelin basic protein gene has been reported in a genetically isolated Finnish population (Pihlaja *et al.*, 2003).

In summary, what has become clear is that MS represents a complex genetic disease with no clear mode of inheritance.

1.3.6 Genes or Germs?

It may be postulated that as populations emerged out of Africa 30,000 to 50,000 years ago, exposure to new microbes resulted in population of individuals with combinations of different genes. The latter provided resistance to novel infectious agents, and when populations came randomly together, this resulted in a hyper-responsive immune system, with subsequent autoimmune disease. MS therefore may be a price an individual pays for protection of the general population. This is analogous to sickle cell and thalassaemia traits (Compston *et al.*, 1998).

1.3.7 Clinical Manifestation

The first attack of this demyelinating disease usually occurs without warning and may be mono- or poly-symptomatic. The commonest presenting symptoms are monocular visual loss, diplopia, vertigo, facial weakness or numbness, ataxia, nystagmus and weakness or numbness of a limb. Remission after the first attack is to be expected and recurrence represents recrudescence of earlier lesions or the effects of new ones.

As previously mentioned, the most common clinical subtype is RRMS, and over a variable period, the patient becomes increasingly handicapped, and the late stage of the disease may be established in 20 to 25 years³. There is no way to predict which patients with MS will remain benign over many years. Axonal transection seems to begin at disease onset and may be clinically silent during the relapsing-

³ Approximately 90% of RRMS patients (Weinshenker *et al.*, 1989)

remitting stage of the disease. Once a threshold for axonal transection is exceeded, MS patients enter an irreversible SPMS stage.

MRI and clinical studies have suggested that SPMS is deterioration due to degenerative rather than inflammatory disease (Confavreux *et al.*, 2000; 2003; Brex *et al.*, 2002; Trojano *et al.*, 2002). MRI studies have shown that while measure of the active inflammatory components of the disease such as contrast-enhancing lesions and T2 lesions correlate with disease course over the early phase of the disease, the correlation during the progressive phase is poor (Brex *et al.*, 2002). Clinical studies have indicated that the extent of inflammatory demyelination and tissue damage may influence the time to an EDSS score of 4 (see Table 1.4), neurodegenerative mechanisms may likely contribute to neuronal decline at more severe stages of the disease (Confavreux *et al.*, 2000; 2003).

In terms of clinical outcome measures, this thesis has used the changes in the Kurtzke Expanded Disability Status Scale (EDSS) (Table 1.4) (Kurtzke, 1983) which is user-friendly (Kurtzke, 1955), reproducible (Kurtzke, 1955), and applicable to all patients (Kurtzke and Berlin, 1954; Kurtzke, 1961). It is widely believed that the EDSS is an impairment scale in its lower range and a disability scale in its upper range (Sharrack and Hughes, 1996). Nonetheless, it is not perfect and the well-recognized disadvantages of EDSS are the poor assessment of cognition, upper limb function and quality of life (Hobart *et al.*, 2000). In addition, previous reliability studies of EDSS have generated variable results.

This inconsistent reliability may reflect the different number of raters used, their level of training or expertise, or the sample size. There is no absolute guideline for this variable and it has been recommended that raters of Kurtzke scales should be trained (Lechner-Scott *et al.*, 1997).

Due to criticism of the EDSS, in 1994, the MS functional score (MSFC) (Cutter *et al.*, 1999) was developed and includes quantitative tests of leg function, arm function and cognitive function. However, the study by Ozakbas *et al.* (2004) comparing the 2 clinical scales concluded that EDSS is still more useful for daily routine practise because the EDSS is more sensitive in identifying changes in functions, and that MSFC is a time consuming process.

Recently, Roxburgh *et al.* (2005) have devised an algorithm; the Multiple Sclerosis Severity Score (MSSS), which corrects EDSS for duration to compare an individual's disability with the distribution of scores in cases having equivalent disease duration. This method has been shown to be more powerful than other tests for detecting different rates of disease progression.

Table 1.4 Kurtzke's Expanded Disability Status Scale (EDSS)

| | | |
|------|---|---|
| 0 | = | Normal neurological examination (all FS normal, mild cerebral signs acceptable) |
| 1.0 | = | No disability, minimal signs in one FS |
| 1.5 | = | No disability, minimal signs in more than one FS |
| 2.0 | = | Minimal disability in one FS |
| 2.5 | = | Minimal disability in two FS |
| 3.0 | = | Moderate disability in one or mild disability in up to 4 FS though fully ambulatory |
| 3.5 | = | Fully ambulatory but with a moderate disability in one FS or 2 others; or moderate disability in 2 FS; or mild disability in 5 FS |
| 4.0 | = | Fully ambulatory without aid; self-sufficient; up and about 12 hours a day despite relatively severe disability in one FS or combination of exceeding the limits of the previous step. Able to walk without aid or rest for 500m. |
| 4.5 | = | Fully ambulatory without aid; up and about much of the day; may otherwise have some limitation of full activity or require minimal assistance. Able to walk without aid or rest for 300m. |
| 5.0 | = | Ambulatory without aid or rest for 200m; disability severe enough to impair full daily activities. |
| 5.5 | = | Ambulatory without aid or rest for 100m; disability severe enough to preclude full daily activities. |
| 6.0 | = | Intermittent or unilateral constant assistance required to walk 100m. |
| 6.5 | = | Constant bilateral assistance to walk 20m without rest. |
| 7.0 | = | Unable to walk 5m even with aid. Essentially restricted to a wheelchair. Transfers alone. |
| 7.5 | = | Unable to walk more than a few steps. May need aid with transfers. |
| 8.0 | = | Restricted to bed or perambulated in wheelchair. Generally has effective use of arms. Out of bed for much of the day. |
| 8.5 | = | Essentially restricted to bed for much of the day. Retain some self-care functions. |
| 9.0 | = | Helpless bed patient; can communicate and eat. |
| 9.5 | = | Totally helpless, unable to communicate effectively or eat/swallow. |
| 10.0 | = | Death due to MS. |

1.3.8 Investigations

1.3.8.1 MRI

MRI is a sensitive imaging technique. Brain MRI (T2 weighted) in MS typically shows periventricular high signal abnormalities; lesions may enhance using gadolinium-DTPA. Areas that are preferentially affected areas include the paraventricular, deep and subcortical white matter of the cerebral hemispheres, the optic nerve and anterior visual pathways, corpus callosum, brainstem, cerebellum and the spinal cord. The white matter lesions are most apparent using the FLAIR (Fast Fluid-Attenuated Inversion Recovery) sequence which increases the sensitivity to 98%.

Clinically silent lesions are common. Asymptomatic cord lesions have been identified in one-third of patients with clinically isolated optic neuritis (O’Riordan *et al.*, 1998a). A ten-year follow-up study has shown a strong correlation between changes in T2 lesion number-load and EDSS in the first five years, but there was a weaker correlation in the second (O’Riordan *et al.*, 1998b). Lesions that are active, i.e. with acute inflammation, tend to enhance with gadolinium (Grossman *et al.*, 1986; Katz *et al.*, 1993; Nesbit *et al.*, 1991; Bruck *et al.*, 1997; Thompson *et al.*, 1991; 1992).

1.3.8.2 CSF analysis

In about 80% of established cases, the CSF is abnormal. There may be a mild mononuclear pleocytosis and a modest increase in total protein, but the gamma globulin fraction is often increased to greater than 12% of the total protein. Over 95% of patients with CDMS have local synthesis of oligoclonal IgG in the CSF, detected by isoelectric focusing (IEF)⁴.

Other central nervous system disorders associated with OCB are human immunodeficiency virus encephalitis, Lyme disease, systemic lupus erythematosus, neuroborreliosis, toxoplasmosis, sarcoidosis, vasculitis, lymphoma, paraneoplastic syndrome, and less frequently Sjogren syndrome, subacute sclerosing panencephalitis.

1.3.8.3 Neurophysiological studies

Lesions that are not clinically manifest may be revealed by visual, auditory and somatosensory evoked potential studies. However they are now rarely needed for diagnosis, except in patients presenting with primary progressive MS.

1.3.10 Treatment

1.3.9.1 Disease modifying therapy

⁴ Oligoclonal bands are IgG resulting from clonal production by activated lymphocytes within the CNS. This is not specific for MS and occurs in other inflammatory, infective and demyelinating disorders.

Three classes of immunomodulating therapies that impact the course of early MS have emerged over the last 2 decades: IFN- β ; glatiramer acetate which is a MHC-binding peptides that engages the T-cell receptor; and the immunosuppressant compound Mitoxantrone.

Two forms of recombinant IFN- β , -1a (Avonex, Rebif) and -1b (Betaferon), are licensed in Europe for the treatment of patients with RRMS. Their efficacy has been proven in several multicentre, controlled trials (The IFNB MS Study Group, 1993; Paty and Li, 1993; Jacobs *et al.*, 1996; PRISMS Study Group, 1998). IFN- β -1b has also been licensed for the treatment of SPMS. The latter is on the basis of a single trial by the European Study Group (1998), which demonstrated a small benefit on progressive disability in this subgroup of patients.

Recombinant beta interferons are produced in different cell systems, which result in some differences in their aminoacid structure, molecular weight, degree of glycosylation, and specific activity (tested against the antiviral activity of the WHO reference standard of human interferon beta) (Revel and Kalinka, 1997). Interferon beta has been shown to reduce relapse frequency and severity by approximately a third (Jacobs *et al.*, 1996; PRISMS, 1998).

The specific mode of action of IFN- β in MS is unknown. IFNs were initially tried in MS for their antiviral effect⁵ (reviewed in Jacobs and Johnson, 1994) and IFN- β turned out to have beneficial effect. Other properties of IFN- β are anti-proliferative actions, although its immunomodulatory effects such as reduced transcription of MHC class II molecules are considered more important (Arnason and Reder, 1996; Yong *et al.*, 1998).

IFN- β is produced by all mammalian cells upon stimulation and is encoded by single genes. A myriad of effects of IFN- β have been described but the relative importance of the variety of actions are not well understood, therefore at present time, the mechanisms of IFN- β treatment remain unknown.

1.3.9.2 Symptomatic Treatment

Three day or five day courses of intravenous methylprednisolone have been shown to speed the recovery of clinical relapses in MS (Milligan *et al.*, 1987). Oral high dose methylprednisolone is also used and has been shown to be as effective as intravenous methylprednisolone when given in a three-week tapering dose.

Relapses are likely to be caused by the traffic of activated, myelin-reactive T cells into the CNS, causing acute inflammation with associated oedema. The ability of high dose steroids to improve the symptoms of an MS relapse suggests that the

⁵ IFNs trigger the synthesis of many host cell protein that contribute to the inhibition of viral replication.



acute oedema and its subsequent resolution may partly underlie the clinical relapse and remission respectively.

Chapter 2

Immunology in Clinically Isolated Syndrome and Multiple Sclerosis

2.1 Historical perspective

In 1868, Charcot noted the accumulation of inflammatory cells in a perivascular distribution within the brain and spinal cord white matter of patients with intermittent episodes of neurological dysfunction (Charcot, 1877). This led to the term *sclérose en plaques disseminées*, or MS.

In 1933, Thomas Rivers demonstrated an autoimmune, demyelinating disease in mammals with immunization of the CNS myelin (experimental autoimmune encephalomyelitis, EAE) (Rivers *et al.*, 1933).

In 1948, Elvin Kabat observed an increase in oligoclonal immunoglobulin in the CSF of patients with MS that provided further evidence of an inflammatory nature of the disease.

Between 1950-2004, at least 30,000 scientific papers on MS have been published.

2.2 Immunology

The hallmark of MS is immune-mediated damage to the oligodendrocyte myelin complex in multiple CNS areas, producing plaques, which are often perivascular. Although a search for a specific virus or antibody in MS has been at best inconclusive. Some assume that a viral infection underlies the cause of MS either

as the primary cause or possibly as the trigger that establishes the pathogenic cascade in MS⁶.

Raised serum anti-viral antibodies and viral RNA and DNA have been detected in MS patients include measles, varicella, mumps, Epstein-Barr virus, MS-associated retrovirus, human herpesvirus 6 etc. (Adams and Imagawa, 1962; Ter Meulen and Stephenson, 1983; Wandinger *et al.*, 2000; Dolei *et al.*, 2002; Challoner *et al.*, 1995).

The serum in MS shows little change in the total level of immunoglobulins, but in most cases, circulating immune complexes can be found (Goust *et al.*, 1978). There is, as yet, no evidence that these can cause demyelination, and demyelination is not a feature of circulating immune complex diseases (Lebowitz, 1983).

2.2.1 Inflammation

Potentially auto-aggressive T-lymphocytes normally occur in the circulation and they are usually controlled by peripheral clonal inactivation (anergy) and suppression. However, in susceptible individuals, an unknown environmental trigger such as viruses can activate these cells. Upon activation, T-lymphocytes express adhesion molecules and attach to endothelial cells.

⁶ It could be further argued that the disease is caused by an unknown virus or is an abnormal reaction to an unidentified virus.

Both serum and CSF levels of soluble vascular cell adhesion molecule (VCAM) are significantly raised in MS patients during an acute exacerbation compared to patients with chronic progressive MS (Matsuda *et al.*, 1995). On reencountering their autoantigen in the CNS these cells are reactivated and initiate a typical cascade of cell-mediated inflammation.

The perivascular inflammatory infiltrates in MS are mainly composed of T-lymphocytes, some B cells and plasma cells and activated macrophages or microglia cells. The active lesions are characterised by perivascular infiltration of oligoclonal T cells, monocytes with occasional B cells and infrequent plasma cells (Prineas and Wright, 1978).

2.2.2 Demyelination

Active demyelination in MS is accompanied by inflammation. Although it is generally believed that inflammation is an obligatory and possible primary feature of demyelination in MS, it has to be emphasised that in exceptional cases – in particular, after profound immunosuppressive therapy, myelin destruction may proceed in spite of a near lack of lymphocytic infiltration. The latter observations implicate a role for endogenous glia (microglia, astrocytes) as sources of mediators.

2.2.3 Axonal Damage

Demyelination with relative preservation of axons and astroglial scarring are the pathological hallmark of MS lesions. Acute axonal damage is found mainly in

lesions with recent or ongoing demyelinating activity. This secondary injury to the axon may be the result of either (1) T cell cytotoxicity, or (2) the failure of local target-derived neurotrophic support from death of myelinating oligodendrocytes.

Axonal loss can apparently precede the onset of substantial demyelination. Axonal loss continues within the active margin of older lesions, but it appears not to be a feature of inactive chronic lesions. The mechanism by which T cells and macrophages transect axons within the acute or active chronic lesion remains unclear. Instead, acute inflammatory mediators, such as proteases, free radicals, cytokines and complement, are more likely to have an effect on axonal integrity. Wallerian degeneration of the distal portion of the axon results in axonal breakdown in about 18 hours, and these events are most likely triggered in the acute lesion (Kapoor *et al.*, 1999; Coleman *et al.*, 1998).

2.2.4 Remyelination

In brain lesions obtained during the early course of the disease, numerous oligodendrocytes may be present, and such lesions may exhibit rapid and extensive remyelination. Such lesions, 'shadow plaques', represent the idea of partial repair of an MS lesion. In some lesions, in particular at site of active myelin destruction, oligodendrocyte loss may be minor and in others, demyelination may be associated with extensive (some suggest up to 70%) or

complete disappearance of these cells (Wolswijk, 1998). This may be due to lack of appropriate trophic factors.

Remyelination is probably more effective in the early stages compared to late stages, and frequent episodes of demyelination in remyelinated areas may lead to progressive destruction of mature oligodendrocytes as well as progenitor cells.

The mechanism by which the inflammatory process is subsequently down regulated is unclear. Oligodendrocytes are depleted by a cytolytic mechanism (Raine *et al.*, 1998) and apoptosis may be a contributing factor.

2.3 Histopathology

Gross examination of brain tissue of individuals with MS reveals multiple sharply demarcated plaques in the CNS white and grey matter with a predilection to the optic nerves and white matter tracts of the periventricular regions, brain stem and spinal cord. Substantial axonal injury with axonal transections is abundant throughout active MS lesions (Trapp *et al.*, 1998).

Approximately 2% of chronic lesions show evidence of acute myelin destruction – inflammatory cell infiltrates and fresh myelin debris within macrophages, as well as progressive loss of oligodendrocytes possibly through lytic or apoptotic mechanisms and marked axonal pathology. In chronic inactive lesions, low grade

axonal degeneration persists (Trapp *et al.*, 1998) and remyelination is often present at the lesion margin.

On a cellular level, lymphocytes may be found in normal appearing white matter beyond the margin of active demyelination. Macrophages are most prominent in the centre of the plaques and are seen to contain myelin and debris. In chronic active lesions, the inflammatory cell infiltrate is less prominent and are largely restricted to the rim of the plaque. Many chronic plaques become fully burnt-out and further immuno-inflammatory response is probably prevented by marked gliosis⁷.

⁷ Astrocytes proliferate within the chronic lesions and become fibrillary (GFAP), laying down astrocytic fibres in parallel packed rows known as isomorphic gliosis.

Chapter 3

Biological Markers in Multiple Sclerosis

According to the NIH Biomarker Definitions Working Group (1998), a biological marker (biomarker) is defined as ‘ *a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.* ’ Surrogate endpoint is a biomarker that is expected to predict clinical benefit/harm, or lack of benefit/harm, based on epidemiologic, therapeutic, pathophysiologic or other scientific evidence. Such a marker has not yet been met with respect to MS.

Currently the most useful secondary outcome measures in clinical studies available in MS are MRI based, encompassing a number of techniques that are sensitive for, and can measure, different components of MS pathology and to detect clinically silent lesions. The finding of intrathecal oligoclonal immunoglobulin bands is useful in clinical practice but the antigen specificity of these oligoclonal bands in MS continues to elude investigators.

Thus there is still a need to develop well-validated, reliable, responsive and acceptable biomarkers so that the management of patients with MS can be improved. This thesis evaluates possible immunological protein markers found in body fluid that may aid in predicting and monitoring neurological decline and thus allow the identification of better biomarkers to be explored.

3.1 MRI

MRI is sensitive for detecting MS lesions and correlates well with clinical relapse (Smith *et al.*, 1993; Thompson *et al.*, 1992; Wiebe *et al.*, 1992) but only poorly with long-term clinical disability as measured by the Kurtzke's EDSS (Kurtzke, 1983) and limited ability to predict the eventual course of MS (Filippi *et al.*, 1995).

A number of new MRI techniques have been developed to examine brain tissue known to be diseased, but in which T2 lesions have not been detected. They include measuring brain volume, MR spectroscopy, MTR imaging and myelin water imaging.

Table 3.1 MRI outcome measures in MS

| |
|--|
| Markers of inflammation |
| New lesion activity (e.g. new T2 lesions, Gd-enhancement) |
| Burden of disease (e.g. T2 lesion volume) |
| <u>Markers of degeneration</u> |
| Atrophy (brain and spinal cord) |
| N-acetyl aspartate (lesions and normal appearing brain tissue) |
| Black holes on T1 (tissue destruction) |
| Magnetisation transfer imaging (lesions and normal appearing brain tissue) |
| Myelin water imaging |

3.2 CSF Oligoclonal Bands

The CSF in MS shows an increase in total immunoglobulins, a κ/λ pattern different from that in plasma, and a number of oligoclonal bands. These oligoclonal bands indicate clones of antibodies which may be directed against specific but unidentified antigens, and have been demonstrated in 95% of MS cases by isoelectric focusing (McClean *et al.*, 1990). They are not specific to MS and are found in other CNS inflammatory and infectious disorders that stimulate antibody production in the CNS (Thompson *et al.*, 1979; Salmi *et al.*, 1983).

3.3 Brain specific proteins

In this thesis, five different types of BSP are studied. Ferritin, a marker of microglial activation; S100B and glial fibrillary acidic protein (GFAP), markers of astrocytic activation and gliosis; and two neurofilament heavy chain phosphoforms (NfH^{SMI34} and NfH^{SMI35}), markers of axonal damage or loss. Anti-MBP and anti-myelin oligodendrocyte glycoprotein (-MOG) are also investigated as potential surrogate endpoints.

Table 3.2 Characteristics of the brain specific proteins

| Brain Specific Proteins | Localization | Functions |
|---|---------------------------------|--|
| Ferritin | Oligodendrocytes (primarily) | Iron binding, growth, ? protect against oxidative insults |
| Glial fibrillary acidic protein | Astrocytes | Structural |
| Neurofilament | Axons | Structural |
| S100B | Astrocytes | ? Trophic, calcium binding |
| Myelin basic protein | Myelin | Structural |
| Myelin oligodendroctye glycoprotein | Myelin | Structural |

3.3.1 Neurofilaments

Neurofilaments, to date, are one of the more promising axonal markers. They are important structural proteins of the neuron and are particularly concentrated in large myelinated axons. However they are, only to a minor extent, expressed in neural cell bodies. Neurofilaments are soluble 10nm diameter triplet proteins, which appear filamentous on electron microscopy and are found in the cytoplasm of neurons but not of other cells in the nervous system.

Neurofilaments have an α -helical rod domain, involved in the formation of oligomers, a N-terminal head domain, involved in the regulation of its assembly, and a C-terminal tail domain which is the main site for phosphorylation. The latter plays a role in protein-protein and protein-organelle interaction (Ching and Liem, 1998). The degree of phosphorylation also determines the diameter of an axon and the rate of neurofilament transport (Ackerley *et al.*, 2003), i.e. the higher the phosphorylation, the broader the diameter (Fuchs and Cleveland, 1998) and the slower the transport rates⁸ (Ackerley *et al.*, 2000; Sanchez *et al.*, 2000; Yabe *et al.*, 2001).

Neurofilaments are composed of three major polypeptides subunits known as: neurofilament light chain (NfL) (molecular weights: 62kDa), NfM or intermediate chain (molecular weight: 102kDa) and heavy chain (NfH) (molecular weight: 200kDa) (Fuchs and Cleveland, 1998). The length of the C-

⁸ Neurofilaments are transported through axons by a process termed slow axonal transport (Ackerley *et al.*, 2003).

terminal tail determines the differences in molecular mass, and they are so-called for their low, middle and high positions on sodium dodecyl sulfate (SDS) gels.

NfL corrects the assembly of neurofilaments and maintains axonal calibre. NfM forms cross-bridges, stabilizes filament networks and helps in longitudinal extension. NfH forms cross bridges and interact with microtubules and other cytoskeletal elements (Al-Chalabi and Miller, 2003). In recent years it has been realized that neurofilaments may also contain the proteins alpha-internexin, peripherin, nestin and vimentin. There are also several interesting neurofilament associated proteins such as plectin and dystonin which appear to integrate neurofilaments into a functioning network with microtubules and microfilaments.

This thesis concentrates on neurofilament heavy chains, in particular, two heavily phosphorylated phosphoforms: NfH^{SMI34} and NfH^{SMI35}. The latter is less phosphorylated than NfH^{SMI34} (Table 3.4) (Goldstein *et al.*, 1987a, 1987b; Sternberger and Sternberger, 1983). NfH is encoded on chromosome 22q12.2 and consists of 1020 amino acids (Lees *et al.*, 1988). Up to nine NfH phosphoforms has been identified to date, and they differ according to the degree of phosphorylation⁹ (Table 3.4). So far however it has been not been possible to develop an ELISA that would allow quantification of all NfH phosphoforms.

⁹ NfH is resistant to protease activity whereas NfL is not (Goldstein *et al.*, 1987; Schlaepfer *et al.*, 1985; Wang *et al.*, 1992)

Table 3.4 illustrates the nomenclature that has been proposed by Sternberger and Sternberger (1983). It is based on the capture antibody, and is to allow differentiation of NfH phosphoforms with immunological methods.

Increased CSF levels of the neurofilament have been reported in neurological disorders such as Alzheimer's disease, amyotrophic lateral sclerosis (Lou Gehrig's disease), giant axon neuropathies, infections and autoimmune CNS disease including MS (Rosengren *et al.*, 1999; Holmberg *et al.*, 1996). It has been speculated that the aberrant transport, processing or expression of neurofilaments could contribute to neurological disease. Recently point mutations in some of the neurofilament proteins have been shown to be causative of certain neurological disorders, the best example being certain types of Charcot-Marie-Tooth disease (Rao and Nixon, 2003; Al-Chalabi and Miller, 2003)

Axonal loss correlates with permanent neurological disability in chronic relapsing experimental allergic encephalomyelitis (CREAE) (Wujek *et al.*, 2002). NfH^{SMI35} levels were significantly lower in CREAE when compared with control animals (Petzold *et al.*, 2003). Two other groups have confirmed this and have observed axonal loss in these animals using immunocytochemical techniques (Wujek *et al.*, 2002; Baker *et al.*, 1990].

In relation to demyelinating diseases, several studies have indicated that patients with MS have a high concentration of NfL in CSF (Lycke *et al.*, 1998; Semra *et*

al., 2002; Haghighi *et al.*, 2004; Malmestrom *et al.*, 2004; Norgren *et al.*, 2003).

The increased CSF NfL chain was found in patients with RRMS and correlated with disability, exacerbation rate and length of time from the start of the previous exacerbations (Lycke *et al.*, 1996). Malmestrom *et al.* (2003) also supported the latter findings, indicating continuous axonal damage during the entire course of the demyelinating disease with the most profound damage during acute relapses (Table 3.3). The highest levels of NfL chain appeared approximately 3 weeks after relapse onset (Malmestrom *et al.*, 2003).

In 2002, two separate studies by Silber *et al.* and Da Silva and Ditto (2002) reported the presence of antibodies against NF proteins in CSF (2002). High levels of intrathecal anti-NfL autoantibodies were detected in progressive MS patients compared to RRMS patients, healthy controls, and those with other neurological disorders (Silber *et al.*, 2002). The elevated levels of antibodies to NfL may be a consequence of ongoing axonal damage, which is further supported by a study that in patients with MS, CSF concentrations of NfL correlate with clinical disability and recent relapses (Lycke *et al.*, 1998). In contrast, antibodies to NfH did not differ between patients with MS and normal controls (Stefansson *et al.*, 1985), and correlated less well with disease duration and disability.

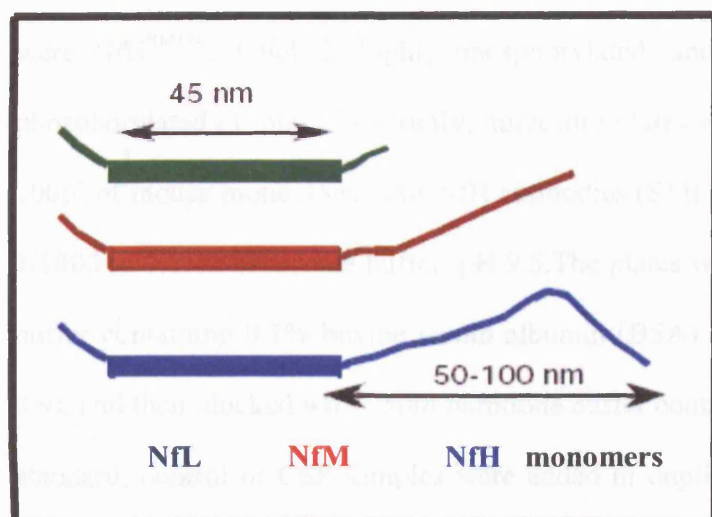
MR investigations have provided evidence for axonal damage in early MS (De Stefano *et al.*, 2002). Indeed, axonal loss may be occurring at the time of clinical onset (Fillipi *et al.*, 2003). The cause of axonal loss in MS is still poorly

understood. The NfL autoantibody index¹⁰ was investigated in a group of 51 MS patients consisting of 21 RRMS, 20 SPMS and 10 PPMS patients, and was found to correlate positively with measures of cerebral atrophy as expressed by parenchymal and ventricular fraction, while the NfH autoantibody index correlated positively only with the ventricular fraction (Eikelenboom *et al.*, 2003). Early axonal damage contributes to cerebral atrophy and clinical disease progression and provides further rationale for early intervention with immunomodulatory MS therapy (Rudick, 1999).

Thus far, neurofilament light chains and autoantibodies against neurofilaments light and heavy chains have been studied in relation to multiple sclerosis. This thesis explores other phosphoforms, namely neurofilament heavy chain (NfH^{SMI35} and NfH^{SMI34}) as potentially useful markers in demyelinating diseases.

¹⁰ NfL antibody index is defined as the CSF to serum ratio of NF antibodies divided by the CSF to serum ratio of albumin.

Figure 3.1 Schematic diagram of neurofilament monomers



3.3.1.1 ELISA method to detect neurofilament heavy chain phosphoforms

A specific ELISA for measuring neurofilament heavy chain phosphoforms was developed by Petzold *et al.* (2003). The 2 subtypes of phosphoforms measured were NfH^{SMI34}, which is highly phosphorylated, and NfH^{SMI35}, which is less phosphorylated (Table 3.4). Briefly, microtitre plates were coated overnight with 100µl of mouse monoclonal anti-NfH antibodies (SMI clones 34 or 35) diluted in 1/5000 in 0.05M carbonate buffer, pH 9.5. The plates were washed with barbitone buffer containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 (pH 8.9), and then blocked with 250µl barbitone buffer containing 1% of BSA. 50µl of standard, control or CSF samples were added in duplicates with 50µl of sample diluent which constituted of 50µl barbitone buffer, 6mM EDTA and 0.1% BSA. After incubating at room temperature for an hour and washed, 100µl of detector antibody: rabbit polyclonal anti-NfH from Affiniti (Exeter, UK) diluted 1/1000 was added then incubated for an hour at room temperature. The wells were washed then horseradish peroxidase (HRP)-labelled swine anti-rabbit antibody diluted 1/1000, were added and incubated an hour at room temperature then washed. 100µl TMB substrate was added and incubated in dark then the reaction was stopped with 50µl 1M hydrochloric acid (HCl) and the absorbance read at 450 and 750nm. The upper reference value for CSF NfH^{SMI35} is 0.73ng/mL. None is available for CSF NfH^{SMI34}.

Table 3.3 Neurofilament studies of MS

| Year | First Author | Body fluid | NF type | Cohort | Observations |
|-------------|---------------------|-------------------|----------------|---|--|
| 1998 | Lycke | CSF | NfL | 60 RRMS 11 healthy control | ↑NFL in MS Correlate with disability, relapse rate |
| 2002 | Semra | CSF | NfL | 35 MS (19 PPMS or SPMS, 16 RRMS) 36 other neurological diseases | ↑NFL in SPMS and PPMS Correlate with disability |
| 2003 | Malmeström | CSF | NfL | 66 MS (41 RRMS, 25 SPMS) 50 healthy control | ↑NFL in relapse RRMS > remission RRMS and SPMS. Overall ↑NFL in MS. |
| 2004 | Haghighi | CSF | NfL | 47 MS 47 healthy siblings | ↑NFL in CDMS ↔NFL in siblings |

↑ = increase; ↔ = no change/constant

Table 3.4 Nomenclature and characteristics of the 9 types of NfH phosphoforms, the immunocytochemical staining pattern, the antibody binding epitope, the antibody type and the commercial (Sternberger monoclonal) and previously published notation for the mouse monoclonal antibodies used in this study.

Types of epitopes:



Extensively phosphorylated








Phosphorylated












Non phosphorylated

Ab *Antibody*

(see next page)

| | | | |
|--|---|---|-------------|
| | Neuronal cell bodies, subpopulation, dendrites and thick axons |  | IgG1 |
| | Neuronal cell bodies, dendrites and thick axons |  | IgG1 |
| | Some axons in normal tissue, more axons in pathological condition |  | IgG1 |
| | Neuronal cell bodies with dendrites and thick proximal axons |  | Ab cocktail |
| | Axonal sprout evoked neurites |  | IgG1 |

| NfH phosphoforms | Immunocytochemical staining pattern | Phosphorylation state | Ab type |
|-----------------------------|---|---|----------------|
| NfH^{SMI32} | Neuronal cell bodies, dendrites, proximal axons and axon-terminal spheroids |  | IgG1 |
| NfH^{SMI33} | Neuronal cell bodies, dendrites, many axons and some macrophages |  | IgM |
| NfH^{SMI34} | Most axons. |  | IgG1 |
| NfH^{SMI35} | Most axons. |  | IgG1 |
| NfH^{SMI37} | Neuronal cell bodies subpopulation, dendrites and thick axons |  | IgM |
| NfH^{SMI38} | Neuronal cell bodies, dendrites and thick axons |  | IgG1 |
| NfH^{SMI310} | Some axons in normal tissue. More axons in pathologic condition |  | IgG1 |
| NfH^{SMI311} | Neuronal cell bodies with dendrites and thick proximal axons |  | Ab cocktail |
| NfH^{NE14} | Axons, spiral ganglion neurones |  | IgG1 |

3.3.2 S100B

S100 was originally discovered by Moore in 1965 and derived its name from the fact that it is soluble in 100% saturated ammonium sulphate at neutral pH. It is an acidic calcium binding protein with molecular weight of 20kDa. It consists of two beta subunits and is located in the cytoplasm of astrocytes and is also expressed in Schwann cells in peripheral nerve. Today there are more than 16 different S100 members and S100B and S100A1 are the most studied (Donato, 2001).

Its function is unknown. S100B is secreted by astrocytes and acts on these cells to stimulate nitric oxide secretion in an autocrine manner (Adami *et al.*, 2001). S100's toxic effects have been suggested to depend on stimulation of calcium influx into responsive cells or nitric oxide release from astrocytes (Hu *et al.*, 1996; 1997). S100B release has been shown to occur via apoptosis. Due to its extracellular effects, it has been viewed as a cytokine (Griffin *et al.*, 1998). It has also been implicated in the regulation of (1) the dynamics of the cytoskeletal constitute (via protein phosphorylation) (2) cell cycle progression (possibly via interactions with transcription factors) and (3) enzymes (Schafer and Heizmann, 1996).

High concentrations of S100B are found in head injury, subarachnoid haemorrhage, rapid parenchymal destruction in epilepsy, CJD, acute brain injury, compression of the spinal cord by tumours, ischaemic disorders and viral or suspected viral infection (Donato, 2001; Sindic, 1982). Its presence in the CSF is due to an immediate release from the cytosol of damaged astrocytes and there is

then a delayed rise in the serum (on day 2) (Petzold *et al.*, 2003). S100 may thus be an index of CNS injury.

In slowly progressive diseases, S100B might play a different role i.e. modulating the inflammatory response through stimulation of inducible nitric oxide synthase (iNOS) – (Donato, 2001; Adami, 2001). Abnormal levels are found in Down's syndrome where its expression is increased in astrocytes and increases progressively with age (Royston *et al.*, 1999). It is also present in plaques in Alzheimer's disease (Griffin *et al.*, 1998) – albeit via an unknown mechanism. Studies on S100B and MS have been extensive: Petzold *et al.* (2003) observed, in post-mortem brain homogenates, that S100B is a marker for the relapsing phase of MS (higher levels in acute versus subacute plaque). Furthermore, RRMS had higher CSF S100B levels compared with SPMS thus S100B could be useful to differentiate between RRMS and progressive MS.

Nonetheless the value of S100B as a marker of disease activity has not been established and the results from several studies are contradictory (Noppe *et al.*, 1986; Lamers *et al.*, 1995, Green *et al.*, 1997, Malmeström *et al.*, 2003).

3.3.2.1 ELISA method to detect S100B

A sensitive and specific in-house ELISA was used for measuring S100B in CSF as described by Green *et al.* (1999). Ninety-six-well plates were coated with monoclonal anti-S100B at a concentration of 9.3µg/ml in 0.05M carbonated buffer, pH 9.5. The plates were washed with 0.67M barbitone buffer containing 5mM calcium lactate, 0.1% BSA and 0.05% Tween and then blocked with 2% BSA for 1 hour and washed again. 50µl of standard, control or CSF samples were added in duplicate and 50µl of 0.67M barbitone buffer containing 5mM calcium lactate. The plates were incubated at room temperature for 1 hour then washed. Rabbit anti-S100 antibody conjugated with HRP polyclonal anti-S100B (Dako, Copenhagen, Denmark) diluted 1/1000 with incubation buffer was added. The plate was incubated for 20 minutes at room temperature in the dark; the colour reaction was stopped with 50µl 1 M HCl and the absorbance read at 450 and 750nm on a Wallac Victor 2. The antigen concentration was calculated from an internal standard curve ranging from 0.01 to 2.5ng/ml. The normal reference range is 0.55ng/mL and less.

Table 3.5 S100B studies of MS

| Year | First Author | Body fluid | Cohort | Observations |
|------|--------------|----------------------------|--|--|
| 1979 | Michetti | CSF | 18 MS 11 neurological or psychiatric diseases | ↑S100B in acute phase of MS |
| 1985 | Massaro | CSF | 10 MS 5 weeks post-relapse | Peak ↑S100B in 3 rd week and detectable in all 5 weeks |
| 1986 | Noppe | CSF | 535 neurological disease: MS, encephalitis, stroke, tumour compression | ↔S100B |
| 1995 | Lamers | CSF | 17 neurological disease | ↑S100B in stroke |
| 1997 | Missler | Plasma | 28MS (17 relapse 11 stable) 120 healthy control | ↑S100B in initial 7 days after relapse. ↔S100B 8 to 28 days post-relapse. |
| 2002 | Petzold | CSF Brain homogenate | 51 MS (20RRMS, 21SPMS, 10PPMS) 51 OND PM brain tissue (12 MS, 8 control) | ↑S100B in RRMS and acute PM lesions |
| 2003 | Malmeström | CSF | 66 MS (41 RRMS, 25 SPMS) 50 healthy control | ↔S100B |

↑ = increase; ↔ = no change/constant

3.3.3 Glial Fibrillary Acidic Protein

GFAP is an intermediate filament (IF) protein of astroglia discovered in 1969. Of note, IF proteins constitute an extremely large multigene family of developmentally and tissue-regulated cytoskeleton proteins.

GFAP has a molecular weight of 50kDa, and is 8-9nm long. It is composed of a head domain, a central rod domain and a carboxy terminal tail domain. It is an insoluble CNS-derived protein and its assembly is regulated by phosphorylation-dephosphorylation of the head domain by altering its charge (Inagaki *et al.*, 1994). However, in CSF, it is secreted in its soluble form and is considered a biomarker of astrogliosis in neurodegenerative disorders.

Its function is thought to be in modulating astrocyte motility and shape by providing structural stability to astrocytic processes. Levels of GFAP are altered during development and aging (Laping *et al.*, 1994; Gomes *et al.*, 1999). It responds dynamically to neurodegenerative lesions and its level is often upregulated after CNS insult. Furthermore, the astroglial response occurs rapidly and can be detected within an hour of a focal mechanical trauma (Eng and Ghinikar, 1994). Thus it is useful as a biologic marker of disease activity, reflecting an increasing rate of astrogliosis.

Increased GFAP levels have been observed in CNS diseases such as AIDS dementia (Eng and Ghinikar, 2000); brain infarction (Aurell *et al.*, 1991); a

variety of viral infections and neuroborreliosis (Dotevall *et al.*, 1999); prion associated spongiform encephalopathies; inflammatory demyelinating disease and neurodegenerative diseases such as Alzheimer's disease (Eng and Ghinikar, 2000).

As a nonspecific biomarker of CNS tissue injury, GFAP has been noted to be upregulated in MS (Noppe *et al.*, 1986; Rosengren *et al.*, 1995; Petzold *et al.*, 2002; Malmeström *et al.*, 2003; Haghighi *et al.*, 2004) (Table 3.6). Astrocytes are perhaps activated in acute MS lesions and participate in gliotic scar formation in chronic lesions, revealing information about the ongoing pathologic processes in MS. In a study by Rosengren *et al.*, (1995), GFAP correlated with increased neurological disability (Table 3.6).

It is not known whether gliosis, i.e. proliferation and migration of glial cells in damaged areas of the CNS, observed in MS has overall beneficial or negative effects on disease progression. Therefore, while GFAP, as a marker, may have a solid biological rationale, its clinical relevance in MS is currently uncertain.

3.3.3.1 ELISA method to detect GFAP

Sandwich ELISA to detect GFAP has been described by Petzold *et al.* (2004). The microtitre plates were coated with mouse monoclonal IgG1 anti-GFAP antibodies (Sternberg Monoclonals, Utherville, MD): 100µl of the capture antibody diluted in 1:1000 in 0.05M carbonate buffer, pH 9.5. The plate is washed

with barbitone buffer containing 0.1% BSA and 0.05% Tween 20. 50µl of barbitone buffer was added in each well followed by 50µl standard, control or CSF sample in duplicates. The plates were incubated at room temperature for an hour then washed. 100µl of HRP-labelled rabbit anti-bovine GFAP diluted 1:1000 barbitone buffer were added to each well, incubated for an hour at room temperature and then washed. The plate was incubated for 20 minutes at room temperature in the dark; the colour reaction was stopped with 50µl 1M HCl and the absorbance read at 450 and 750nm on a Wallac Victor 2. The normal CSF reference range is 0.09ng/mL.

Table 3.6 GFAP studies of MS

| Year | First Author | Body fluid | Cohort | Observations |
|-------------|---------------------|-------------------------|--|--|
| 1986 | Noppe | CSF | 535 neurological disease: MS, encephalitis, stroke, tumour compression | ↑GFAP dementia/syringomyelia |
| 1995 | Rosengren | CSF | 13 RRMS 5 healthy control | ↑GFAP in MS GFAP correlate with neurological dysfunction. |
| 2002 | Petzold | CSF Brain homogenate | 51 MS (20RRMS, 21SPMS, 10PPMS) 51 OND PM brain tissue (12 MS, 8 control) | ↑GFAP in PM MS tissue |
| 2003 | Malmeström | CSF | 66 MS (41 RRMS, 25 SPMS) 50 healthy control | ↑GFAP in SPMS |
| 2004 | Haghighi | CSF | 47CDMS 47 healthy siblings | ↑GFAP in CDMS ↔GFAP in siblings |

↑ = increase; ↔ = no change/constant

3.3.4 Ferritin

About one third of iron in the brain is bound to ferritin, which has traditionally been thought of as the intracellular storage protein for iron.

Ferritin is a large spherical protein made up of heavy and light chain subunits, each of 21 and 19kDa, respectively. Brain ferritin (of about 450kDa) contains a higher proportion of heavy to light chain subunits and a lower proportion of iron atoms per molecule than non brain ferritin. The heavy chain form of ferritin may allow for more rapid mobilization of iron than the light chain form, which is associated with longer storage of iron.

Apart from the choroids plexus which stains strongly for iron, the other principle cells that stain for iron within the brain are oligodendrocytes. Iron also stains in microglia, astrocytes, neuronal cell bodies, and possibly even axons and myelin sheaths (Koeppen, 2003). Iron uptake is maximal during rapid brain growth which coincides with the peak myelinogenesis. Conversely iron deficiency has been associated with hypomyelination at development. Iron is thus vital for myelination but its continuous requirement for myelin maintenance is less clear.

Ferritin has been presented as a general marker for normal and activated microglia. The accumulation of iron and ferritin in microglia in diseased brain is not necessarily associated with an increase of total brain iron (e.g. total brain iron is not increased in Alzheimer's disease). During stresses like hypoxia, oligodendrocytes can increase their synthesis of ferritin, to protect cells against oxidative damage, by sequestering iron which is toxic in a free or loosely bound

state. In normal adult human brain tissue, ferritin binds predominantly to white matter and has the potential to deliver approximately 2000 times more iron than transferrin. Of note, iron levels in white matter are higher than in grey matter.

CSF ferritin has been shown to be largely non-glycosylated (compared with blood) and high levels have been associated with diverse conditions including: subarachnoid haemorrhage, intraparenchymal cerebrovascular events including haemorrhage and infarction, miscellaneous CNS infections and circulatory disorders associated with vasculitis (Keir *et al.*, 1993).

The normal distribution of ferritin binding sites is altered in and around plaques from periventricular white matter isolated from MS brains (Hulet *et al.*, 1999). Ferritin levels were significantly elevated in the CSF of chronic progressive active MS patients compared to normal individuals (Levine *et al.*, 1999). This could be a defense mechanism to protect against iron induced oxidative damage. There was no difference between normal individuals and RRMS patients (whether they were active or stable).

3.3.4.1 ELISA method to detect ferritin

Assay plates were coated overnight at 4°C with 1 in 200 dilution (55µL rabbit anti-human ferritin serum (Dako Ltd., high Wycombe; product number A133) to 11ml of 0.05M carbonate buffer, pH 9.5). First antibody was decanted and 200µL 0.05% BSA was added and rinsed with solution and then decanted again.

Blocking solution (1% bovine serum albumin) was added and left at room temperature for an hour to saturate all unoccupied protein binding sites. Wells were replaced with 40µL sample diluent prepared with 2ml blocking solution and 18ml Tween solution. Fifty microlitres of standard/CSF/brain homogenate sample was added. Plates were covered with cling film and incubated for 2 hours at room temperature, and then decanted and washed. Eleven microlitres of detector antibody peroxidase-conjugated rabbit anti-human ferritin (Dako Ltd., High Wycombe; product P145) was added to 11mL of sample diluent. One hundred microlitres of this were added to each well at room temperature for an hour. Plates were washed and the colour was developed with *o*-phenylenediamine. The optical density was read in a Wallac ELISA plate reader at a measuring wavelength of 492nm against a reference wavelength of 405nm. The mean normal CSF ferritin concentration is 5.6µg/L.

Table 3.7 Ferritin studies of MS

| Year | First Author | Body fluid (Human) | Cohort | Observations |
|-------------|---------------------|-------------------------------|---|--|
| 1989 | Valberg | Serum | 49 MS 49 healthy control | ↑ferritin |
| 1999 | LeVine | CSF | 44 chronic progressive MS 42 control | ↑ferritin in chronic progressive MS ↔ferritin in RRMS |
| 2002 | Petzold | CSF | 51 MS (20RRMS, 21SPMS, 10PPMS) | ↑Ferritin in SPMS and in all lesion stages in PM |
| | | Brain homogenate | 51 other neurological disorders PM brain tissue (12 MS, 8 control) | |

↑ = increase; ↔ = no change/constant

3.3.5 Anti-myelin antibodies

There are many reports demonstrating low affinity antimyelin autoantibodies by ELISA in sera and CSF of patients with MS, though their role either in the disease's pathogenesis or in predicting outcome is still not defined. However, high affinity anti-MBP or MOG antibodies appear to be more difficult to detect in the serum or CSF of patients with MS while anti-MOG antibodies can be found in MS CNS plaque tissue [Genain *et al.*, 1999].

3.3.5.1 Anti-Myelin Basic Protein

MBP accounts for about 30% of central myelin protein. It is not however a CNS myelin specific protein as it is also expressed in the peripheral nervous system as well as cells of the immune system (Pribyl *et al.*, 1996). Anti-MBP antibody response is a relatively common sequel to any CNS injury¹¹ (Frick and Stickl, 1980; Paterson *et al.* 1981; Newcombe *et al.*, 1985; Baig *et al.*, 1991; Solders *et al.*, 1992; Wang *et al.*, 1992b; Olsson *et al.*, 1993). Anti-MBP response also appears to accumulate with time¹².

3.3.5.2 Anti-Myelin Oligodendocyte Glycoprotein

MOG is a 218-amino acid glycoprotein and constitutes of 0.01-0.05% of the total membrane protein (Brunner *et al.*, 1989). It is exclusively expressed in oligodendrocytes (Gardinier *et al.*, 1992) and is located on the outer lamellae of oligodendroglial membranes and myelin.

MOG is sequestered from the immune system by the endothelial blood-brain barrier and lack any direct lymphoid drainage from the CNS compartment. However it is apparent that immune sequestration does not render MOG uniformly immunogenic in all patients, as only 40-50% of MS and other

¹¹ *Healthy subjects; OND - Lyme neuroborreliosis, syphilis, cerebrovascular diseases; other inflammatory neurological disorders - ON, subacute sclerosing panencephalitis, motor neurone disease, hereditary motor and sensory neuropathy; other non-neurological disorders - carcinoma, rheumatoid arthritis.*

¹² *In contrast to anti-MOG-Ig which does not differ with time and subsets of MS patients.*

inflammatory neurological disorders samples was anti-MOG-Ig antibody positive (Reindl *et al.*, 1999).

Overall, MOG is highly immunogenic and MOG immunization induces severe relapsing EAE in rodents and marmoset (Stefflerl *et al.*, 2000). The immunodominant target for this demyelinating autoantibody response is directed against the extracellular Ig domain of the protein (Adelmann *et al.*, 1995; Genain *et al.*, 1995). Furthermore, T cells proliferate to MOG more than to other myelin antigens in patients with MS (Berger *et al.*, 2003).

MOG may be dependent on synergy with a T-cell mediated inflammatory response in the CNS. Circulating pathogenic anti-MOG antibodies are unable to induce demyelination *per se*. Only when blood brain permeability is enhanced can antibody enter the CNS, bind to the myelin surface and initiate demyelination (Litzenburger *et al.* 1998).

Recent data suggest that its autoimmune response is in part regulated by polymorphisms in the MOG gene, implicating the genetic and immunological stratification of patients with MS (Gomez-Lira *et al.* 2002). Anti-MOG antibodies fix complement and are bound to disintegrating myelin in acute MS lesions. Enhanced T- and B-cells responses to MOG have been reported in MS (Sun *et al.*, 1991; Xiao *et al.*, 1991; Kerlero de Rosbo *et al.*, 1993; Wallstrom *et al.*, 1998) (Table 3.9). Berger *et al.* (2003) used the first 102 N-terminal amino acids of recombinant human MOG, expressed in *Escherichia coli*, as the antigen in their Western blot analyses and previous ELISAs (Berger *et al.*, 2003). His group

found that the antibodies are present in the serum and CSF of a third of patients at the time of their first attack of MS (Berger *et al.*, 2003).

The anti-MOG-Ig antibody response appears to be established early in the course of MS and does not differ significantly between the different patient subsets, i.e. CSF anti-MOG index is persistently higher in all stages of MS. However, it is unlikely that MOG is the only autoantigen expressed on the myelin surface that can be targeted by a pathogenic autoantibody response in MS.

3.4 Summary

In this chapter, conventional MRI, traditional CSF oligoclonal bands and some novel brain specific proteins have been reviewed.

Early pathological changes in MS may contribute to later long term disability. Therefore prevention of early abnormality may be the best way to prevent disability. But the main question is which changes in early MS should be targeted? For this reason, it would be desirable to study CIS patients with BSP, as BSP could provide further insight into pathological mechanisms in early disease.

Chapter 5 asks what insight can be learnt about pathological mechanisms in ON with BSP for neuronal loss (NfH) and myelination (ferritin). Are these BSPs more abnormal in patients with ON who subsequently convert to CDMS or in those with non-MS-ON?

Chapter 5 asks what is the key determinant of poor relapse recovery. Again, BSPs could provide valuable insights. Does higher levels of NfH predict poor outcome? Do steroids improve axonal loss?

There is also a need to correlate histopathology with BSP to gain further understanding about what BSP abnormality means. This data is presented in chapter 6.

Questions also remain as to whether serum BSPs could be useful as surrogate markers. Long-term disability develops over decades and the effect of therapies on preventing long-term disability cannot be assessed with clinical endpoints. Therefore it is necessary to find surrogate markers which are:

- 1) markers for pathology (e.g. axonal loss) that cause disability,
- 2) that predicts long term disability, and
- 3) are abnormal in early MS.

Two studies were done in the present thesis, asking questions about surrogates. Chapter 7 asks whether S100B increase over time in PPMS – investigating any usefulness of serum S100B as a surrogate marker. Chapter 8 evaluates whether anti-myelin antibodies predict conversion of CIS to CDMS, in light of the recent positive findings by Berger and colleagues (2003).

| First Author | Body fluid | Subjects | Results | Comment |
|----------------------|-------------------|---|--|---|
| Lampasona 2004 | Serum | 87 MS patients 12 encephalomyelitis 47 healthy control | +ve anti-MOG IgM in 40/87 MS patients and in control subjects. +ve anti-MOG IgG and IgM in all groups and subgroups. | Anti-MOG Ab are not disease specific |
| Mantegazza 2004 | Paired serum/CSF | 262 MS patients; 175 RRMS, 43 PPMS, 44 SPMS; 131 OND, 307 healthy controls (sera only) | +ve serum anti-MOG Ab in 13.7% MS patients, 13.7% of OND patients, 6.2% of controls. +ve CSF anti-MOG antibodies in 11.4% MS patients, 18.9% OND patients. | Anti-MOG Ab are not disease specific |
| Berger 2003 | Serum | 103 patients with CIS (positive findings on cerebral MRI, and oligoclonal bands in CSF). | 9/39 seronegative Ab patients had a relapse within mean time 45.1+/-13.7 months. 21/22 patients with Ab against both MOG and MBP had a relapse within 7.5+/-4.4 months, 35/42 patients with anti-MOG Ab had a relapse within 14.6+/-9.6 months. The adjusted hazard ratio for the development of CDMS was 76.5 (95% CI, 20.6 to 284.6) among the patients who were seropositive for both Abs. | +ve <u>anti-MOG and anti-MBP</u> Ab had relapses more often and earlier than patients without these antibodies. Anti-MOG and anti-MBP together allows the prediction of early conversion to CDMS. |
| Markovic. 2003 | CSF | 44 MS patients 51 controls 11 with OIND 40 with NIND. | +ve anti-MOG Ab in 30% MS patients was significantly ↑ than NIND (8%, p=0.02), but not compared to OIND group (55%, p=0.228). +ve anti-MOG Ab was similar in patients with clinically active and stable MS (32% and 26%, respectively; p=0.921). | Anti-MOG Ab has potential role in the subset of patients with MS. |
| Kennel De March 2003 | Serum | 44 MS patients 30 controls | ↑ anti-MOG Ab levels in MS patients. | Anti-MOG humoral immunity has a role in the pathophysiology of MS. |
| Tejada-Simon 2002 | Serum | 20 MS patients; 14 RRMS, 6 chronic progressive MS 10 healthy subjects | Anti-MOG Ab derived from MS patients displayed a skewed reactivity pattern, even though the occurrence and titres of serum anti-MOG Ab were only slightly ↑ in MS patients. | Anti-MOG antibodies are present within demyelinating lesions of MS |
| Egg 2001 | Serum | 261 MS patients | 72% had anti-MOG Ab, 59% were anti-MBP +ve. Patients who actually suffered from a relapse were significantly more often anti-MOG and anti-MBP IgG3 +ve than those in remission. | ↑ anti-MOG levels in MS patients and is associated with relapse. |
| Reindl 1999 | Serum CSF | 130 MS patients, 32 patients with OIND, 30 patients with NIND, 10 patients with rheumatoid arthritis- sera only | +ve anti-MOG-Ig: 38% of MS patients 53% in OIND 3% in rare in NIND 10% in rheumatoid arthritis. | Anti-MOG Ab are common in CNS inflammation. However, in OIND these antibody responses are transient, whereas they persist in MS. |
| Karni 1999 | Paired CSF/plasma | 31 patients with MS 31 OND, 28 healthy controls (sera only) | ↑CSF anti-MOG Ab in patients with MS and OND compared with controls. ↑ plasma anti-MOG Ab in MS patients > OND and controls. | ↑ anti-MOG Ab is not specific for MS because a similar appearance was also demonstrated in patients with OND. |
| Xiao 1991 | Paired CSF/plasma | 30 MS patients, 30 with OND, 30 with headache | +ve anti-MOG Ab in CSF of 7 MS patients, 2 with OND, 1 with tension headache. No anti-MOG IgG antibodies were demonstrable in plasma | The significance of CSF anti-MOG Ab remains to be defined. |

Table 3.8 Anti-MOG antibody studies in MS

+ve=positive ↑=elevated

PART 2

BRAIN SPECIFIC PROTEINS IN PATIENTS WITH DEMYELINATING DISEASES AND IN POST MORTEM BRAINS WITH MULTIPLE SCLEROSIS

Chapter 4

Cerebrospinal Fluid Levels of Brain Specific Proteins in Optic Neuritis

4.1 Introduction

The aim of this study is to evaluate the levels of CSF BSP in subjects with ON who are at high risk of progression to MS. It is important to study such patients, as early treatment in MS may be the most effective way at preventing long-term disability. It would therefore be desirable to understand the pathological mechanism that occur early but which may be associated with later disability. The study was achieved in association with the group in the Division of Neurology and Department of Ophthalmology in Karolinska Institute, Huddinge University Hospital, Stockholm. With the above hypothesis in mind, CSF from the ON cohort obtained from Stockholm was investigated for NfH, S100B and ferritin.

Acute monosymptomatic ON can be the first presentation of MS. It is an inflammatory demyelinating condition, and long-term follow-up indicates that up to 75% of subjects with ON will develop CDMS (Hickman *et al.*, 2002; Francis *et al.*, 1987; Rizzo *et al.*, 1988; Brex *et al.*, 2002). Between 50-70% of subjects with monosymptomatic ON have clinically silent MS-like lesions on brain MRI, and 60-70% have the local synthesis of oligoclonal IgG bands typically seen in MS, (provided sensitive techniques are used) (Soderstrom *et al.*, 1998). Intrathecal immunoglobulin synthesis (e.g. oligoclonal IgG bands), in monosymptomatic

patients is also strongly predictive for the future development of CDMS (Soderstrom *et al.*, 1998; Keir *et al.*, 1993).

As mentioned previously, BSP are detectable in the CSF in a number of differing neuro-pathological processes. In particular, ferritin a marker of microglial activation (Keir *et al.*, 1993), S100B a marker of astrocytic activation (Green *et al.*, 1997) and two neurofilament heavy chain phosphoforms (NfH^{SMI34} and NfH^{SMI35}) putative markers of axonal degeneration (Petzold *et al.*, 2003). The neurofilaments differ in their degree of phosphorylation; NfH^{SMI34} is more extensively phosphorylated than NfH^{SMI35} (Petzold *et al.*, 2003).

4.2 Aims

The hypothesis is that CSF BSP levels in subjects with ON who remain monosymptomatic (without eventual progression to MS), would differ from levels in subjects with ON destined to develop further episodes of CNS inflammation. To test this hypothesis the levels of ferritin, a microglial activation marker, S100B, a marker of astrocyte activation, and NfH^{SMI34} and NfH^{SMI35} in the CSF of ON patients were determined, comparing those at high risk of progression to MS (MRI positive and OCB positive) with those who are MRI and OCB negative.

Secondly, BSP levels were compared in those who developed CDMS on follow up with those who remained monosymptomatic.

A third objective was to compare cross-sectional differences in levels of BSP in all subjects with ON, including those with MS, to those with other neurological diseases.

4.3 Methods

4.3.1 Subjects

Forty one subjects who had acute monosymptomatic unilateral ON were consecutively recruited from the Stockholm Optic Neuritis Study. The outlines of this study and the protocol for clinical examinations, CSF and MRI investigations have been described in detail elsewhere (Soderstrom *et al.*, 1998). In all patients, blood tests, lumbar puncture and MRI were performed within four weeks from onset of visual symptoms of ON.

Twenty one subjects had intrathecal IgG synthesis, i.e. oligoclonal IgG bands in CSF which were absent in a corresponding serum sample, and a brain MRI fulfilling the Paty criteria (Paty *et al.*, 1988) for being highly suggestive of MS thus representing “MS-type ON”. One subject had oligoclonal IgG bands in CSF but not in serum, but did not have a MRI performed. Nineteen subjects showed no signs of intrathecal immunoglobulin synthesis (absence of oligoclonal IgG bands and normal IgG-index) and had a normal brain MRI thus representing “non-MS-type ON”.

Routine blood tests, a screen for collagen vascular disease and serologic tests for Lyme neuroborreliosis and neurosyphilis in CSF were normal or negative for all patients.

Seventeen subjects with other neurological diseases (OND) served as controls. The clinical features of those with OND are summarized in Table 4.1.

4.3.2 *Follow-up*

None of the ON patients received oral or intravenous corticosteroids as acute treatment or during the follow-up. Eighteen of the patients initiated treatment with intramuscular interferon-beta-1a 30 mcg once weekly (AvonexTM) during the follow-up period. The decision to initiate this disease modifying treatment was based on clinical judgment and patient acceptance.

The patients were monitored closely during the first six months and thereafter regularly every six months. They were instructed to contact the department if they experienced any ophthalmological or neurological disturbances. Eight subjects with MS-type ON subsequently developed new neurological signs at median of 18 (11-25) months and were diagnosed with CDMS according to Poser criteria (Poser *et al.*, 1983). All of them had been started on intramuscular interferon-beta-1a 30 mcg once weekly (AvonexTM).

The Ethics Committee of the Karolinska Institute at Huddinge University Hospital, Stockholm, Sweden, approved the study protocol and informed consent was obtained from all patients.

4.3.3 CSF analysis

Total protein, ferritin, S100B, phosphorylated and extensively phosphorylated neurofilament heavy chain (NfH^{SMI35} and NfH^{SMI34} respectively) were quantified in the CSF of all the subjects.

CSF samples were kept frozen at -40°C until the time of analysis.

4.3.4 Oligoclonal Bands, Total Protein, S100B and Ferritin

Oligoclonal bands in CSF and serum were determined using isoelectric focusing and immunofixation using a standard method (Keir *et al.*, 1990). Total protein concentration was measured using the Lowry method. S100B was quantified in CSF by ELISA as previously described by Green *et al.*, without EDTA in the washing solution (Green *et al.*, 1997). Ferritin was determined in CSF by ELISA with the normal range being equal or less than 12ng/mL (Keir *et al.*, 1993).

4.3.5 Neurofilament Heavy Chains (NfH^{SMI35} and NfH^{SMI34})

The two neurofilament heavy chain phosphoforms were detected in CSF by sandwich ELISA assay as described by Petzold *et al.* (2003).

4.3.6 Statistical analysis

All CSF tests were performed with researchers blinded to the clinical diagnosis and MRI results. Statistical analyses: median, interquartile range and significance of group differences (Mann-Whitney U tests) were evaluated using the SPSS software package (version 11.0 for Windows). Probability values less than 0.05 were considered significant. Three subgroup analyses were undertaken;

1. Subjects who developed CDMS (ON(CDMS)) versus those that are negative for both CSF/serum OCB and have normal MRI (non-MS-type ON).
2. Subjects with MS-type ON and non-MS-type ON;
3. All subjects with optic neuritis (ON total) and those with other neurological diseases (OND);

Relationships between parameters were assessed using Spearman rank correlation coefficient (r).

The degree of phosphorylation of the neurofilament phosphoforms was expressed as a ratio of NfH^{SMI34} to NfH^{SMI35}. A ratio of less than 1 is thought to indicate a relative decrease in NfH phosphorylation a putative marker of damaged axons (see Figure 4.1).

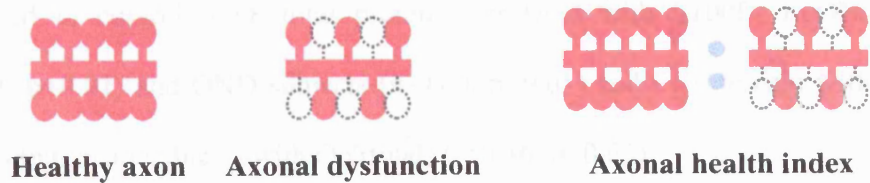


Figure 4.1 Schematic diagram of the degree of phosphorylation of healthy axons vs. damaged axons and the significance of the ratio for neurofilament phosphoforms.

4.4 Results

The demographics for all subjects and for the six separate group types are described in Table 4.2. There were no significant differences in any of the subgroups in relation to sex and age. CSF oligoclonal IgG bands were present in 61% of all subjects with ON (total) and 100% in those with ON (CDMS) and by definition in 100% of subjects with MS-type-ON. None of the subjects with non-MS-type ON and OND was positive for CSF oligoclonal IgG bands.

4.4.1 ON (total) vs. OND (Table 4.3)

NfH^{SMI34} was significantly elevated in subjects with ON compared to controls (Figure 4.2). No such difference was found for NfH^{SMI35} in ON (total) vs. OND. The NfH^{SMI34:SMI35} ratio was higher in all subjects with ON when compared to OND (Figure 4.3). S100B levels were similar in all subjects studied. Lower levels of CSF ferritin were observed in all subjects with ON (total) compared to those with OND. The CSF total protein was higher in all subjects with ON (total)

compared to control. CSF total protein correlated with S100B in ON(total) ($r=0.41$, $p=0.01$) and OND subjects ($r=0.60$, $p=0.01$) and CSF ferritin with CSF total protein in all subjects with ON(total) ($r=0.36$, $p=0.03$).

4.4.2 ON (CDMS) vs. Non-MS-type ON

There were no significant differences in the levels of CSF brain specific proteins when ON subjects that subsequently went on to develop CDMS were compared with ON subjects with normal MRI and negative CSF/serum OB.

4.4.3 MS-type ON vs. Non-MS-type ON (Table 4.3)

When comparing MS-type ON with non-MS-type ON, the total protein in CSF was higher in the former subgroup but the difference was not statistically significant ($p=0.08$). CSF NfH^{SMI34} and NfH^{SMI35} levels as well as the degree of phosphorylation did not differ between MS-type ON and non-MS-type ON. Ferritin and S100B levels were similar in both groups. There were no correlations between age, ferritin, S100B, neurofilament heavy chain phosphoforms and total protein in MS-type ON and non-MS type ON.

4.5 Discussion

Neurofilament heavy chain levels and the NfH^{SMI34: SMI35} ratio, thought to be a relative index of NfH phosphorylation status, were increased in the CSF of subjects with optic neuritis whereas levels of S100B did not differ between

subjects with optic neuritis and neurological controls. CSF ferritin was significantly lower in these two groups but the levels were within the normal limits.

Neurofilament heavy chain is thought to be a marker of axonal pathology and its release into the CSF in optic neuritis may indicate that acute axonal damage and/or loss is occurring in this condition. There are different phosphoforms of neurofilament heavy chain (Goldstein *et al.* 1983), and this study shows that extensively phosphorylated neurofilament heavy chains are released into the CSF in optic neuritis. Extensively phosphorylated isoforms of NF are found in large healthy axons compared to the less phosphorylated isoforms that are indicative of axonal pathology (Trapp *et al.*, 1988). Therefore raised levels of extensively phosphorylated isoforms indicate damage and release of NfH from healthy axons. This is what one would expect in subjects presenting with their initial attack. In comparison in subjects with more established MS in which significant damage has already occurred to the CNS. Petzold *et al.* (2002) have shown that the ratio of extensively phosphorylated to phosphorylated NfH (NfH^{SMI34:SMI35}) to be reduced in patients with progressive and advancing MS compared to patients with non-advancing relapsing disease. They have also observed high levels of CSF NfH^{SMI35} in CSF of patients with subarachnoid haemorrhage, amyotrophic lateral sclerosis, space occupying lesions and disc prolapses (Petzold *et al.*, 2003).

Those with ON who are at high risk of progression to MS (subjects with white matter lesions on MRI and local synthesis of oligoclonal bands) had CSF BSP levels similar to those at low risk. Furthermore, a retrospective comparison of subjects who subsequently went on to develop CDMS with those who did not, revealed no difference in baseline CSF BSP levels. It is of relevance that 86% of the subjects with MS-type ON which include all of the subjects who subsequently developed CDMS were on intramuscular interferon-beta-1a 30 mcg once weekly (AvonexTM). This disease modifying therapy may have delayed conversion to CDMS and/or slowed down axonal damage or atrophy, hence a source of confounding error in the lack of difference seen.

Another explanation for the lack of difference is that although levels of lumbar CSF BSP may be indicative of recent pathology they may not provide information about ongoing inflammation. In this sense, it should be noted that CSF examination was performed within a month of symptom onset. Furthermore, the pathology of optic neuritis, irrespective of the risk for further attacks is likely to be similar, and involve axonal loss. Therefore levels of BSP would be similar in all cases of ON. In one study, no differences were noted for a number of cytokines measured by *in situ* hybridization between MS-type ON vs. non MS-type ON, concluding that the inflammatory markers in blood and CSF was more related to the inflammation per se (in the optic nerve) than to MS (Kivisakk *et al.*, 1997).

Axonal damage has been observed in patients with ON (ophthalmic examination reveals subtle atrophy of the retinal nerve fibre layer (Frison *et al.*, 1974)) and in an experimental model of optic neuritis where the presence of axonal cytoskeletal changes has been detected (Zhu *et al.*, 1999). Acute inflammation may be an important cause of axonal loss in both MS and EAE (Ferguson *et al.*, 1997; Trapp *et al.*, 1988) and therefore it is likely that these mechanisms also exist in ON. In MRI studies, low concentration of the metabolite N-acetyl aspartate is thought to be a marker of axonal loss or damage and has been found occurring focally at the earliest phase of the disease. This reduction however was not seen in NAWM demonstrating that widespread axonal changes are not yet detectable in the early clinical stage (Brex *et al.*, 1999; Hickman *et al.*, 2001). This may explain the significant difference in neurofilament levels detected between subjects with optic neuritis and controls, and at the same time, the lack of detectable difference seen between those with acute monosymptomatic ON and those that progressed to develop CDMS.

The lack of difference in S100B levels between subjects with ON and other neurological controls suggests that the astroglial activation, which occurs in the optic nerve as a result of inflammation, cannot be detected in the lumbar CSF.

CSF ferritin was not raised in subjects with ON. This finding is similar to that reported in RRMS (Petzold *et al.*, 2002). In comparison CSF ferritin is raised in SPMS. A number of mechanisms have been proposed for the alterations observed

in CSF ferritin levels, and these include an elevation seen in association with microglial activation. Disorders associated with intracranial haemorrhage and necrosis also results in an increase in CSF ferritin (Keir *et al.*, 1993). Finally, oligodendrocytes require iron to synthesize myelin [Hulet *et al.*, 1999] and ferritin binding is decreased in MS lesions (suggesting that oligodendrocytes bind myelin) (Connor *et al.*, 1996). It is plausible therefore that reduced CSF ferritin may occur with remyelination.

The observation that ferritin levels were depressed in this cohort of ON subjects is intriguing. One explanation is that the neurological controls had mildly elevated levels. An alternative explanation is that remyelination could be accounting for the ferritin reduction. A phase of active remyelination is likely to occur in the weeks following optic neuritis and this change would therefore coincide with the timing of the CSF examination in this study. These observations will need to be confirmed.

This study has shown that following ON, hyperphosphorylated neurofilament heavy chain is increased while ferritin may be reduced in the CSF, a month following symptom onset. This suggests, as expected, that acute axonal damage, of “healthy axons”, is a feature of optic neuritis and that remyelination may also be occurring at this stage. BSP measured in CSF may be useful markers of acute pathological change. Whether changes in CSF levels of BSP are capable of predicting outcome in demyelinating disease requires further study.

Table 4.1 Characteristics of subjects with other neurological disease

| Subjects | Age (yrs) | Sex | Diagnosis |
|----------|-----------|-----|----------------------------------|
| 1 | 73 | F | Normal pressure hydrocephalus |
| 2 | 29 | M | Depression |
| 3 | 32 | F | Tension headache |
| 4 | 36 | F | Tension headache |
| 5 | 65 | F | Normal pressure hydrocephalus |
| 6 | 54 | F | Tension headache |
| 7 | 52 | M | Normal pressure hydrocephalus |
| 8 | 23 | F | Tension headache |
| 9 | 69 | F | Frontotemporal atrophy |
| 10 | 27 | F | Tension headache |
| 11 | 23 | F | Benign intracranial hypertension |
| 12 | 51 | F | Blepharospasm |
| 13 | 68 | F | Parkinson's Disease |
| 14 | 43 | M | Parkinson's Disease |
| 15 | 23 | F | Atrophy of cerebellum |
| 16 | 67 | F | Normal pressure hydrocephalus |
| 17 | 21 | F | Hereditary ataxia |

Yrs, years; M, male; F, female.

Table 4.2 Clinical characteristics of subjects

| | OND (n=17) | ON(total) (n=41) | ON(CDMS) (n=8) | MS-type ON (n=21¹) | Non-MS- type ON (n=19) |
|--------------------|-----------------------------|-----------------------------------|---------------------------------|--|---|
| Male:Female | 3:14 | 13:28 | 2:6 | 4:17 | 8:11 |
| Age (yrs) | 36.0 (32.0-44.0) | 36.0 (31.0-43.5) | 43.0 (25.0-6.0) | 36.0 (29.5-42.0) | 38.0 (32.0-44.0) |

MS-type ON, subjects with positive MRI and CSF oligoclonal bands; Non-MS-type ON, subjects with normal MRI and negative CSF oligoclonal bands; ON(total),all subjects with optic neuritis; ON(CDMS), optic neuritis with clinically definite MS; OND, other neurological disorders; n,number of subjects; yrs,years.

Age expressed as median (interquartile range).

*One subject had oligoclonal IgG bands in CSF but not in serum, but did not have a MRI done.

Table 4.3 CSF characteristics expressed as median (interquartile range)

| BSP markers | OND (n=17) | ON(total) (n=41) | ON(CDMS) (n=8) | MS-type ON (n=21[*]) | Non-MS-type ON (n=19) |
|------------------------------------|--|--|----------------------------|--|----------------------------------|
| OB; | Not done | 22:19 | 8:0 | 21:0 | 0:19 |
| Pos:Neg | | 54 | 100 | 100 | 0 |
| % | | n=41 | n=8 | n=21 | n=19 |
| Total protein (g/L) | 0.41 ^a (0.36-0.48) n=16 | 0.50 ^a (0.39-0.60) n=39 | 0.55 (0.43-0.55) n=8 | 0.58 (0.44-0.61) n=21 | 0.47 (0.35-0.56) n=17 |
| Ferritin (ng/mL) | 7.0 (6.0-10.0) n=11 | 6.0 (4.0-6.0) n=37 | 6.0 (4.0-6.0) n=8 | 6.0 (4.0-6.0) n=19 | 6.0 (4.0-6.5) n=17 |
| S100B (ng/mL) | 0.57 (0.41-0.83) n=17 | 0.56 (0.41-0.72) n=41 | 0.55 (0.43-0.60) n=8 | 0.54 (0.40-0.76) n=21 | 0.57 (0.41-0.71) n=19 |
| NfH^{SMI34} (ng/mL) | 0.03 ^b (0.00-0.13) n=14 | 0.21 ^b (0.00-0.87) n=37 | 0.06 (0.00-0.50) n=8 | 0.14 (0.00-0.93) n=18 | 0.25 (0.00-0.94) n=18 |
| NfH^{SMI35} (ng/mL) | 0.17 (0.14-0.23) n=17 | 0.14 (0.11-0.18) n=41 | 0.15 (0.09-0.18) n=8 | 0.14 (0.12-0.17) n=21 | 0.15 (0.11-0.22) n=19 |
| NfH^{SMI34: SMI35} | 0.20 ^c (0.00-0.75) n=14 | 1.90 ^c (0.00-6.37) n=36 | 0.70 (0.07-4.20) n=7 | 1.08 (0.00-6.72) n=17 | 1.90 (0.00-6.03) n=18 |

ON(total), all subjects with optic neuritis; ON(CDMS), optic neuritis with clinically definite MS;

MS-type-ON, subjects positive for MRI and oligoclonal bands; non-MS-type-ON, subjects with

normal MRI and negative for oligoclonal bands; pos, positive for CSF oligoclonal bands;

neg, negative for CSF oligoclonal bands.

(continuation Table 4.3)

P values calculated by Mann-Whitney U Test.

^{a,b,c}Significant P value is taken at 0.05 level.

*One subject had oligoclonal IgG bands in CSF but not in serum, but did not have a MRI done.

Figure 4.2 Box-and-whiskers plot of levels of extensively phosphorylated neurofilaments in subjects with optic neuritis (n=41) and those with other neurological disorders (n=17) was significantly different (**P<0.05).

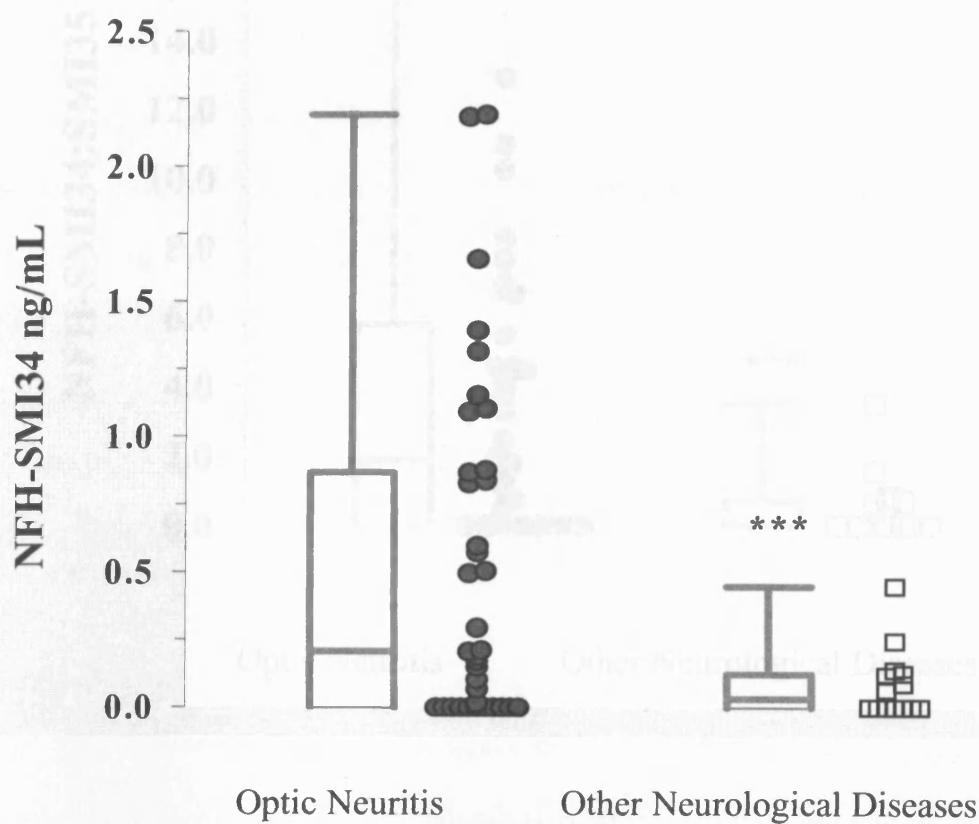
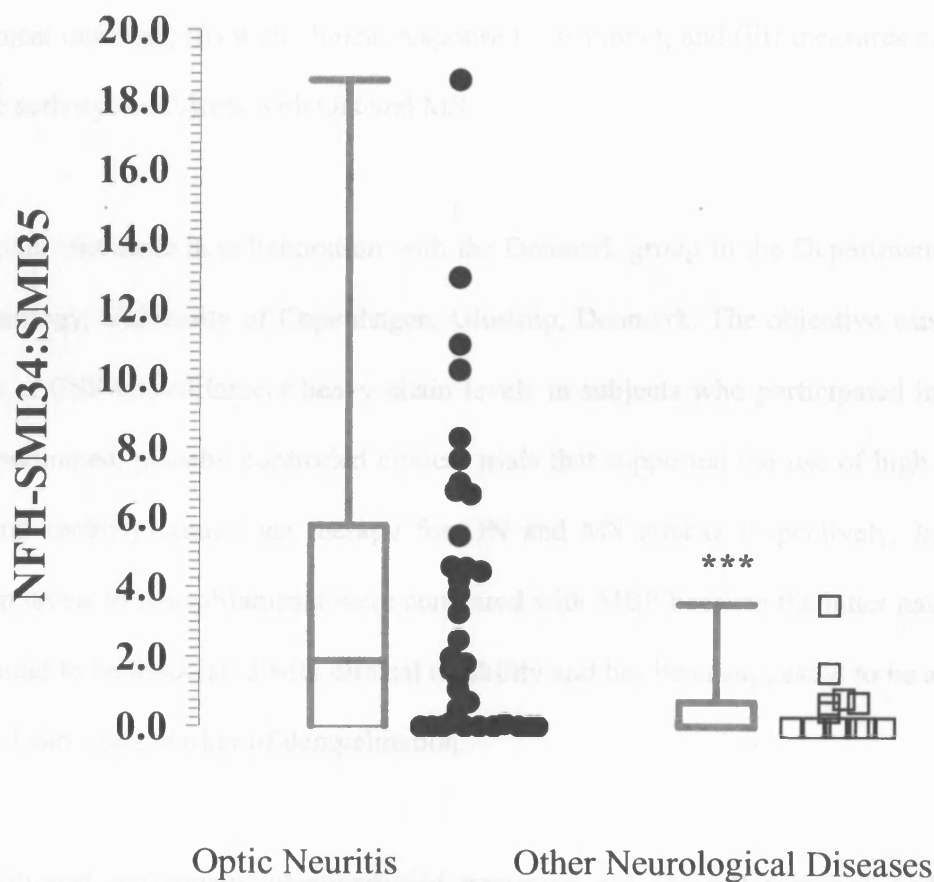


Figure 4.3 Box-and-whiskers plot levels of the ratio $\text{NfH}^{\text{SMI34}}:\text{NfH}^{\text{SMI35}}$ in subjects with optic neuritis (n=36) and that with other neurological disorders (n=14) was significantly different ($***P<0.05$).



Chapter 5

Cerebrospinal fluid levels of neurofilament heavy chain in acute optic neuritis and multiple sclerosis patients treated with oral high-dose methylprednisolone

5.1 Introduction

Following from the previous chapter, it is important to confirm the findings and to determine whether CSF neurofilament heavy chain levels were associated with, (1) clinical outcome, (ii) with clinical response to treatment, and (iii) measures of disease activity in subjects with ON and MS.

This study was done in collaboration with the Denmark group in the Department of Neurology, University of Copenhagen, Glostrup, Denmark. The objective was to look at CSF neurofilament heavy chain levels in subjects who participated in two randomized, placebo controlled clinical trials that supported the use of high-dose oral methylprednisolone therapy for ON and MS attacks respectively. In addition levels of neurofilaments were compared with MBP because the latter has been found to be associated with clinical disability and has been suggested to be a potential surrogate marker of demyelination.

As mentioned previously, glucocorticoid treatment reduces inflammation and accelerates recovery in some diseases. The efficacy of oral high-dose methylprednisolone in attacks of optic neuritis (ON) and multiple sclerosis (MS)

however has been controversial. Furthermore, acute inflammation may be an important cause of axonal loss and acute axonal damage has been observed in subjects with ON and MS (Trapp *et al.* 1988; Lycke *et al.* 1998; Semra *et al.* 2002).

As mentioned in detail in chapter 3, neurofilament heavy chain is a highly phosphorylated axonal protein found in all neuronal processes. It controls axonal caliber by forming cross bridges and by interacting with microtubules, thereby preserving the morphological integrity of axons and the conduction velocity of nerve impulses (Hoffman *et al.*, 1987). Its release into CSF in ON and MS may indicate that acute axonal loss is occurring in these conditions. High concentration of MBP has been found to be associated with clinical disability in patients with MS and ON (Thompson *et al.*, 1985; Barkhof *et al.*, 1992a; Sellebjerg *et al.*, 1998b), suggesting its potential role as a surrogate marker in demyelinating disease.

5.2 Aims

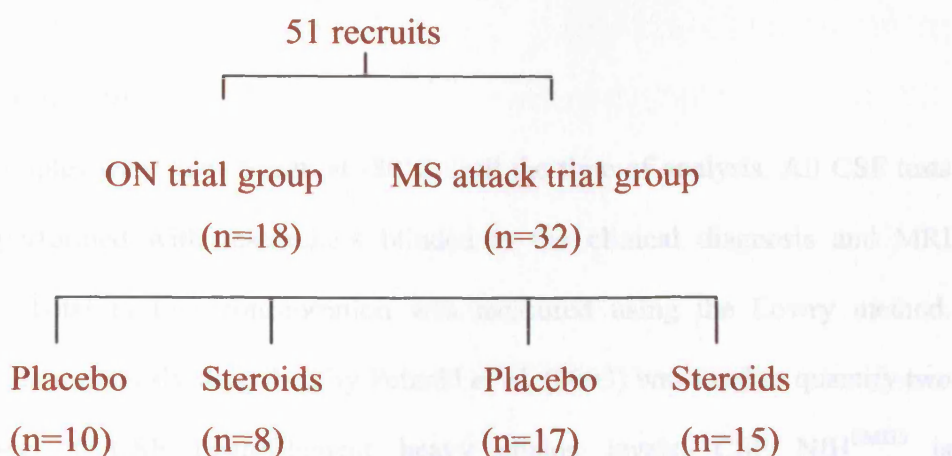
This study looks at CSF neurofilament heavy chain levels in a subgroup of subjects who participated in two randomized, placebo-controlled trials that supported the use of oral high dose methylprednisolone in acute ON and attacks of MS (Sellebjerg *et al.* 1998a; 1999). The objective of this study is to determine whether CSF neurofilament heavy chain levels were associated with clinical response to treatment and measures of disease activity including MBP.

5.3 Methods

5.3.1 Patients and examination

Fifty subjects who gave informed consent to participate in two double blind, randomised, placebo-controlled clinical trials were included in a repeated lumbar puncture study (Sellebjerg *et al.*, 1998a; 1999; 2000). Eighteen patients were included in the ON trial (Sellebjerg *et al.*, 1999) and 32 subjects were included in the MS attack trial (Sellebjerg *et al.*, 1998a). Eight subjects with ON were treated with oral methylprednisolone and 15 subjects with MS attacks also received treatment with oral methylprednisolone: 500mg once daily for 5 days, then weaned daily according to the following regime: 400mg, 300mg, 200mg, 100mg, 64mg, 48mg, 32mg 16mg, 8mg and 8 mg once daily, i.e., a total treatment duration of 15 days. Twenty-seven subjects had oral placebo.

Figure 5.1 Number of subjects who received treatment



Subjects in the ON trial received scores on the visual function system of the Kurtzke EDSS (Kurtzke, 1983) and those in the MS attack trial received scores on the Kurtzke EDSS. Their clinical disability was assessed pre-treatment, and at 1, 3, 8 and 52 weeks post-treatment. Lumbar puncture was performed prior to treatment and a week after completing treatment.

5.3.2 MRI

MRI of the brain was performed a median of one day prior to treatment (interquartile range 0-2 days) (Sellebjerg *et al.*, 2003). Quantitative analysis of the enhancing lesion volume (ELV) was measured, and T2-weighted MRI was classified as normal or being highly suggestive of MS (Sellebjerg *et al.*, 2003; Paty *et al.*, 1988).

The regional scientific ethics committee approved the study.

5.3.3 CSF tests

CSF samples were kept frozen at -80°C until the time of analysis. All CSF tests were performed with researchers blinded to the clinical diagnosis and MRI results. Total protein concentration was measured using the Lowry method. ELISA as previously described by Petzold *et al.* (2003) was used to quantify two subtypes of CSF neurofilament heavy chains levels. CSF NfH^{SMI35} is phosphorylated and NfH^{SMI34} is extensively phosphorylated neurofilament heavy chain (Petzold *et al.*, 2003). A ratio of NfH^{SMI34} to NfH^{SMI35} was also used..

Sellebjerg and colleagues had determined the CSF MBP levels in their laboratory in Denmark using a previously described method (Sellebjerg *et al.* 1998b).

5.3.4 Statistical analyses

The statistical analysis was performed in conjunction with Dr Daniel Altmann (Medical Statistics Unit, London School of Hygiene & Tropical Medicine, London). Ordered logistic regression with proportional odds assumption (Greene 2000) was carried out in the MS and ON attack trial groups separately to examine associations between change in EDSS as an outcome and a neurofilament level predictor variable; EDSS change was recoded before analysis by collapsing categories with 5 or less patients: for change 8 week - baseline these were [-3 to -1.5], [-1, -0.5], [0], [0.5 to 3], for 52 week – baseline [-3.5 to -1.5], [-1, -0.5], [0 to 5], and for 52 -8 week [-4 to -1], [-0.5], [0], [0.5 to 2]. In the ON attack trial group the 8 week – baseline categories were [-5, -4], [-3 to -1]; and the 52 week – baseline and 52 - 8 week categories were [-5, -4], [-3, -2], [-1, 0] and [-1, 0], [1] respectively. The neurofilament predictors examined for CSF NfH and CSF NfH were 3 week level, and the change in level 3 week-baseline. Potential confounding by age, gender, MS duration, symptom duration and steroid treatment was examined by introducing these terms as covariates in the regression. There was no evidence in any of the models that the proportional odds assumption was not satisfied.

The two trial groups were combined in an analysis of a dichotomised EDSS change variable: patients were classified according to whether they were in the more or less deteriorating (approximate) half of their respective group. EDSS changes 3 week – baseline, 8 week – baseline, 52 week – baseline and 52 week – 8 week were dichotomised. The thresholds used for 3 week - baseline were EDSS change ≤ -0.5 in the MS group and ≤ -3 in the ON attack group; for 8 week – baseline the respective thresholds were ≤ -1 and ≤ -4 ; for 52 week – baseline respectively ≤ -0.5 and ≤ -3 ; and for 52 week – 8 week respective ≤ -0.5 and ≤ 0 . The binary variables were analysed by logistic regression on the neurofilament predictors, with covariates as above for examination of potential confounding.

The neurofilament variables were somewhat skewed, and the difference in these variables between treated and untreated patients (both trial groups combined) were assessed using the Wilcoxon rank sum test. Spearman rank correlation was used to examine associations between these variables and baseline enhanced lesion volume. Association between these variables and the three categories of T2-weighted MRI abnormality were examined by a non-parametric test for trend (Cuzick, 2001). Spearman rank correlation was also used to examine associations between neurofilaments and MBP. Statistical analysis was performed using Stata 7.0 (Stata Corporation) and SPSS 11.5.

5.4 Results

5.4.1 CSF neurofilament heavy chain levels and clinical disability

5.4.1.1 MS attack trial group

The result of the ordered logistic regression to examine associations between CSF neurofilament levels and change in clinical outcome are shown in Table 5.1. There was evidence that absolute level of CSF NfH^{SMI35} predicted greater 0 to 8 week EDSS deterioration (odds ratio of being in worse adjacent category of deterioration per unit increase in CSF NfH^{SMI35} was 4.24, 95% confidence interval 1.04 to 17.30; p=0.044). In addition, there was evidence that greater clinical deterioration from 0 to 52 weeks was predicted by greater increase compared to baseline in 3 week CSF NfH^{SMI34} (odds ratio 1.76, 95% confidence interval 1.04 to 2.98; p=0.035); and similar evidence for an association of this predictor with late EDSS deterioration 8 to 52 weeks (odds ratio 2.24, 95% confidence interval 1.08, 4.64; p=0.030). No evidence of any association was seen between the ratio of NfH^{SMI34} to NfH^{SMI35} and clinical disability. There was no evidence of confounding by age, gender, MS duration, symptom duration or treatment.

5.4.1.2 ON attack trial group

In the ON trial group, there was no evidence of any association between the CSF neurofilament heavy chain levels and changes in EDSS.

5.4.2 Effect of treatment on clinical outcome

When both MS and ON trial groups were combined, there is some evidence that patients treated with steroids had less EDSS deterioration between baseline and week 3 (odds ratio that treated patients were in the more deteriorating half of group was 0.30, 95% confidence interval 0.09 to 0.97; $p=0.045$). However, there was no evidence of a treatment difference in the more long-term EDSS changes.

5.4.3 Effect of treatment on CSF neurofilament levels

Steroid treatment had no effect on the levels of CSF neurofilament heavy chains in the combined ON and MS group.

5.4.4 CSF neurofilament heavy chain levels and MRI measures (Table 5.2)

Baseline CSF NfH^{SMI34} and NfH^{SMI35} were both significantly positively correlated with baseline ELV. The level of CSF NfH^{SMI35} at week 3 was also significantly positively correlated with baseline ELV, whereas there was only a borderline significant and reduced positive correlation between CSF NfH^{SMI34} measured at week 3 and baseline ELV. There was also borderline evidence of a positive correlation between ELV and the CSF NfH^{SMI34}:NfH^{SMI35} ratio at baseline and at week 3.

T2-weighted MRI abnormality was associated with higher values of CSF NfH^{SMI35} at baseline and week 3 ($p=0.005$ and $p=0.002$ respectively), but not with the week 3 – baseline change. There was no evidence of association with CSF

NfH^{SMI34} or the ratio NfH^{SMI34}:NfH^{SMI35} at either time point, nor with changes in these.

5.4.5 CSF neurofilament heavy chain levels and MBP

Figures 5.2 and 5.3 illustrate that CSF NfH^{SMI35} at baseline and week 3 were both statistically significantly positively correlated with MBP measured at baseline ($r=0.56$ $p=0.006$ and $r=0.77$ $p<0.001$) and week 3 ($r=0.63$ $p<0.001$ and $r=0.68$ $p<0.001$). CSF NfH^{SMI34} at week 3 correlated with the concentration of MBP at week 3 ($r=0.58$ $p=0.004$). It is known that methylprednisolone treatment results in a marked decrease in the CSF concentration of MBP [Barkhof *et al.* 1992; Sellebjerg *et al.* 2003]. Nonetheless additional analysis in the steroid treated group did not observe any correlation between the degree of MBP decrease and the amount of increase in levels of CSF neurofilament heavy chain.

5.5 Discussion

This study suggests that complex changes in CSF neurofilament phosphoform levels seen after the initial attack relate to clinical outcome in the subsequent two month to one year period in a cohort of subjects with clinically definite MS. As a consequence one is able to evaluate the health of the axons prior to damage, the extent of the damage, and the potential for recovery or non-recovery over the subsequent months.

In the MS attack trial group, high levels of CSF neurofilaments are predictive of poorer clinical outcome and of continuing deterioration between 8 weeks and one

year post-relapse. These new findings suggest that the degree of axonal damage from a relapse can determine functional impairment in the longer term. Although there was no statistical evidence of an association between clinical outcome and CSF neurofilament levels when the ON trial group was taken into account separately, one must bear in mind that the ON sample size is small and that the patients with ON have no spinal cord disease. CSF BSP levels are more likely to be representative of what is occurring locally, i.e. in the spinal cord rather than in the optic nerve that is distal to the CSF outflow pathways of the IVth ventricle. Of note, when both groups were combined, we observe a similar pattern to that seen in the MS attack trial group alone.

The present study also shows that CSF neurofilament heavy chain levels correlate with MBP, gadolinium enhancing lesion volume and T2-weighted MRI abnormalities in subjects with ON and MS. High concentrations of the structural myelin protein, MBP in CSF is implicated in ongoing inflammation and has been found to be associated with clinical disability in patients with demyelinating disease (Thompson *et al.*, 1985; Barkhof *et al.*, 1992a; Sellebjerg *et al.*, 2003), and steroid treatment results in suppression of gadolinium-enhancement and the a decrease in the concentration of MBP in CSF (Thompson *et al.*, 1985; Barkhof *et al.*, 1992a; Sellebjerg *et al.*, 2003). However, current results show no change in clinical EDSS outcome with steroid treatment at one year which raises the question of how reliable EDSS is as a clinical measure.

An important question is what effects anti-inflammatory therapy such as methylprednisolone has on axonal markers found in the CSF? The treatment effect observed in the patient subgroups included in the present study confirm the earlier results which showed that after treatment with oral methylprednisolone in both ON and MS attack trial groups (Sellebjerg *et al.*, 1998a, 1999), the chance of worse clinical outcome is less, with a 70% reduction in odds estimated from our analysis, suggesting that steroids hastens recovery.

Axonal damage appears to be more marked in the most acute of lesions and so strategies that reduce inflammation would be expected to be neuroprotective. We have found that steroid treatment has no effect on the levels of CSF neurofilament heavy chain levels therefore the observed short term recovery post-steroid in both ON and MS group is independent of the release of neurofilaments.

Many studies have confirmed that axonal damage is found in MS plaques (Trapp *et al.*, 1988; Kornek and Lassmann 1999; Ferguson *et al.*, 1997). MRI and spectroscopic methods provide accurate means of assessing axon loss and have been shown to correlate with disability in MS (Matthews *et al.*, 1998; Grimaud *et al.*, 1999; Paolillo *et al.*, 2000; Pelletier *et al.*, 2001; Fischer *et al.*, 2000). The findings in this study support the latter view as well as suggesting that acute inflammation in these demyelinating diseases results in axonal pathology. Furthermore, my results are in agreement with the notion that axonal degeneration may continue for some time after the resolution of inflammation.

The relationship between the two different subtypes of neurofilament heavy chain is not understood. NfH^{SMI34} is more phosphorylated than NfH^{SMI35} and the latter is thought to be indicative of axons subjected to chronic injury. Thus a ratio of NfH^{SMI34} to NfH^{SMI35} is thought to represent a putative index of 'axonal health'. This hypothesis however was not confirmed in this study. Nonetheless, neurofilament deficient axons have the ability to regrow and remyelinate albeit at a slower rate (reviewed by Medana and Esiri, 2003). Anti-inflammatory therapy has minimal effect on relapse response. Perhaps, therapeutic intervention should consider targeting axonal recovery that seems to be associated with future disability.

In conclusion the results of the present study provide evidence that the severity of axonal damage determines clinical outcome and confirms previous reports that acute inflammation in MS results in axonal pathology.

Table 5.1 Results of ordered logistic regression in multiple sclerosis trial group examining association between EDSS change and neurofilament levels.

| Variable | | P value ^a |
|---------------------------------------|--|----------------------|
| EDSS change week 8 minus baseline | CSF NfH ^{SMI34} at week 3 | 0.504 |
| | Change in CSF NfH ^{SMI34} at week 3 minus baseline | 0.968 |
| | CSF NfH ^{SMI35} at week 3 | 0.044 |
| | Change in CSF NfH ^{SMI35} at week 3 minus baseline | 0.503 |
| EDSS change week 52 minus baseline | CSF NfH ^{SMI34} at week 3 | 0.080 |
| | Change in CSF NfH ^{SMI34} at week 3 minus baseline | 0.035 |
| | CSF NfH ^{SMI35} at week 3 | 0.566 |
| | Change in CSF NfH ^{SMI35} at week 3 minus baseline | 0.243 |
| EDSS change week 52 minus week 8 | CSF NfH ^{SMI34} at week 3 | 0.080 |
| | Change in CSF NfH ^{SMI34} at week 3 minus baseline | 0.030 |
| | CSF NfH ^{SMI35} at week 3 | 0.336 |
| | Change in CSF NfH ^{SMI35} at week 3 minus baseline | 0.254 |

NfH^{SMI35}, phosphorylated neurofilament; NfH^{SMI34}, extensively phosphorylated neurofilament; NfH^{SMI34: SMI35}, ratio of extensively phosphorylated neurofilament to phosphorylated neurofilament; week 3, one week post-treatment.

^aThe P-value tests the null hypothesis that odds of being in a higher category of EDSS change do not vary with values of the neurofilament predictor. P-values rejecting null hypothesis give evidence that higher predictor levels are associated with greater clinical deterioration.

Table 5.2 Spearman rank correlation coefficients were used to examine associations between CSF neurofilament heavy chain levels and baseline T1 weighted MRI gadolinium area (enhancing lesion volume).

| Variable | Spearman correlation coefficient | P-value |
|--|----------------------------------|--------------------|
| CSF NfH ^{SMI34} at baseline | 0.50 | 0.005 ^a |
| CSF NfH ^{SMI34} at week 3 | 0.33 | 0.06 |
| CSF NfH ^{SMI35} at baseline | 0.53 | <0.01 ^b |
| CSF NfH ^{SMI35} at week 3 | 0.65 | <0.01 ^c |
| NfH ^{SMI34} :NfH ^{SMI35} at baseline | 0.39 | 0.07 |
| NfH ^{SMI34} :NfH ^{SMI35} at week 3 | 0.42 | 0.05 ^d |

NfH^{SMI34}:SMI35, ratio of extensively phosphorylated neurofilament to phosphorylated neurofilament

P-values calculated by Spearman rank correlation.

^{a,b,c,d} Significant P value is taken at 0.05 level.

Figure 5.2 The association between neurofilament and myelin basic protein at baseline in the placebo group.

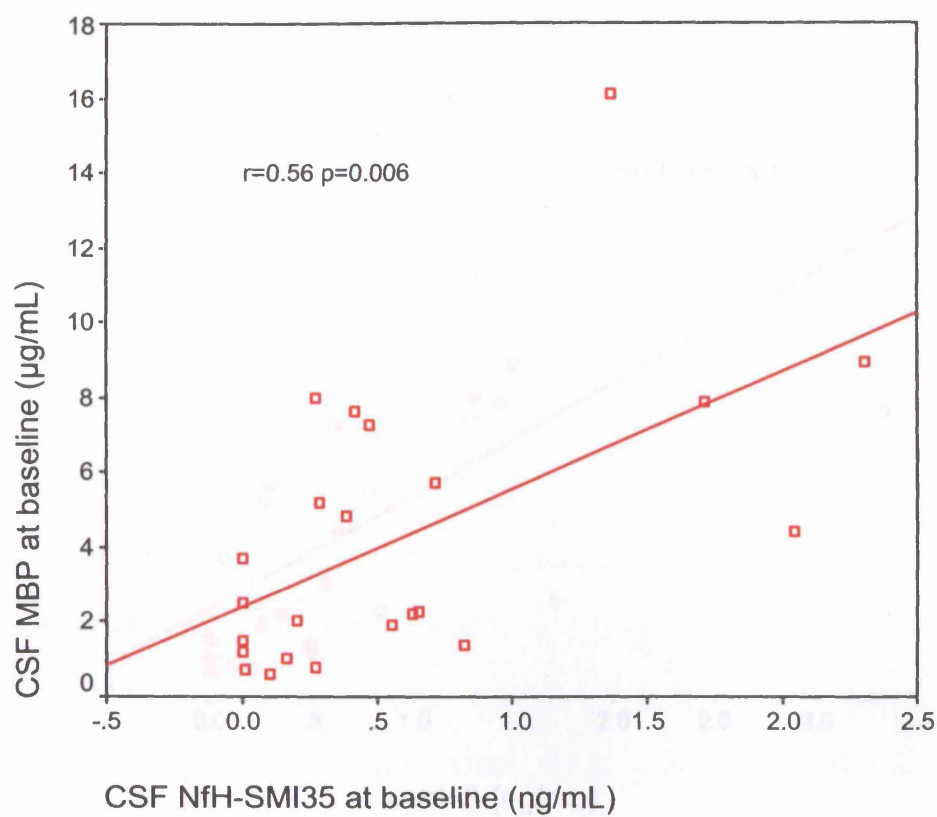
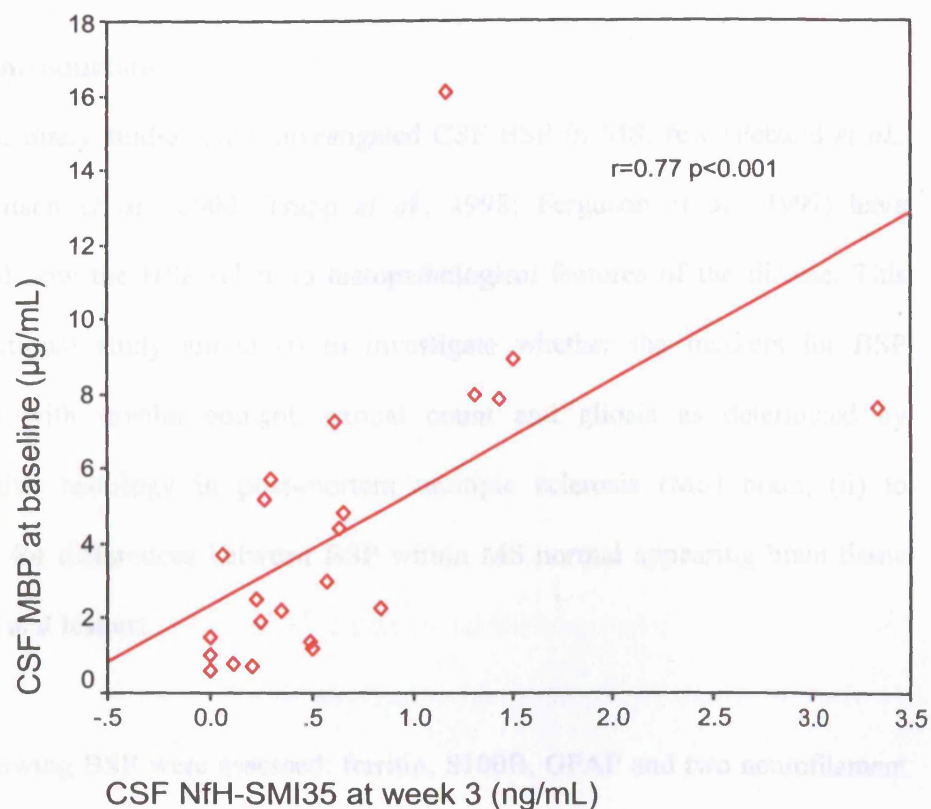


Figure 5.3 The association between neurofilament measured after treatment and baseline MBP in the placebo group.



Chapter 6

A comparison of the brain specific proteins and histopathology in post mortem multiple sclerosis brain

6.1 Introduction

Although many studies have investigated CSF BSP in MS, few (Petzold *et al.*, 2002; Bitsch *et al.*, 2000; Trapp *et al.*, 1998; Ferguson *et al.*, 1997) have described how the BSP relate to histopathological features of the disease. This cross-sectional study aimed (i) to investigate whether the markers for BSP correlate with myelin content, axonal count and gliosis as determined by quantitative histology in post-mortem multiple sclerosis (MS) brain, (ii) to evaluate for differences between BSP within MS normal appearing brain tissue (NABT) and lesions.

The following BSP were assessed: ferritin, S100B, GFAP and two neurofilament heavy chain phosphoforms (NfH^{SMI34} and NfH^{SMI35}). As previously mentioned, ferritin is a marker for normal and activated microglia and also a marker for oligodendrocyte activity. It is indirectly involved in myelin production, as oligodendrocytes are the predominant iron-containing cells in the brain. During stress such as hypoxia, oligodendrocytes increase their synthesis of ferritin and thereby protect cells against oxidative damage (Cole, 2003; Arosio and Levi, 2002). S100B is secreted by astrocytes and it is implicated in the regulation of cytoskeletal dynamics principally via protein phosphorylation and cell cycle

progression, most likely by interacting with transcription factors and enzymes (Schafer and Heizmann 1996, Donato 1999). GFAP is a useful biological marker of astrocytic activity, and it is upregulated in astrogliosis (Eng and Ghinikar, 1994). Finally, neurofilament heavy chain is a highly phosphorylated axonal protein found in all neuronal processes. NfH^{SMI35} is phosphorylated and NfH^{SMI34} is extensively phosphorylated neurofilament heavy chain (Petzold *et al.*, 2003). Neurofilaments control axonal caliber by forming cross bridges and by interacting with microtubules, thereby preserving the morphological integrity of axons and the conduction velocity of nerve impulses (Al-Chalabi and Miller, 2003).

The objective of this study was to determine whether (i) neurofilaments and S100B quantified *in vitro* relate to axonal marker count; (ii) GFAP (ELISA) levels correlate with the extent of gliosis quantified histologically; and (iii) ferritin levels relate to myelin content in post-mortem brain tissue of patients with MS.

6.2 Materials and Methods:

Fresh post- mortem brain slices (10mm thick) of 14 subjects with MS (12 women and 2 men) were obtained from the UK Multiple Sclerosis Tissue Bank at the Charing Cross Hospital, Imperial College School of Medicine, London, UK. The median age (and interquartile range) of the patients was 57 years (49-73), with median disease duration of 29 years (18-34). The post-mortem interval was 46 hours (41-59). Sixty-seven macroscopically visible plaques were sampled along with 109 samples of brain tissue from normal appearing grey matter (NAGM;

n=54) and white matter (NAWM; n=55). The samples were homogenized and then analysed for BSP using ELISA technique. For the comparison of lesions with NAWM using BSP and histological techniques, material from lesions and the adjacent NAWM were obtained from 11 MS brain slices (28 tissue samples in total). Three patients were excluded from this analysis because they had no macroscopic lesions but all 14 brain slices were used in the subsequent analysis of NAWM and lesions using BSP markers alone – although in the 3 brain without lesions, only samples from NABT were obtained).

6.2.1 Protein extraction

Brain tissue (wet weight between 1 and 2 g) were finely cut and suspended in barbitone buffer containing 0.1% bovine serum albumin. Samples were homogenized on ice by sonification for 60 seconds. Myelin protein was extracted by adding di-isopropyl ether in the proportions of 1:500, centrifuged at 20,000 x g and the lipid layer removed. The supernatant was stored at -80°C until further analysis. Total protein concentration was measured using the Lowry method (Peterson 1979).

6.2.2 Brain specific protein analyses

Levels of S100B, GFAP, ferritin and the two neurofilament heavy chain phosphoforms (NfH^{SMI35} and NfH^{SMI34}) were quantified in brain tissue preparation by sandwich ELISA technique as previously described by Green *et al.* (1997), Petzold *et al.* (2004), Keir *et al.* (1993), and Petzold *et al.* (2003). The dilutions used were as follows: S100B in a dilution of 1:1000 without EDTA in

the washing solution, GFAP in a dilution of 1:5000, ferritin in a dilution of 1:100 and NfH^{SMI35} and NfH^{SMI34} in a dilution of 1:5000. All values of BSP are described as ratios to total protein.

6.2.3 *Histology studies*

The histological studies were performed by Klaus Schmierer. In these, the myelin content, extent of gliosis and axonal count in lesions and areas of NAWM were assessed. NABT were visually co-registered, in which the brain slice was divided using a 5mm deep iron angle with the cut surface corresponding to the centre of the MR imaging plane. T2 weighted MRI guidance was used to ascertain NABT and to record any suspected lesion on MRI that was macroscopically visible. Stereotactic co-registration was used for lesions. Tissue blocks of approximately 2.25cm³ in volume and centred on each region of interest (ROI) were dissected. The blocks were then cut in half using a 5mm deep iron angle resulting in two blocks of equal thickness with the cut surface corresponding to the centre of the MR imaging plane. The macroscopically visible lesion of the tissue of the cut surface was recorded.

All dissected blocks were marked with a notch at a known position, usually on the ventral cut surface (figure 6.1) and processed for paraffin embedment. Sections were stained with Haematoxylin and Eosin (H&E), Luxol-Fast blue (LFB), and Bielschowsky's silver impregnation (BIEL) (figure 6.2).

Klaus Schiemerer then classified the tissue sections according to any evidence of lesion activity, demyelination or remyelination. The lesion stage¹³ was categorized as either

1. Active (inflammation throughout the lesion)
2. Chronic active (hypocellular centre, inflammation only at the rim of the lesion), or
3. Chronic inactive (no inflammation).

Demyelinated lesions were defined as clearly distinct, sharply demarcated areas of myelin loss on LFB stained slides. Remyelinated lesions were defined as clearly distinct, sharply demarcated areas with decreased intensity of LFB staining compared to the surrounding NAWM and uniformly thin myelin sheaths (in relation to axon diameter) throughout the lesion i.e. shadow plaques (Prineas *et al.*, 2002).

Klaus Schiemerer counted the axons on BIEL-stained slides using a 21 bar (bar length: 13 μ m) quadrate grid graticule (size: 160 μ m³) and a final magnification of x1250. Random points were superimposed to lesions and surrounding NAWM. On each slide the total number of bars intersecting axons were counted in 12-16 areas of both the lesion and surrounding NAWM (Bruck *et al.*, 1997). The counts were then averaged for each lesion and respective NAWM ROI. The average axon count within the lesions divided by the average axon count in the respective

¹³ The same staging category was previously used by Trapp *et al.* (1998) and van der Valk and de Croot (2000).

NAWM provided the relative measure of axonal loss for each lesion (expressed in percent).

Myelin content was quantified in lesions and NAWM by assessing transmittance (Tr), defined as the transmitted light divided by the incident light, on LFB-stained sections using a Leica Q500MC digital image analyser with a 256 grey scale resolution (Leica Cambridge Ltd., UK), which was mounted on a Zeiss photomicroscope 3 (Carl Zeiss, Jena, FRG). The program was set in RGB mode, the white level was kept constant at 75% of the maximum, and a final magnification of x125 was used. Within every ROI (i.e. a lesion or an equally sized region of using a field size of $400 \times 385 \mu\text{m}^2$) the values obtained from each area were averaged, and then divided by the light intensity transmitted through the object slide (away from the tissue section) to result in $\text{Tr}_{\text{myelin}}$ ¹⁴ values for lesions and NAWM (Nabors *et al.*, 1988; Gentleman *et al.*, 1999).

Gliosis in lesions and NAWM was classified on GFAP-stained slides quantitatively in lesions and the surrounding NAWM in the same manner as for myelin content and expressed as $\text{Tr}_{\text{gliosis}}$.

The thickness of LFB- and GFAP-stained sections was assessed using a stereological microscope and a final magnification of x1250. In order to control

¹⁴ A high value of $\text{Tr}_{\text{myelin}}$ reflects low myelin content.

for possible systematic errors in the measurements of Tr, on each slide, three to five measurements were performed and averaged.

6.2.4 Statistical Analyses

All post mortem brain homogenate analyses were performed with researchers blinded to the histopathology results. Statistical analyses: comparisons between groups were made with two sample T-tests and relationships between parameters were made with Spearman correlation coefficients, using the SPSS software package (version 11.0 for Windows). All levels of BSPs presented are ratios of BSP to total protein, e.g. ferritin:total protein, GFAP:total protein, S100B:total protein, NfH^{SMI34}:total protein and NfH^{SMI35}:total protein. Probability values less than 0.05 were considered significant. Multiple comparisons were done when investigating the relationships of BSP, therefore, after Bonferroni's correction, p values of less than 0.01 were considered to indicate statistical significance.

6.3 Results

6.3.1 BSP vs. histopathology (Table 6.1)

Twenty eight brain tissue samples from eleven brain slices were available for BSP and histopathology quantitative studies. Thirteen/28 brain tissue homogenates from 9 coronal brain slices were obtained from lesions that could be matched with 13 brain tissue samples from the adjacent macroscopic NAWM. 2/28 brain tissue samples from two brain slices were excised from NAWM only.

GFAP (ELISA) inversely correlated with axonal density ($r=-0.51$, $p=0.01$) and the number of axons ($r=-0.46$, $p=0.01$) (see table 6.1, figures 6.3 and 6.4). There was borderline evidence that S100B inversely correlated with axonal density ($r=-0.33$, $p=0.09$) (table 6.1). There was no evidence of an association between neurofilament heavy chain levels and axonal density or between neurofilament heavy chain levels and axonal count. GFAP did not correlate with the extent of gliosis detected histopathologically.

6.3.2 BSP in normal appearing brain tissue vs. lesions

A total of 176 brain tissue homogenates were excised from 14 post-mortem brains. Sixty-seven of the 176 (38%) brain tissue specimens were from macroscopically visible lesions and 109/176 (62%) were from NABT. Of the latter, 55/176 (31%) constituted NAWM and 54/109 (31%) NAGM. Fifty seven/176 (32%) samples of lesions were excised from the white matter and 10/176 (10%) from the grey matter.

Table 6.2 illustrates that ferritin levels was highest in NABT and lowest in lesions, particularly lesions in the grey matter. The ferritin levels in grey matter lesions is significantly lower than the levels in NAWM, NAGM and WM lesions (all p values <0.01).

Higher levels of S100B were detected in white matter in comparison to GM, with the highest levels in macroscopically detectable WM lesions and the lowest level

in GM lesions. This astroglial protein was sevenfold higher in WM lesions than in GM lesions (p-value<0.001). There was no significant difference between levels of S100B in macroscopically normal white matter and WM lesions.

Similarly, levels of GFAP (ELISA) were highest in WM lesions and lowest in GM lesions. However, the difference between GFAPs levels in WM lesions and NAWM was nearly threefold (p<0.05).

There was no significant difference in NfH^{SMI34} and NfH^{SMI35} levels in NABT and macroscopically detectable lesions.

6.3.3 Relationships between BSPs (ELISA) (Table 6.3)

In NAGM and NAWM, S100B is associated with ferritin (r=0.65 p<0.01, r=0.49 p<0.01) and GFAP (ELISA) (r=0.40 p<0.01, r=0.49 p<0.01 respectively). There is also evidence that in NABT, ferritin correlates with GFAP (ELISA) and NfH^{SMI34} (r=0.65 p<0.01, r=0.51 p<0.01 (in NAGM); r=0.47 p<0.01, r=0.6 p<0.01 (in NAWM)), and that the axonal marker NfH^{SMI34} correlates with NfH^{SMI35} (r=0.49 p<0.01 (in NAGM); r=0.54 p<0.01 (in NAWM)).

In WM and GM lesions, the astroglial marker S100B is associated with GFAP (ELISA). Similar to NABT, ferritin correlates with GFAP (ELISA) and NfH^{SMI34} in white matter lesions (r=0.50 p<0.01, r=0.41 p<0.01 respectively). In GM lesions, NfH^{SMI34} correlates with NfH^{SMI35} (r=0.83 p<0.01).

6.4 Discussion

This study suggests that axonal loss in MS as measured using histopathological techniques is more pronounced in the vicinity of reactive gliosis as measured via ELISA though there was no evidence suggesting this by using histology alone. S100B, an astrocytic protein, was also more abundant in regions of axonal loss. However, neurofilament heavy chain levels did not correlate with the axonal count and density; GFAP (ELISA) did not correlate with the extent of gliosis; and ferritin was not associated with myelin content; possible reasons for these negative results will be discussed below. Of note, BSP were able to detect differences between GM and WM (ferritin) and between lesions and normal appearing brain tissue in GM (ferritin and S100B) and in WM (GFAP and NfH^{SMI34}).

The presence of axonal injury in the vicinity of reactive gliosis is intriguing. Astrocytic proliferation and astrogliosis occur in response to CNS insult (Penkowa *et al.* 2003; Eng *et al.*, 2000), and acute inflammatory changes in MS lesions are associated with axonal transection (Trapp *et al.*, 1998). However the areas of astrocytic activation as indicated by the GFAP levels were predominantly in WM lesions, less in NAWM, and less in the grey matter. Therefore one possible explanation for the link between GFAP and axonal density is that acute inflammatory insult results in both processes. The histological analysis of the samples used for this study has shown that the extent of gliosis did not differ between lesions and NAWM (Schmierer *et al.*, 2004), rather that fibrillary gliosis

was present throughout MS white matter (Allen *et al.*, 2001; Schmierer *et al.*, 2004). Further studies are required to resolve these conflicting findings.

We have also found abundant ferritin in the NAGM and particularly low levels in lesions. In normal adult human brain tissue, the total ferritin concentration has been analyzed separately in white and grey matter from the cerebrum and there are no marked differences between the two tissues (Connor *et al.* 1992). It is mainly contained within oligodendrocytes (Gerber and Connor, 1989; Gelman *et al.* 1992; Kaneko *et al.*, 1989; Bradbury, 1997) but is also a marker for normal and activated microglia. It is also significantly elevated in the CSF of chronic progressive active MS patients i.e. patients who had deterioration of their disease status within the past 12 months, compared to normal individuals (Levine 1999), and the elevated ferritin levels in normal brain tissue could be a possible defense mechanism to protect against iron induced oxidative damage (Cole, 2003; Arosio and Levi, 2002). We have also observed elevated ferritin in GM in comparison to WM (both in lesions and normal appearing brain tissue). Possible reasons for this include that ferritin iron contributes to the neurodegenerative pathway of MS i.e. increased toxicity, which is also seen in Parkinson's disease and Alzheimer's disease (Bartzokis *et al.*, 2004). In vivo MRI that measure and track brain ferritin iron level is required to confirm this finding (Bartzokis *et al.*, 2004).

The presence of S100B in CSF is normally due to an immediate release from the cytosol of astrocytes and an upregulation of its release has been implicated as an

index of CNS injury (Petzold *et al.*, 2003). A high concentration of this astrocytic protein is seen in subarachnoid haemorrhage, rapid parenchymal destruction in epilepsy, Creutzfeldt-Jakob disease, acute brain injury, compression of the spinal cord by space-occupying lesions, ischaemic disorders and viral or suspected viral infection (Donato 2001, Sindic 1982). We have found a high concentration of S100B in white matter lesions and a significantly lower level in grey matter lesions. Of note, GM lesions have been shown to be less inflammatory than WM lesions and difference in the pathology between GM and WM lesions might explain the differences in GFAP that we have observed (Bo *et al.*, 2003a; 2003b; 2004).

Significantly higher levels of extensively phosphorylated neurofilament heavy chain (NfH^{SMI34}) are found in NAWM compared to white matter lesions. This supports the hypothesis that axons are healthier within NABT compared to axons traversing lesions (Lim *et al.*, 2004).

The lack of a correlation between neurofilaments and axonal count; GFAP (ELISA) and gliosis, and ferritin and myelin content may be due to methodological limitations of this study. The MS lesions were not sampled using MRI-guidance, the tissue block is 5mm below the site of tissue sampling, and a proportion of abnormalities detected by post-mortem MRI may not be macroscopically visible (van Waesberghe *et al.*, 1999; Bo *et al.*, 2004). The brain

slices were also not from the same anatomical region and this will almost certainly have confounded our results.

In conclusion, this study has shown a relationship between GFAP levels as measured by ELISA and the degree of axonal loss detected in the same tissue sample. In addition, GFAP was elevated and NfH^{SMI34} reduced in WM lesions in comparison to NAWM. These data provide further evidence for a relationship between axonal injury and reactive gliosis.

Figure 6.1 Brain slice indicating ventral and dorsal sides with arrow pointing to plaques which were sampled on either side. Adjacent NABT were also sampled.

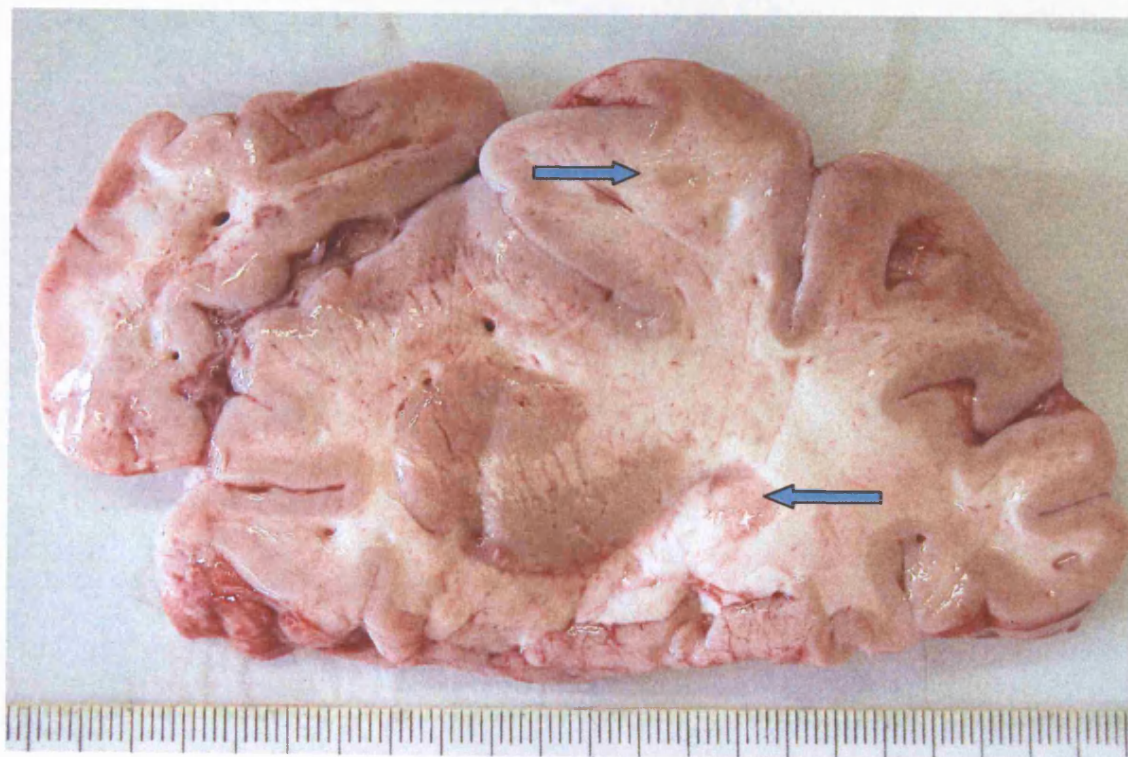
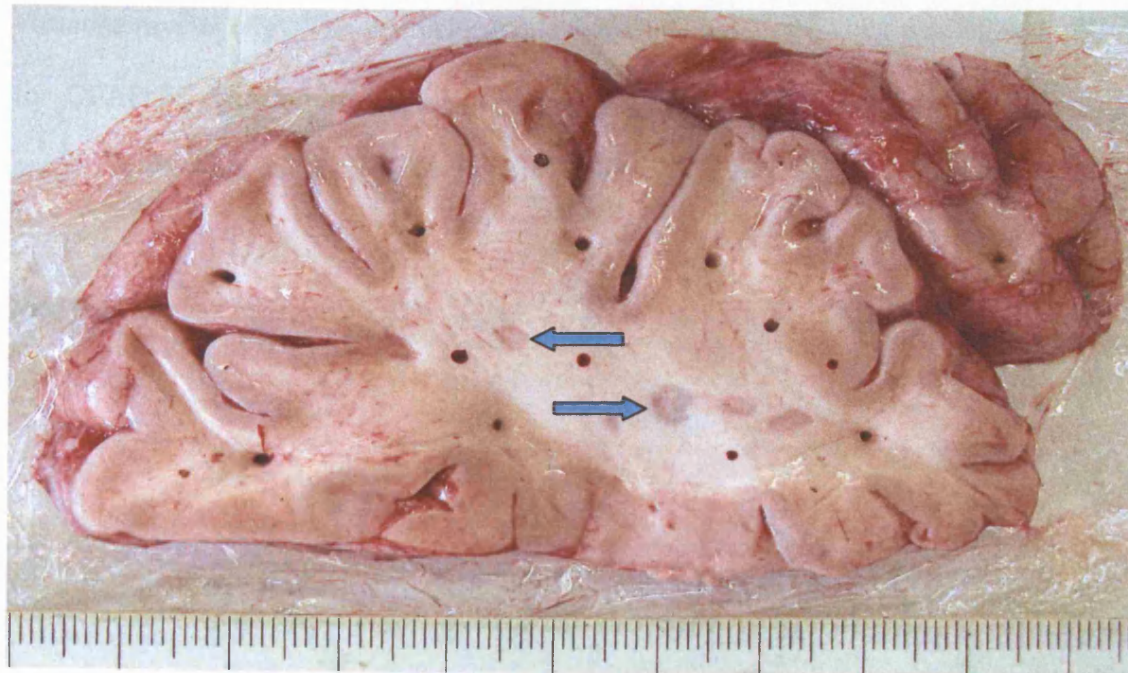
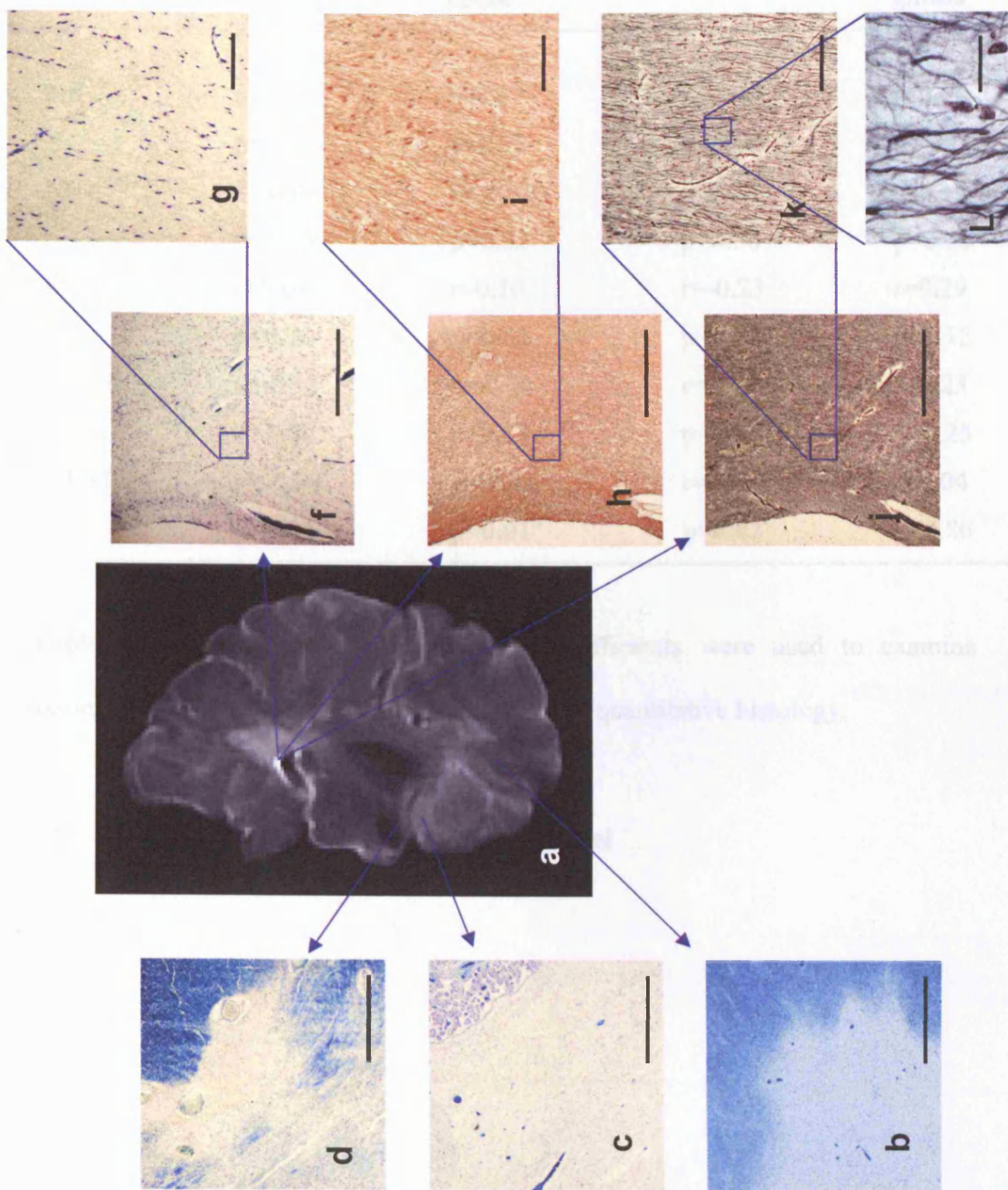


Figure 6.2 MRI T2-weighted image (a) was done 5mm beneath the surface where samples were excised. This brain slice revealed 5 areas of high signal on T2-weighted MRI (b-d). All the bluish images are stained for Luxol fast blue to visualise myelin (f,g). The demyelinated lesion as shown on the right was stained for GFAP for gliosis(h,i) and Bielschowsky's silver stain for axons (j-L). The section in the right corner is magnified for counting of axons (L).

(See next page)



| Variables | Axonal density | Axonal lesion count | Myelin content | Area of gliosis |
|----------------------|---|---|--|------------------|
| NfH ^{SMI34} | r=0.06 p=0.77 | r=0.07 p=0.72 | r=-0.11 p=0.58 | r=0.05 p=0.82 |
| NfH ^{SMI35} | r=-0.04 p=0.85 | r=-0.05 p=0.82 | r=0.11 p=0.58 | r=0.18 p=0.38 |
| Ferritin | r=0.08 p=0.70 | r=0.10 p=0.62 | r=-0.23 p=0.25 | r=0.29 p=0.15 |
| S100B | r=-0.33 p=0.09 | r=-0.30 p=0.12 | r=0.11 p=0.57 | r=0.23 p=0.25 |
| GFAP | r=-0.50 p=0.01^a | r=-0.46 p=0.01^b | r=0.43 p=0.02^c | r=0.04 p=0.86 |

Table 6.1 Spearman rank correlation coefficients were used to examine associations between brain specific proteins and quantitative histology.

a,b,c Significant P value is taken at 0.05 level

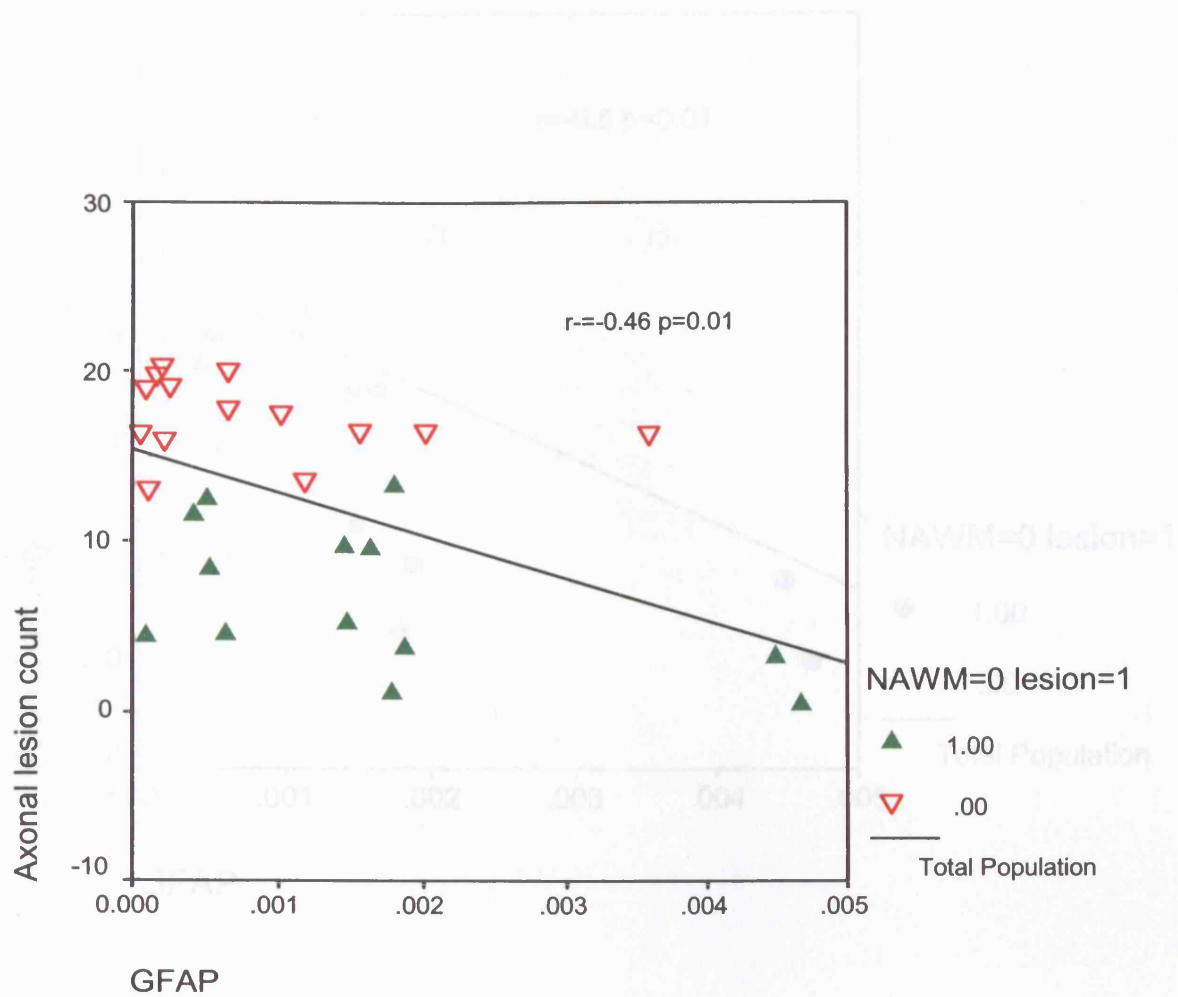
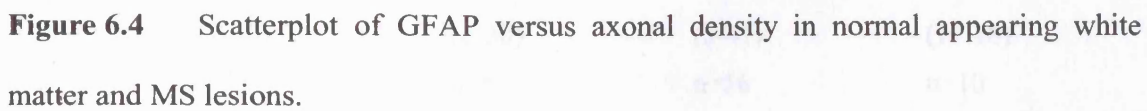


Figure 6.3 Scatterplot of GFAP versus axonal count in normal appearing white matter and lesions.



| | NAWM n=55 | NAGM n=54 | Lesions in WM n=57 | Lesions in GM n=10 |
|----------------------|-----------------------|----------------------|-----------------------|-----------------------|
| Ferritin | 10 (4-18) n=46 | 12 (5-26) n=45 | 4 (1-23) n=51 | 4 (2-6) n=10 |
| | | | | |
| S100B | 19 (8-38) n=55 | 7 (3-14) n=53 | 21 (7-88) n=56 | 1 (1-16) n=10 |
| | | | | |
| GFAP | 5 (1-16) n=55 | 3 (1-6) n=54 | 14 (4-34) n=56 | 2 (1-13) n=10 |
| | | | | |
| NfH ^{SMI34} | 25 (8-61) n=55 | 17 (9-31) n=52 | 18 (9-33) n=51 | 11 (9-31) n=10 |
| NfH ^{SMI35} | 20 (12-33) n=55 | 11 (6-30) n=54 | 22 (5-41) n=56 | 13 (11-18) n=10 |

Table 6.2 Brain specific proteins to total protein ratios in multiple sclerosis brain tissues [median (interquartile range, number)]. All values have been multiplied by 10^4 .

Significant P value is taken at 0.05 levels.

| <i>S100B</i> | <i>Ferritin</i> | <i>GFAP</i> | <i>NfH</i> <i>SMI34</i> | <i>NfH</i> <i>SMI35</i> | <i>Ferritin</i> | <i>GFAP</i> | <i>NfH</i> <i>SMI34</i> | <i>NfH</i> <i>SMI35</i> | <i>GFAP</i> | <i>NfH</i> <i>SMI34</i> | <i>NfH</i> <i>SMI35</i> | <i>NfH</i> <i>SMI34</i> | <i>NfH</i> <i>SMI35</i> |
|---------------------|---|--------------------------|----------------------------|---|-----------------|---|---|---|-------------|---|---|----------------------------|---|
| NAGM | r=0.65 p=0.00 n=45 | r=0.49 p=0.00 n=53 | r=0.30 p=0.03 n=52 | r=0.09 p=0.51 n=53 | | r=0.65 p=0.00 n=45 | r=0.51 p=0.00 n=45 | r=0.07 p=0.64 n=45 | | r=0.38 p=0.01 n=52 | r=0.28 p=0.04 n=54 | | r=0.49 p=0.00 n=52 |
| NAWM | r=0.40 p=0.01 n=46 | r=0.49 p=0.00 n=55 | r=0.023 p=0.09 n=55 | r=0.35 p=0.01 n=55 | | r=0.47 p=0.00 n=46 | r=0.60 p=0.00 n=46 | r=0.45 p=0.00 n=46 | | r=0.30 p=0.03 n=55 | r=0.39 p=0.00 n=55 | | r=0.54 p=0.00 n=55 |
| WM lesions | r=0.16 p=0.28 n=51 | r=0.38 p=0.00 n=55 | r=0.23 p=0.10 n=51 | r=-0.04 p=0.77 n=56 | | r=0.50 p=0.00 n=50 | r=0.41 p=0.00 n=51 | r=0.25 p=0.08 n=51 | | r=0.26 p=0.07 n=50 | r=0.22 p=0.10 n=55 | | r=0.16 p=0.27 n=51 |
| GM lesions | r=-0.46 p=0.19 n=10 | r=0.82 p=0.00 n=10 | r=0.24 p=0.51 n=10 | r=-0.06 p=0.88 n=10 | | r=-0.27 p=0.45 n=10 | r=0.32 p=0.37 n=10 | r=0.37 p=0.29 n=10 | | r=0.42 p=0.23 n=10 | r=0.10 p=0.78 n=10 | | r=0.83 p=0.00 n=10 |

Table 6.3 Non parametric Spearman associations between different brain specific protein to total protein ratios in NAGM, NAWM, lesions in WM and lesions in GM.

After Bonferroni's correction, p values < 0.01 is taken as significant

Highlighted values are statistically significant

PART 3

SPECIFIC MARKERS IN PROGRESSIVE

DISEASE AND CLINICALLY ISOLATED

SYNDROMES

Chapter 7

Serum S100B in primary progressive multiple sclerosis patients treated with interferon-beta-1a

7.1 Introduction

This study of serum S100B in PPMS is based on data obtained from a randomized, controlled trial of Interferon β -1a in subjects with PPMS. The key questions were whether S100B levels were associated with either disability or MRI findings in PPMS and whether Interferon β -1a has an effect on their S100B levels. So far these questions have been answered with regards to RRMS and SPMS, and as yet, not with PPMS.

Several studies suggest that S100B has a role in the pathogenesis of multiple sclerosis. Phenotypically and functionally similar T cells specific against S100B can be detected in the peripheral blood of MS patients making S100B a putative candidate auto-antigen in MS (Schmidt, 1998). Furthermore, S100B may act as a cytokine (Donato, 2001; Massaro *et al.*, 1985; Michetti *et al.*, 1980) and in vitro studies show that, at high levels, S100 can induce the neuronal expression and secretion of pro-inflammatory interleukin-6. In addition, elevated levels of S100B have been detected in the CSF of MS patients during acute phases or exacerbations of the

disease (Massaro *et al.*, 1985) and it has therefore been proposed that elevated S100B may be indicative of active cell injury (Michetti *et al.*, 1980).

Interferon- β is effective in reducing relapse rate in RRMS (The IFNB Multiple Sclerosis Study Group, 1993; Jacobs *et al.*, 1996; PRISMS Study Group, 1998) and SPMS (European Study Group on IFNB, 1998) but the mechanisms behind the beneficial action of IFN β are not fully understood. Two potential sites of action are on cytokine production (Dayal *et al.*, 1995; Gayo *et al.*, 1999; Panitch *et al.*, 1987) and on the entry of leukocytes into the CNS (Leppert *et al.*, 1996; Lou *et al.*, 1999; Stuve *et al.*, 1996; Uhm *et al.*, 1999).

7.2 Aims

In this clinically negative phase II study (Leary *et al.*, 2003); the aim was to assess the effect of IFN β -1a on serum levels of S100B at 3-month intervals in subjects with primary progressive MS (PPMS). The key questions were whether serum S100B levels correlated with disability or MRI findings in patients with PPMS, and whether IFN- β has an effect on levels of serum S100B.

7.3 Methods

7.3.1 Patients and examination

Fifty patients with PPMS were recruited in a phase II trial of IFN β -1a (Avonex®, Biogen) and were assessed three monthly over a study period of 2 years. Fifteen of these patients were treated with IFN β -1a 30 μ g intramuscularly (im) weekly (IFN30), 15 received IFN β -1a 60 μ g im weekly (IFN60) and 20 with placebo. IFN β -1a was reduced to half dose in 5 subjects receiving 60 μ g im weekly, and in 2 subjects receiving IFN β -1a 30 μ g im weekly. Seven subjects withdrew from treatment (Leary *et al.*, 2003) (Figure 7.1).

Neurological examination was performed at each visit and disability was measured using Kurtzke's expanded disability status scale (EDSS). Progression was defined as a sustained (3 months apart) increase of at least 1.0 on the EDSS scale between 0 to 5 and 0.5 for subjects with EDSS score of 5.5 and above.

Fourteen healthy subjects served as controls.

7.3.2 MR imaging and analyses

MRI was performed at baseline and 6 monthly for 2 years. Only baseline and year 2 data were included in this study. Brain and spinal cord atrophy, ventricular volume, T1 and T2 lesion load were measured as described in Leary *et al.* [2003] study.

7.3.3 Serum S100B levels

Serum samples were centrifuged and stored at -20°C. Serum S100B levels were quantified using a modified ELISA method as described in chapter 3.

7.3.4 Statistical analyses

Median, interquartile range and significance of group differences (Mann-Whitney U tests) were evaluated. Changes of serum level over time were examined using variance components regression models of serum response variable on time as predictor, with random subject-specific intercepts and fixed common slopes. Curvature was assessed using a quadratic term in time; modification of curve over time by treatment was assessed using additional terms for treatment and treatment by time interaction in the model. Two sets of treatment terms were used: i) indicators of assigned weekly dose ii) average weekly dose over follow-up (including changes to dose regime) as continuous variable. Modification of the curve over time by MRI variable values were similarly examined using terms for MRI variable and MRI variable by time interaction.

Direct associations between serum level and MRI/clinical variables were examined by regression models of 24 month serum on 24

month MRI variable, adjusting for baseline serum and MRI values (this type of model takes into account change from baseline), with additional terms for treatment and treatment by MRI variable interaction, the latter to assess possible modifications of the relationship by treatment.

Softwares used were the SPSS software package (version 11.0 for Windows) and Stata 7.0 (Stata Corporation, 2001).

7.4 Results

7.4.1 Serum S100B between subjects with PPMS and controls

The median and interquartile ranges for all subjects are described in Table 7.1. There were no significant differences between any of the groups in relation to age. When comparing S100B levels at baseline of subjects with PPMS and controls, the difference was not statistically significant ($p=0.3$).

7.4.2 Serum S100B change over time (Figures 7.2, 7.3 and 7.4)

There was no change over time in the serum S100B levels. The shape of the serum trajectory did not vary between the treatment regimes, i.e. placebo vs. IFN30 vs. IFN60.

7.4.3 Serum S100B versus Clinical and MRI parameters (Table 7.2)

There was no evidence that the 24-month serum S100B values were associated with either changes in the T1 or T2 loads, or ventricular or cord volumes at 24 months, after adjusting for the baseline values of each subject. There was no correlation with disease progression on the EDSS. There was also no evidence that these relationships were modified by treatment assignment (intention-to-treat analysis) (Table 7.2) or the overall average dose, which included the changes to treatment regime (non-intention-to-treat analysis) (Table 7.2).

7.5 Discussion

These results suggest that serum S100B levels in patients with PPMS were not affected by intramuscular IFN β -1a and that there was no observable change in S100B over time. Furthermore, we did not observe any correlation between S100B levels and clinical disability or between S100B and quantitative MRI measures.

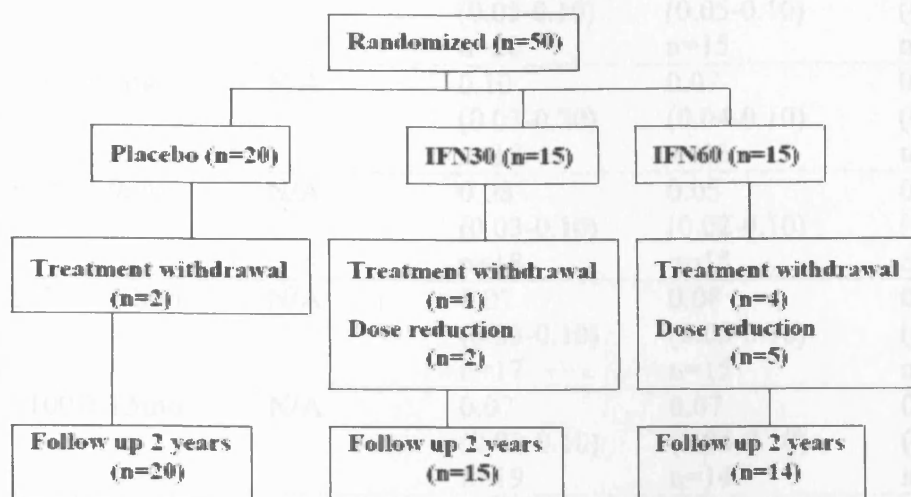
This study therefore suggests that serum S100B would be ineffective as a surrogate marker in PPMS. In addition, as there is evidence that S100B elevation in MS is related to inflammatory activity (Massaro *et al.*, 1985; Michetti *et al.*, 1980; Petzold *et al.*, 2002a), this study has shown that S100B was not sensitive to

disease progression in PPMS. This adds weight to the view that PPMS is less inflammatory.

Nonetheless it is necessary to devise surrogate markers in PPMS to aid the development of effective therapeutic intervention. It is possible that such markers would need to be less related to acute inflammation and dependent on other neuropathology such as axonal loss and regeneration.

Figure 7.1 Fifty subjects with PPMS were randomised in a phase II trial of Interferon β -1a and were assessed 3 monthly over a 2-year study period.

n=number of subjects with PPMS



| | Control (n=14) | Placebo (n=20) | IFN30 (n=15) | IFN60 (n=15) |
|-------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Male:Female | 6:8 n=14 | 15:5 n=20 | 10:5 n=15 | 7:8 n=15 |
| Age (years) | 32 (29-44) n=14 | 43 (36-51) n=20 | 51 (39-53) n=15 | 52 (43-54) n=15 |
| S100B-0mo | 0.08 (0.08-0.10) n=14 | 0.09 (0.02-0.10) n=20 | 0.06 (0.04-0.10) n=12 | 0.07 (0.04-0.10) n=14 |
| S100B-3mo | N/A | 0.08 (0.05-0.10) n=20 | 0.06 (0.05-0.10) n=15 | 0.07 (0.04-0.10) n=14 |
| S100B-6mo | N/A | 0.10 (0.03-0.20) n=18 | 0.07 (0.04-0.10) n=15 | 0.07 (0.04-0.10) n=14 |
| S100B-9mo | N/A | 0.08 (0.03-0.10) n=18 | 0.05 (0.02-0.10) n=15 | 0.06 (0.04-0.10) n=14 |
| S100B-12mo | N/A | 0.07 (0.03-0.10) n=17 | 0.08 (0.05-0.10) n=15 | 0.08 (0.04-0.10) n=14 |
| S100B-15mo | N/A | 0.07 (0.02-0.10) n=19 | 0.07 (0.04-0.10) n=14 | 0.09 (0.04-0.10) n=14 |
| S100B-18mo | N/A | 0.07 (0.04-0.10) n=18 | 0.06 (0.02-0.09) n=13 | 0.09 (0.03-0.20) n=14 |
| S100B-21mo | N/A | 0.06 (0.05-0.10) n=18 | 0.08 (0.04-0.10) n=14 | 0.08 (0.04-0.20) n=13 |
| S100B-24mo | N/A | 0.07 (0.02-0.10) n=18 | 0.07 (0.05-0.10) n=15 | 0.06 (0.04-0.10) n=13 |

Table 7.1 Age and serial serum S100B levels expressed as median (interquartile range).

n=number of subjects; mo, months; N/A, non-applicable.

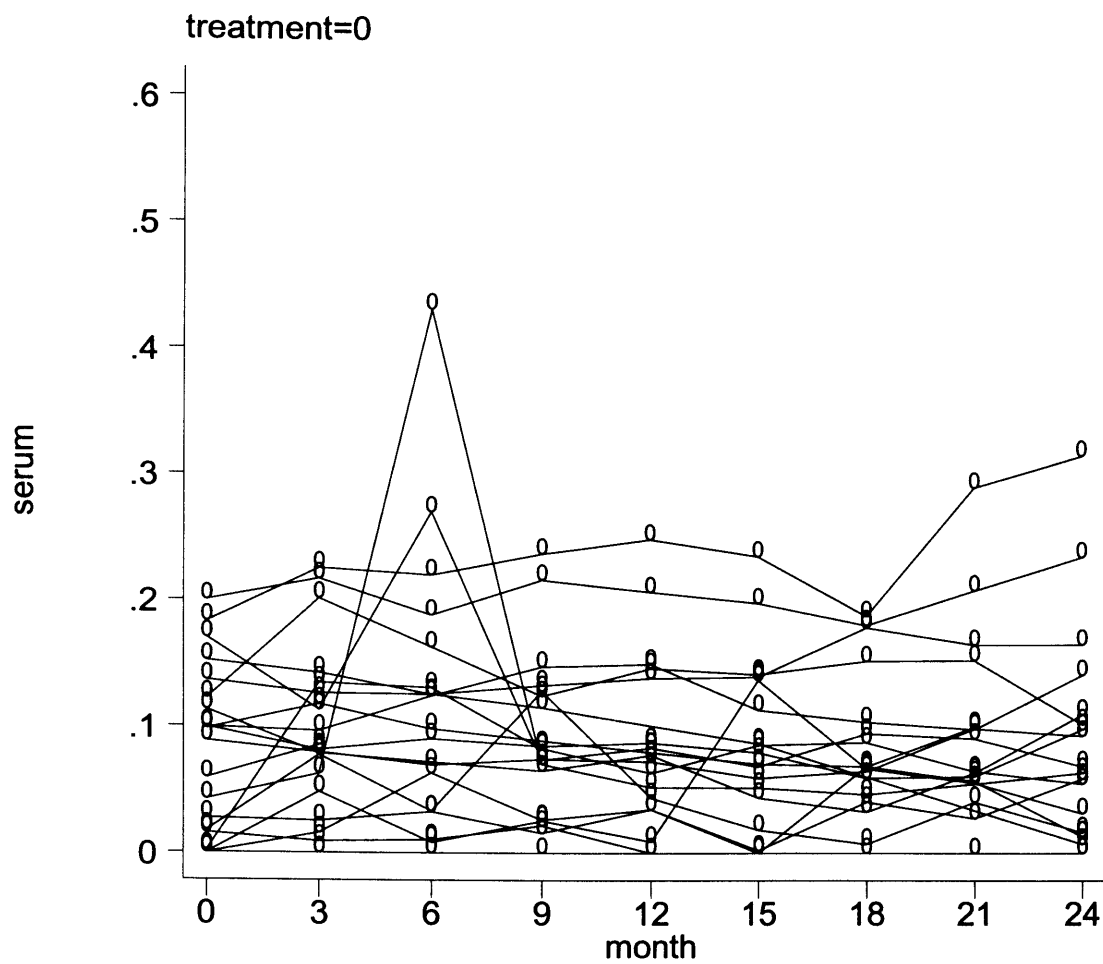


Figure 7.2 Three-monthly serum S100B levels trajectory of each subject with PPMS who were given placebo weekly for 2 years.

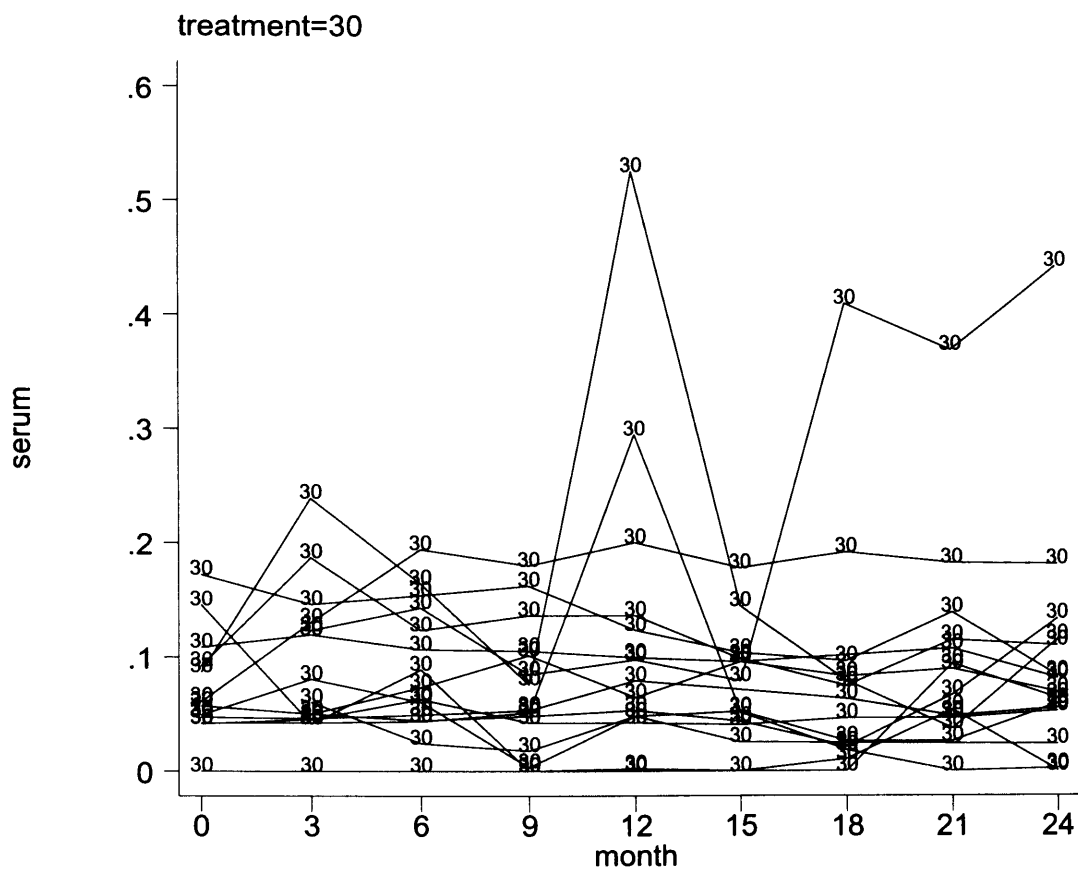


Figure 7.3 Three-monthly serum S100B levels trajectory of each patient with PPMS who received IFN β -1a 30 μ g intramuscularly weekly for 2 years.

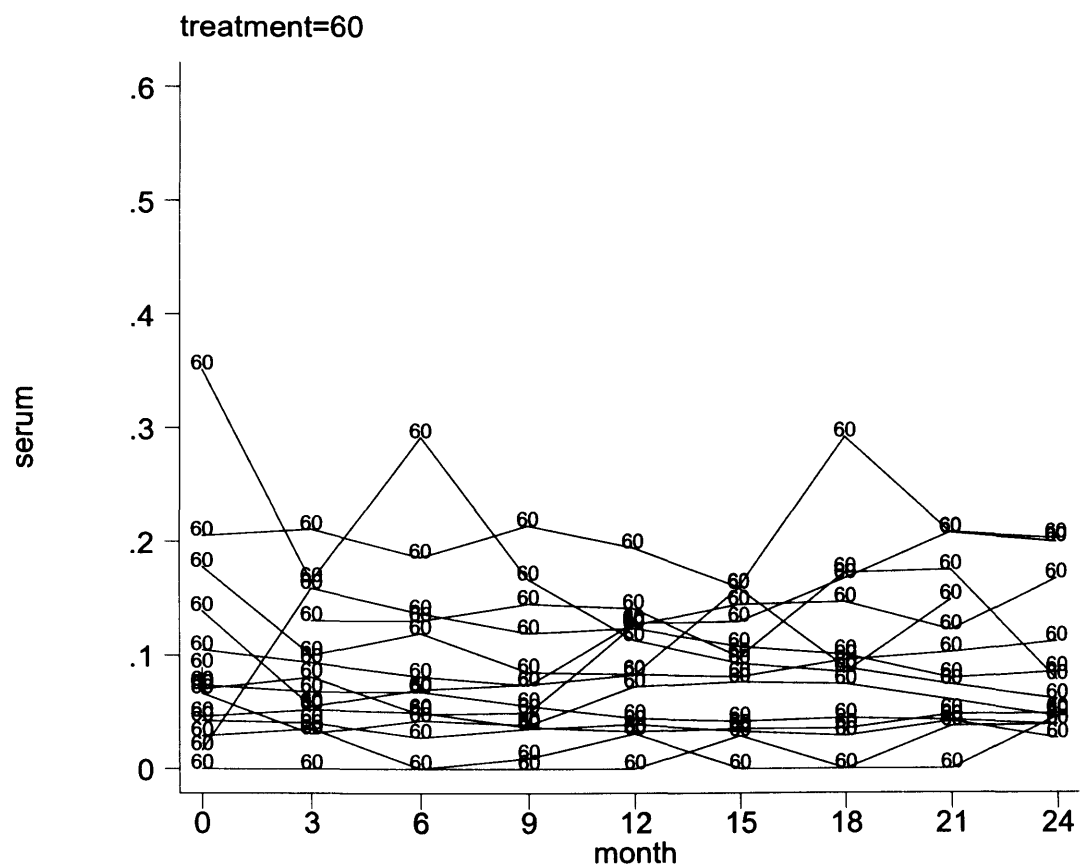


Figure 7.4 Three-monthly serum S100B levels trajectory of each patient with PPMS who received IFN β -1a 60 μ g intramuscularly weekly for 2 years.

Serum S100B versus Clinical and MRI variables

| Variable | Coefficient | P-value | 95% Confidence Interval (CI) | P-value for treatment modification* : Assignment average dose | |
|-----------------------------|---------------------|---------|---------------------------------------|---|------|
| 24 month T1 load | -4×10^{-6} | 0.35 | $-1 \times 10^{-5}, 4 \times 10^{-6}$ | 0.76 | 0.59 |
| 24 month T2 load | -3×10^{-6} | 0.16 | $-7 \times 10^{-6}, 1 \times 10^{-6}$ | 0.57 | 0.89 |
| 24 month ventricular volume | 7×10^{-7} | 0.75 | $-3 \times 10^{-6}, 5 \times 10^{-6}$ | 0.46 | 0.24 |
| 24 month cord volume | -2×10^{-3} | 0.54 | $-9 \times 10^{-3}, 5 \times 10^{-3}$ | 0.58 | 0.88 |

Table 7.2

Estimated mean change in 24-month serum S100B associated with unit increase in mean value of T1 and T2 lesion load, ventricular and spinal cord volume, adjusted for baseline values of both serum S100B and of MRI parameters. Baseline adjustment ensures that the coefficient assesses the ‘effect’ of the 24-month MRI parameters value relative to its baseline.

* Test of treatment interactions with row variable.

Chapter 8

Do anti-myelin antibodies allow earlier diagnosis of multiple sclerosis?

8.1 Introduction

Recently, Berger *et al.* (2003) have determined that in a cohort of patients with CIS, and an abnormal MRI study at baseline, who were seropositive for the anti-myelin antibodies, had more frequent relapses than those who were seronegative, and that these antibodies predicted the conversion to MS (Berger *et al.*, 2003). In collaboration with Berger and colleagues (Department of Neurology, University of Innsbruck, Austria), this study investigated whether the presence of anti-MBP and anti-MOG antibodies in the peripheral blood would predict conversion to MS according to the McDonald (2001) and Poser (1983) criteria, in a separate cohort of patients with CIS.

A possible pathogenetic mechanism responsible for conversion of a CIS to CDMS is antibody-mediated demyelination. MBP is one of the most important proteins of the myelin sheath, and MOG is exclusively expressed at the outer surface of myelin sheath and oligodendrocyte plasma membrane (Brunner, 1989). Enhanced T- and B-cell responses to MBP and MOG has been reported in multiple sclerosis (MS) (Reindl *et al.*, 1999; Egg *et al.*, 2001;

Kerlero de Rosbo *et al.*, 1990; Xiao *et al.*, 1991; Sun *et al.*, 1991; Wallström *et al.*, 1998), but the role of these responses in disease pathogenesis is uncertain.

8.2 Aims

The objective of this study is to determine whether the presence of serum and plasma antibodies against MOG and MBP in patients presenting with a CIS compatible with demyelination predicts early conversion to MS.

8.3 Methods

8.3.1 Patients

Patients with CIS were recruited from either Moorfields Eye Hospital or the National Hospital for Neurology and Neurosurgery, London. A CIS was defined as a single event of acute onset in the central nervous system suggestive of demyelination. The diagnosis of MS was based on the new McDonald MRI criteria (2001) and CDMS using the more established Poser criteria (1983). Clinical and MRI assessments were done less than 3 months from clinical onset, and again after 3 months and 1 year (patients are also being followed at 3 years but 3 year data is not included in this report because of the small number who had anti-myelin antibody testing). The design of this prospective CIS follow-up study and

the protocol for clinical examination and MRI investigations has been described in detail elsewhere (Dalton *et al.*, 2002).

8.3.2 *Antimyelin antibody analysis*

Blood samples were centrifuged and stored at -80°C. Berger and colleagues had analysed the anti-MOG and anti-MBP antibodies in their laboratory using western blotting as previously described (Reindl *et al.*, 1999; Egg *et al.*, 2001) with minor modifications (Berger *et al.*, 2003).

8.3.3 *Statistical Analysis*

All blood tests were performed with researchers blinded to the clinical diagnosis and MRI results. Statistical analyses were evaluated using the SPSS software package (Version 11.0 for Windows). The characteristics of the patients, their clinical disability and MRI measures according to their antibody status were compared using median, interquartile range and Fischer rank exact test. The relative risk of the development of clinically definite MS was measured using Cox-regression. Probability values less than 0.05 were considered significant.

8.4 **Results**

A total of forty-seven patients had baseline anti-MOG and anti-MBP antibodies analyzed. Thirty-five of the 47 patients were

female and 12 were male; their median age at the onset of disease was 31 years (28-36). Forty-six of the patients presented with clinically isolated ON and 1 patient had spinal cord syndrome. The median EDSS score was 1.0 (1.0 to 1.0). The characteristics of the patients and their disease are shown in Table 8.1. All 47 were followed up after 3 months and 35 after one year.

Thirteen/47 patients (27.7%) were seropositive for both anti-MOG and anti-MBP antibodies, 13 (27.7%) were seropositive for only anti-MOG antibodies, 11 (23.4%) were seropositive for anti-MBP antibodies alone and 10 (21.3%) were seronegative for both anti-MOG and anti-MBP antibodies.

Table 8.2 shows the number of patients who converted to MS at 3 months and 12 months based on both McDonald and Poser criteria. After 3 months, 13/47 (28%) had developed MS according to the McDonald criteria; after one year, 20/35 (57%) had MS. Tables 8.3 and 8.4 illustrate the cumulative total that converted to MS compared to those that did not according to the McDonald and Poser criteria respectively at 3 and 12 months.

Antibody status does not relate to the patients who subsequently developed MS or CDMS according to either the McDonald or Poser criteria respectively. Furthermore, Cox-regression analysis

(Figure 8.1) showed that antibody status did not predict risk of MS ($p=0.27$).

Thirteen of the 47 patients had normal baseline T2-weighted MRI brain. Five/13 (38.5%) were seropositive for both anti-myelin antibodies, 4 (30.8%) had antibodies against MOG and 1 (7.7%) had antibodies against MBP. Three of the 13 patients (23%) were seronegative for both antibodies.

There was no evidence that antibody status related to T2-weighted or gadolinium enhanced brain or spinal cord MRI findings at baseline, or with new MRI lesions at 3 months and 1 year. There was no association between antibody status and EDSS assessed at baseline.

8.5 Discussion

This study shows that anti-myelin antibodies did not predict the development of MS or CDMS within one year after a first demyelinating event in this cohort of CIS patients. There was also no association between antibody status and baseline clinical or MRI variables.

Although this study is relatively small, recruitment was prospective and based on clinical findings alone (thus including subjects with a normal MRI). Analysis of serum was also performed blinded to clinical and MRI findings. A limitation is that follow up was for only 12 months; however the new McDonald criteria were used, which increased the rate of early diagnosis such that 57% had developed MS after one year. The McDonald criteria incorporate the surrogate MRI markers in predicting conversion from CIS to MS, and have been shown to have a high specificity for the subsequent development of CDMS (Dalton *et al.*, 2002; Tintore *et al.*, 2003). The old Poser criteria for diagnosing CDMS were employed in parallel for comparison and yielded similar lack of association with anti-myelin antibody findings. The same method was used for detecting anti-myelin antibodies as was reported in the original study of Berger *et al.* (2003).

Although this study did not reproduce the original findings reported by Berger *et al.* (2003), there are differences between the study design e.g. patients with a normal baseline MRI study were included and the follow-up is of a shorter duration of time. This study was also confined, with a single exception, to patients with isolated ON; one cannot exclude the possibility that different anti-

myelin antibody profiles might occur in other CIS cohorts e.g. isolated brain stem or spinal cord syndromes. Of note, the results remain unchanged when the one patient with clinical isolated spinal cord syndrome who is seronegative for both anti-myelin antibodies is excluded from the cohort.

Nevertheless, the findings do, however, raise doubt whether anti-MOG and anti-MBP antibodies will prove to be reliable as markers of prognosis and also question whether these antibodies are involved in the early pathogenesis of MS. On the contrary this study has found no evidence that the antibody status related to the early disease course or to changes in MRI measures in brain and spinal cord at 3 and 12 months. Further studies in other early MS or CIS cohorts are recommended.

| Variable | Negative for Anti-MOG and Anti-MBP | Positive for Anti-MBP and Negative for anti-MOG | Positive for Anti-MOG and Negative for Anti-MBP | Positive for Anti-MOG and Anti-MBP |
|---|------------------------------------|---|---|------------------------------------|
| No. of patients | 10 | 11 | 13 | 13 |
| Female sex –no. (%) | 8 (80) | 8 (73) | 9 (69) | 10 (77) |
| Age – yrs | 33 (30-38) | 33 (27-35) | 28 (25-36) | 31 (29-37) |
| Types of symptoms at presentation – no. (%) | | | | |
| Optic neuritis | | | | |
| Spinal cord syndrome | 9 (90) | 11 (100) | 13 (100) | 13 (100) |
| | 1 (10) | 0 (0) | 0 (0) | 0 (0) |
| Median no. of lesions on T ₂ -weighted MRI brain at baseline | 18 (0-33) | 4 (3-45) | 1 (0-10) | 5 (0-11) |
| Median no. of lesions on T ₁ -weighted gadolinium enhanced MRI | 0 (0-2) | 1 (0-2) | 0 (0-0) | 0 (0-1) |
| Median no. of lesions on T ₂ -weighted MRI spinal cord at baseline | 0 (0-0) | 1 (0-4) | 0 (0-0.5) | 0 (0-0) |
| Clinical disability | | | | |
| | 1 (10) | 1 (9) | 1 (8) | 0 (0) |
| EDSS=0 | 9 (90) | 10 (91) | 11 (84) | 10 (77) |
| EDSS=1 | 0 (0) | 0 (0) | 0 (0) | 1 (8) |
| EDSS=1.5 | 0 (0) | 0 (0) | 1 (8) | 2 (15) |
| EDSS=2 | | | | |

Table 8.1

Characteristics of the 47 patients and their disease, according to the antibody status.

| | McDonald MS | | Poser MS | |
|------------------|-------------|----------|----------|----------|
| | Positive | Negative | Positive | Negative |
| Follow-up | | | | |
| 3 months (n=47) | 13 (28) | 34 (72) | 3 (6) | 44 (94) |
| 12 months (n=35) | 20 (57) | 15 (43) | 9 (32) | 26 (74) |

Table 8.2 Conversion to clinically definite multiple sclerosis according to McDonald and Poser criteria.

MS=multiple sclerosis; n=number of patients.

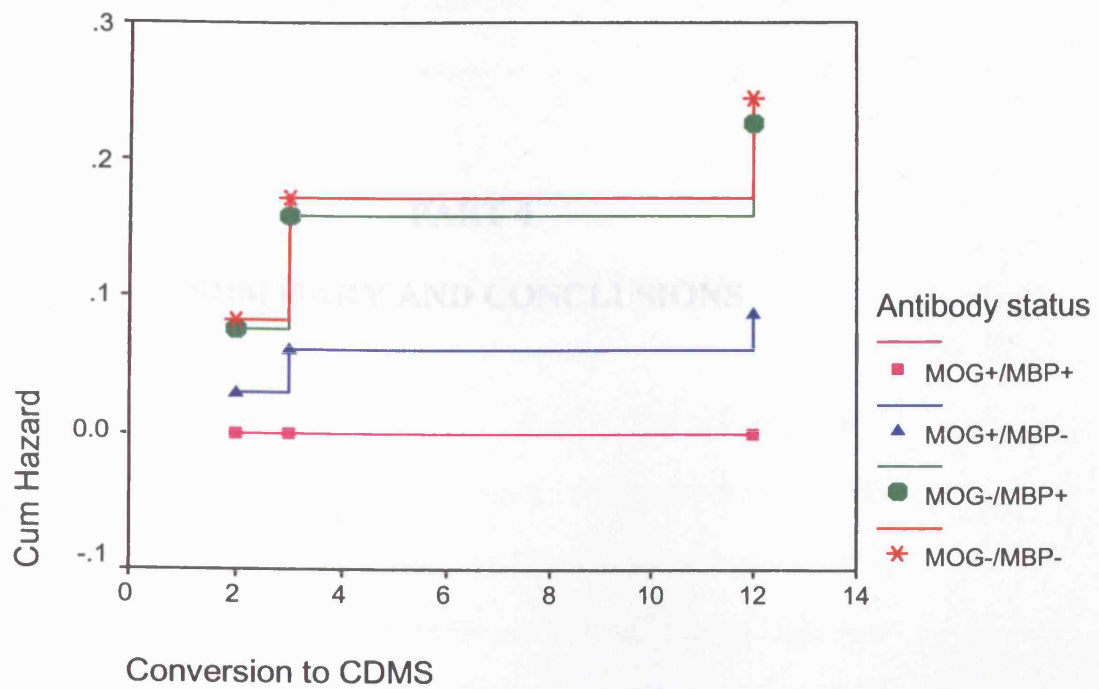
| Follow-up | McDonald MS | | Poser MS | |
|--|------------------------|------------------------|----------------------|-----------------------|
| | Positive | Negative | Positive | Negative |
| 3 months (n=47) | 13 (28) | 34 (72) | 3 (6) | 44 (94) |
| 12 months (n=35) | 20 (57) | 15 (43) | <u>9 (26)</u> | 26 (74) |
| Cumulative total who converted to MS at following time-points | 3 months | 12 months | 3 months | 12 months |
| | Positive n=13 (28%) | Positive n=20 (57%) | Positive n=3 (6%) | Positive n=9 (26%) |
| Cumulative no. with anti-MBP ab alone | 4 (31) | 5 (25) | 2 (67) | 2 (22) |
| Cumulative no. with anti-MOG ab alone | 2 (15) | 3 (15) | 0 (0) | 1 (11) |
| Cumulative no. with both antibodies | 3 (23) | 5 (25) | 0 (0) | 2 (22) |
| Cumulative no. without antibody | 4 (31) | 7 (35) | 1 (33) | 4 (44) |

Table 8.3

Conversion to clinically definite multiple sclerosis according to McDonald and Poser criteria and the cumulative total that converted to MS compared to those that did not according to each criteria.

No., n=number of patients; ab=antibody

Figure 8.1 Hazard Function of the risk of CDMS according to antibody status



PART 4

SUMMARY AND CONCLUSIONS

Chapter 9

Summary and Conclusions

The thesis looks at whether brain specific proteins have any role as potential surrogate markers in demyelinating diseases, in particular multiple sclerosis.

The first study consists of 41 subjects who had acute ON and 17 subjects with other neurological diseases served as controls. Twenty one subjects with ON had white matter lesions on MRI and intrathecal synthesis of oligoclonal IgG bands consistent with being at high risk of progression to MS; eight of whom later were diagnosed with CDMS. Levels of S100B, ferritin and two neurofilament heavy chain phosphoforms (NfH^{SMI34} and NfH^{SMI35}) were analysed using ELISA technique. A putative index of 'axonal health' was expressed as a ratio of NfH^{SMI34} to NfH^{SMI35}. NfH^{SMI34} and the NfH^{SMI34: SMI35} were significantly elevated in subjects with ON compared to controls. No significant differences in levels of CSF BSP were seen between ON subjects with CDMS plus those at high risk of progression to MS and ON subjects with normal MRI and negative CSF analysis. In conclusion, there is evidence of axonal damage in subjects who present with ON, which is independent of the diagnosis of CDMS.

The findings of the next study are CSF neurofilament heavy chain levels, thought to be markers of axonal pathology, predict clinical outcome and correlate with gadolinium enhancing lesion volume and CSF MBP in subjects with ON and MS. Again the study supports the view that acute inflammation in ON and MS results in axonal pathology and that the latter has a role in determining functional impairment.

The third study provides preliminary evidence for a relationship between axonal injury and reactive gliosis. The data shows a relationship between GFAP levels as measured via ELISA and the degree of axonal loss detected in the same tissue sample. In addition, GFAP was elevated and NfH^{SM134} reduced in WM lesions in comparison to NAWM. Further studies are required to confirm these findings.

In Part 3, serial serum S100B levels were measured using an ELISA method. The results demonstrated that serum S100B is not related to either disease progression or MRI findings in subjects with primary progressive MS given Interferon β -1a. Furthermore there is no correlation between S100B levels and the primary and secondary outcome measures.

The final study shows that anti-myelin antibodies do not predict conversion to multiple sclerosis. In view of previous positive study by Berger *et al.* 2003, further studies should be done to clarify this issue.

In conclusion, acute inflammatory demyelination (optic neuritis and multiple sclerosis) is associated with acute axonal damage and loss which supports previous pathology work by Trapp *et al.* 1998. Furthermore, axonal markers such as neurofilaments predicts clinical outcome and ongoing deterioration in patients with MS. Axonal changes occur in the vicinity of gliosis indicating that inflammation, demyelination and repair are closely linked. In the search for potential markers, neurofilaments appear the most promising, and future studies need to confirm the findings.

REFERENCES

Ackerley S, Grierson AJ, Brownlees J, Thornhill P, Anderton BH, Leigh PN, Shaw CE, Miller CC. Glutamate slows axonal transport of neurofilaments in transfected neurons. *J Cell Biol.* 2000;150:165-176.

Ackerley S, Thornhill P, Grierson AJ, Brownlees J, Anderton BH, Leigh PN, Shaw CE, Miller CC. Neurofilament heavy chain side arm phosphorylation regulates axonal transport of neurofilaments. *J Cell Biol.* 2003;161:489-495.

Adami C, Sorci G, Blasi E, Agneletti AL, Bistoni F, Donato R. S100B expression in and effects on microglia. *Glia* 2001;33:131-142.

Adams JM, Imagawa DT. Measles antibodies in multiple sclerosis. *Proc Soc Exp Biol Med.* 1962;111:562-566.

Adelmann M, Wood J, Benzel I, Fiori P, Lassmann H, Matthieu JM, Gardinier MV, Dornmair K, Linington C. The N-terminal domain of the myelin oligodendrocyte glycoprotein (MOG) induces acute demyelinating experimental autoimmune encephalomyelitis in the Lewis rat. *J Neuroimmunol.* 1995;63:17-27.

Al-Chalabi A, Miller CCJ. Neurofilaments and neurological disease. *Bioessays.* 2003;25:346-355.

Allen IV, McQuaid S, Mirakhur M, Nevin G. Pathological abnormalities in the normal-appearing white matter in multiple sclerosis. *Neurol Sci.* 2001;22:141-144.

Arnason BG, Dayal A, Qu ZX, Jensen MA, Genc K, Reder AT. Mechanisms of action of interferon-beta in multiple sclerosis. *Springer Semin Immunopathol.* 1996;18:125-148.

Arosio P, Levi S. Ferritin, iron homeostasis, and oxidative damage. *Free Radic Biol Med.* 2002;33:457-463.

Aurell A, Rosengren LE, Karlsson B, Olsson JE, Zbornikova V, Haglid KG. Determination of S-100 and glial fibrillary acidic protein concentrations in cerebrospinal fluid after brain infarction. *Stroke.* 1991;22:1254-1258.

Baig S, Olsson T, Hojeberg B, Link H. Cells secreting antibodies to myelin basic protein in cerebrospinal fluid of patients with Lyme neuroborreliosis. *Neurology.* 1991;41:581-587.

Baker D, Oneill JK, Gshmeissener SE, Wilcox CE, Butter C, Turk JL. Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J. Neuroimmunol.* 1990;28:261-270

Barkhof F, Frequin ST, Hommes OR, Lamers K, Scheltens P, van Geel WJ, Valk J. A correlative triad of gadolinium-DTPA MRI, EDSS, and CSF-MBP in relapsing multiple sclerosis patients treated with high-dose intravenous methylprednisolone. *Neurology*. 1992a;42:63-67.

Barkhof F, Scheltens P, Frequin ST, Nauta JJ, Tas MW, Valk J, Hommes OR. Relapsing-remitting multiple sclerosis: sequential enhanced MR imaging vs clinical findings in determining disease activity. *AJR Am J Roentgenol*. 1992b;159:1041-1047.

Barkhof F, Filippi M, Miller DH, Scheltens P, Campi A, Polman CH, Comi G, Ader HJ, Losseff N, Valk J. Comparison of MR imaging criteria at first presentation to predict conversion to clinically definite MS. *Brain* 1997;120:2059-2069

Bartzokis G, Tishler TA, Shin IS, Lu PH, Cummings JL Brain ferritin iron as a risk factor for age at onset in neurodegenerative diseases. *Ann N Y Acad Sci*. 2004;1012:224-236.

Beck RW, Cleary PA, Anderson MM Jr, Keltner JL, Shults WT, Kaufman DI, Buckley EG, Corbett JJ, Kupersmith MJ, Miller NR, et al. A randomized, controlled trial of corticosteroids in the treatment of acute optic neuritis. *N Engl J Med* 1992;326:581-588.

Beck RW, Cleary mPA, Trobe JD et al. The effect of corticosteroids for acute optic neuritis on the subsequent development of multiple sclerosis. N Engl J Med 1993;329:1764-1769.

Beck RW. The optic neuritis treatment trial: three-year follow up results. Arch Ophthalmol 1995;113:136-137.

Berger T, Rubner P, Schautzer F, Egg R, Ulmer H, Mayringer I, Dilitz E, Deisenhammer F, Reindl M. Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. N Engl J Med 2003;349:139-145.

Bitsch A, Schuchardt J, Bunkowski S, Kuhlmann T, Bruck W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain. 2000;123:1174-1183.

Bo L, Vedeler CA, Nyland H, Trapp BD, Mork SJ. Intracortical multiple sclerosis lesions are not associated with increased lymphocyte infiltration. Mult Scler. 2003a;9:323-331.

Bo L, Vedeler CA, Nyland H, Trapp BD, Mork SJ. Subpial demyelination in the cerebral cortex of multiple sclerosis patients. J Neuropathol Exp Neurol.

2003b;62:723-732.

Bo L, Geurts JJ, Ravid R, Barkhof F. Magnetic resonance imaging as a tool to examine the neuropathology of multiple sclerosis. *Neuropathol Appl Neurobiol.* 2004;30:106-117.

Bradbury MW. Transport of iron in the blood-brain-cerebrospinal fluid system. *J Neurochem.* 1997;69:443-454.

Brex PA, Ciccarelli O, O'Riordan JI, Sailer M, Thompson AJ, Miller DH. A longitudinal study of abnormalities on MRI and disability from multiple sclerosis. *N Engl J Med* 2002;346:158-164.

Brex PA, Gomez-Anson B, Parker GJ, Molyneux PD, Miszkiel KA, Barker GJ, MacManus DG, Davie CA, Plant GT, Miller DH. Proton MR spectroscopy in clinically isolated syndromes suggestive of multiple sclerosis. *J Neurol Sci.* 1999;166:16-22.

Bruck W, Bitsch A, Kolenda H, Bruck Y, Stiefel M, Lassmann H. Inflammatory central nervous system demyelination: correlation of magnetic resonance imaging findings with lesion pathology. *Ann Neurol.* 1997;42:783-793.

Brunner C, Lassmann H, Waehnel TV, Matthieu JM, Linington C. Differential ultrastructural localization of myelin basic protein, myelin/oligodendroglial glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult rats. *J Neurochem.* 1989;52:296-304.

Challoner PB, Smith KT, Parker JD, MacLeod DL, Coulter SN, Rose TM, Schultz ER, Bennett JL, Garber RL, Chang M, et al. Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. *Proc Natl Acad Sci USA.* 1995;92:7440-7444.

Charcot J. Lectures on the diseases of the nervous system. The New Sydenham Society. London, United Kingdom 1877 pp157-222.

Ching G, Liem R. Roles of head and tail domains in alpha internexin's self-assembly and coassembly with the neurofilament triplet proteins. *J Cell Sci* 1998;111:321-333.

Cole GM. Ironic fate: can a banned drug control metal heavies in neurodegenerative diseases? *Neuron.* 2003;37:889-890.

Coleman MP, Conforti L, Buckmaster EA, Tarlton A, Ewing RM, Brown MC, Lyon MF, Perry VH. An 85kb tandem triplication in the slow Wallerian degeneration mouse. *Proc Natl Acad Sci USA.* 1998;95:9985-9990.

Compston A, Ebers G, Lassman H, McDonald I, Matthews B, Wekerle Hartmut.

McAlpine's Multiple Sclerosis. Edinburgh: Churchill Livingstone, 3rd edn 1998.

Confavreux C, Vukusic S, Moreau T, Adeleine P. Relapses and progression of disability in multiple sclerosis. N Engl J Med. 2000;343:1430-1438.

Confavreux C, Vukusic S, Adeleine P. Early clinical predictors and progression of irreversible disability in multiple sclerosis: an amnesic process. Brain. 2003;126:770-782.

Connor JR, Menzies SL. Relationship of iron to oligodendrocytes and myelination. Glia 1996;17:83-93.

Connor JR, Snyder BS, Beard JL, Fine RE, Mufson EJ. Regional distribution of iron and iron-regulatory proteins in the brain in aging and Alzheimer's disease. J Neurosci Res. 1992;31:327-335.

Cutter GR, Baier ML, Rudick RA, Cookfair DL, Fischer JS, Petkau J, Syndulko K, Weinshenker BG, Antel JP, Confavreux C, Ellison GW, Lublin F, Miller AE, Rao SM, Reingold S, Thompson A, Willoughby E. Development of a multiple sclerosis functional composite as a clinical trial outcome measure. Brain. 1999;122 :871-882.

Cuzick J. A Wilcoxon-type test for trend. *Statistics in Medicine* 1985; 4:87-90.

Da Silva JS, Dotti CG. Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. *Nat Rev Neurosci.* 2002;3:694-704.

Dalton CM, Brex PA, Miszkiel KA, et al. Application of the New McDonald Criteria to patients with clinically isolated syndromes suggestive of multiple sclerosis. *Ann Neurol* 2002;52:47-53.

Dayal A, Jensen M, Lledo A, Arnason B. Interferon-gamma-secreting cells in multiple sclerosis patients treated with interferon beta-1b. *Neurology* 1995;45:2173-2177.

De Groot CJ, Bergers E, Kamphorst W, Ravid R, Polman CH, Barkhof F, van der Valk P. Post-mortem MRI-guided sampling of multiple sclerosis brain lesions: increased yield of active demyelinating and (p)reactive lesions. *Brain.* 2001;124:1635-1645.

De Stefano N, Narayanan S, Francis SJ, Smith S, Mortilla M, Tartaglia MC, Bartolozzi ML, Guidi L, Federico A, Arnold DL. Diffuse axonal and tissue injury in patients with multiple sclerosis with low cerebral lesion load and no disability. *Arch Neurol* 2002; 59:1565-1571.

Dolei A, Serra C, Mameli G, Pugliatti M, Sechi G, Ciroto MC, Rosati G, Sotgiu S. Multiple sclerosis-associated retrovirus (MSRV) in Sardinian MS patients. *Neurology*. 2002;58:471-473.

Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol* 2001;33:637-668.

Dotevall L, Hagberg L, Karlsson JE, Rosengren LE. Astroglial and neuronal proteins in cerebrospinal fluid as markers of CNS involvement in Lyme neuroborreliosis. *Eur J Neurol*. 1999;6:169-178.

Egg R, Reindl M, Deisenhammer F, Linington C, Berger T. Anti-MOG and anti-MBP antibody subclasses in multiple sclerosis. *Mult Scler* 2001;7:285-9.

Eikelenboom MJ, Petzold A, Lazeron RH, Silber E, Sharief M, Thompson EJ, Barkhof F, Giovannoni G, Polman CH, Uitdehaag BM. Multiple sclerosis: Neurofilament light chain antibodies are correlated to cerebral atrophy. *Neurology*. 2003;60:219-223.

Eng LF, Ghimikar RS. GFAP and astrogliosis. *Brain Pathol*. 1994;4:229-37.

Eng LF, Ghirmikar RS, Lee YL. Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). *Neurochem Res.* 2000; 25:1439-51.

European Study Group on Interferon β -1b in Secondary Progressive Multiple Sclerosis. Placebo-controlled multicentre randomised trial of interferon β -1b in treatment of secondary progressive multiple sclerosis. *Lancet* 1998;352:1491-1497.

Evangelou N, Esiri MM, Smith S, Palace J, Matthews PM. Quantitative pathological evidence for axonal loss in normal appearing white matter in multiple sclerosis. *Ann Neurol.* 2000;47:391-395.

Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. *Brain* 1997;120:393-399.

Filippi M, Bozzali M, Rovaris M, Gonen O, Kesavadas C, Ghezzi A, Martinelli V, Grossman RI, Scotti G, Comi G, Falini A. Evidence for widespread axonal damage at the earliest clinical stage of multiple sclerosis. *Brain* 2003; 126:433-437.

Fisher E, Rudick RA, Cutter G, Baier M, Miller D, Weinstock-Guttman B, Mass MK, Dougherty DS, Simonian NA. Relationship between brain atrophy and disability: an 8-year follow-up study of multiple sclerosis patients. *Mult Scler.* 2002;6: 373-377.

Foroozan R, Buono LM, Savino PJ, Sergott RC. Acute demyelinating optic neuritis. *Curr Opin Ophthalmol*. 2002;13:375-380.

Francis DA, Compston DA, Batchelor JR, McDonald WI. A reassessment of the risk of multiple sclerosis developing in patients with optic neuritis after extended follow-up. *J Neurol Neurosurg Psychiatry* 1987;50: 758-765.

Frederickson JL, Larsson HB, Olesen J. Correlation of magnetic resonance imaging and CSF findings in patients with acute monosymptomatic optic neuritis. *Acta Neurologica Scandinavica* 1992;86:317-322.

Frick E, Stickl H. Antibody-dependent lymphocyte cytotoxicity against basic protein of myelin in multiple sclerosis. *J Neurol Sci*. 1980;46:187-197.

Frisen L., Hoyt WF. Insidious atrophy of retinal nerve fibers in multiple sclerosis. Fundoscopic identification in patients with and without visual complaints. *Arch. Ophthalmol* 1974;92:91-97.

Fuchs E, Cleveland DW. A structural scaffolding of intermediate filaments in health and disease. *Science*. 1998;279:514-519.

Gardinier MV, Amiguet P, Linington C, Matthieu JM. Myelin/oligodendrocyte glycoprotein is a unique member of the immunoglobulin superfamily. *J Neurosci Res*. 1992;33:177-187.

Gayo A, Mozo L, Suarez A, Tunon A, Lahoz C, Gutierrez C. Interferon beta-1b treatment modulates TNF alpha and IFN gamma spontaneous gene expression in MS. *Neurology* 1999;52:1764-1770.

Gelman BB, Rodriguez-Wolf MG, Wen J, Kumar S, Campbell GR, Herzog N. Siderotic cerebral macrophages in the acquired immunodeficiency syndrome. *Arch Pathol Lab Med.* 1992;116:509-516.

Gerber MR, Connor JR. Do oligodendrocytes mediate iron regulation in the human brain? *Ann Neurol.* 1989;26:95-98.

Genain CP, Cannella B, Hauser SL, Raine CS. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med.* 1999;5:170-175.

Genain CP, Nguyen MH, Letvin NL, Pearl R, Davis RL, Adelman M, Lees MB, Linington C, Hauser SL. Antibody facilitation of multiple sclerosis-like lesions in a nonhuman primate. *J Clin Invest.* 1995;96:2966-2974.

Gentleman SM, McKenzie JE, Royston MC, McIntosh TK, Graham DI. A comparison of manual and semi-automated methods in the assessment of axonal injury. *Neuropathol Appl Neurobiol.* 1999;25:41-47.

Goldstein MS, Sternberger LA, Sternberger NH. Microheterogeneity ("neurotypy" of neurofilament proteins. Proc Natl Acad Sci USA 1983;80:3101-3105.

Goldstein MS, Sternberger N, Sternberger L. Phosphorylation protects neurofilaments against proteolysis. J Neuroimmunol 1987;14:149-160.

Gomes FC, Paulin D, Moura Neto V. Glial fibrillary acidic protein (GFAP): modulation by growth factors and its implication in astrocyte differentiation. Braz J Med Biol Res. 1999; 32:619-631.

Gomez-Lira M, Morreto G, Bodamini D, Benedetti MD, Pignatti PF, Rizutto N, Salviato A. Myelin oligodendrocyte glycoprotein polymorphisms and multiple sclerosis. J Neuroimmunol. 2002;133:241-243.

Goodin DS. Corticosteroids and optic neuritis. Neurology 1993;43:632-633.

Goust JM, Chenais F, Carnes JE, et al. Abnormal T cell subpopulations and circulating immune complexes in the Guillain-Barre syndrome and multiple sclerosis. Neurology. 1978;28:421-425.

Green AJ, Keir G., Thompson EJ. A specific and sensitive ELISA for measuring S-100b in cerebrospinal fluid. *J Immunol Methods* 1997;205:35-41.

Greene WH. *Econometric Analysis*. Upper Saddle River, NJ: 4th edition. Prentice-Hall 2000.

Griffin WS, Sheng JG, McKenzie JE, Royston MC, Gentleman SM, Brumback RA, Cork LC, Del Bigio MR, Roberts GW, Mrak RE. Life-long overexpression of S100beta in Down's syndrome: implications for Alzheimer pathogenesis. *Neurobiol Aging*. 1998;19:401-405.

Grimaud J, Barker GJ, Wang L, Lai M, MacManus DG, Webb SL, Thompson AJ, McDonald WI, Tofts PS, Miller DH. Correlation of magnetic resonance imaging parameters with clinical disability in multiple sclerosis: a preliminary study. *J Neurol* 1999;246:961-967.

Grossman RI, Gonzalez-Scarano F, Atlas SW, Galetta S, Silberberg DH. Multiple sclerosis: gadolinium enhancement in MR imaging. *Radiology*. 1986;161:721-725.

Haghighi S, Andersen O, Oden A, Rosengren L. Cerebrospinal fluid markers in MS patients and their healthy siblings. *Acta Neurol Scand*. 2004;109:97-99.

Haines JL, Ter-Minassian M, Bazyk A, Gusella JF, Kim DJ, Terwedow H, Pericak-Vance MA, Rimmler JB, Haynes CS, Roses AD, et al. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatibility complex. The Multiple Sclerosis Genetics Group. *Nat Genet.* 1996;13:469-471.

Hickman SJ, Dalton CM, Miller DH, Plant GT. Management of acute optic neuritis. *Lancet* 2002;360:1953-1962.

Hickman SJ, Brex PA, Brierley CM, Silver NC, Barker GJ, Scolding NJ, Compston DA, Moseley IF, Plant GT, Miller DH. Detection of optic nerve atrophy following single episode of unilateral optic neuritis by MRI using a fat-saturated short-echo fast FLAIR sequence. *Neurorad.* 2001;45:123-128

Hobart J, Freeman J, Thompson A. Kurtzke scales revisited: the application of psychometric methods to clinical intuition. *Brain.* 2000;123:1027-1040.

Hoffman PN, Cleveland DW, Griffin JW, Landes PW, Cowan NJ, Price DL. Neurofilament gene expression: a major determinant of axonal caliber. *Proc Natl Acad Sci USA.* 1987;84:3472-3466.

Hohol MJ, Olek MJ, Orav EJ, Stazzone L, Hafler DA, Khoury SJ, Dawson DM, Weiner HL. Treatment of progressive multiple sclerosis with pulse

cyclophosphamide/ methylprednisolone: response to therapy is linked to the duration of progressive disease. *Mult Scler.* 1999;5:403-409.

Holmberg B, Rosengren L, Karlsson JE, Johnels B. Increased cerebrospinal fluid levels of neurofilament protein in progressive supranuclear palsy and multiple-system atrophy compared with Parkinson's disease. . *J Neurochem.* 1996; 67: 2013–2018.

Hu J, Ferreira A, Van Eldik LJ. S100beta induces neuronal cell death through nitric oxide release from astrocytes. *J Neurochem.* 1997;69:2294-2301.

Hu J, Van Eldik LJ. S100 beta induces apoptotic cell death in cultured astrocytes via a nitric oxide-dependent pathway. *Biochim Biophys Acta.* 1996;1313:239-245.

Hulet SW, Powers S, Connor JR. Distribution of transferrin and ferritin binding in normal and multiple sclerotic human brains. *J Neurol Sci* 1999;165:48-55.

Inagaki M, Nakamura Y, Takeda M, Nishimura T, Inagaki N. Glial fibrillary acidic protein: dynamic property and regulation by phosphorylation. *Brain Pathol.* 1994;4:239-243.

Jacobs LD, Cookfair DL, Rudick RA, Herndon RM, Richert JR, Salazar AM,

Fischer JS, Goodkin DE, Granger CV, Simon JH, et al. Intramuscular interferon-beta-1a for disease progression in relapsing-remitting multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Ann Neurol* 1996;39:285-294.

Jacobs L, Johnson KP. A brief history of the use of interferons as treatment of multiple sclerosis. *Arch Neurol*. 1994;51:1245-1252.

Jacobs LD, Kaba SE, Miller CM, Priore RL, Brownschidle CM. Correlation of clinical magnetic resonance imaging, and cerebrospinal fluid findings in optic neuritis. *Ann Neurol* 1997;41:392-398.

Kabat EA, Moore DH, Landow H. An electrophoretic study of the protein components in cerebrospinal fluid and their relationship to the serum proteins. *Journal of Clinical Investigation* 1942;21:571-577.

Kaneko Y, Kitamoto T, Tateishi J, Yamaguchi K. Ferritin immunohistochemistry as a marker for microglia. *Acta Neuropathol (Berl)*. 1989;79:129-136.

Kapoor R, Davies M., Smith KJ. Temporary axonal conduction block and axonal loss in inflammatory neurological disease. A potential role for nitric oxide? *Ann NY Acad Sci*. 1999;893:304-308.

Karni A, Bakimer-Kleiner R, Abramsky O, Ben-Nun A. Elevated levels of antibody to myelin oligodendrocyte glycoprotein is not specific for patients with multiple sclerosis. *Arch Neurol.* 1999;56:311-315.

Keir G., Luxton RW, Thompson EJ. Isoelectric focusing of cerebrospinal fluid in immunoglobulin G: an annotated update. *Ann Clin Biochem* 1990;27:436-443.

Keir G., Tasdemir N., and Thompson EJ. Cerebrospinal fluid ferritin in brain necrosis: evidence for local synthesis. *Clinica Chimica Acta* 1993;216:153-166.

Kennel De March A, De Bouwerie M, Kolopp-Sarda MN, et al. Anti-myelin oligodendrocyte glycoprotein B-cell responses in multiple sclerosis. *J Neuroimmunol.* 2003;135:117-125.

Kerlero de Rosbo N, Honegger P, Lassmann H, Matthieu JM. Demyelination induced in aggregating brain cell cultures by a monoclonal antibody against myelin/oligodendrocyte glycoprotein. *J Neurochem.* 1990;55:583-587.

Kerlero de Rosbo N, Milo R, Lees MB, Burger D, Bernard CC, Ben-Nun A. Reactivity to myelin antigens in multiple sclerosis. Peripheral blood lymphocytes respond predominantly to myelin oligodendrocyte glycoprotein. *J Clin Invest.* 1993;92:2602-2608.

Khoury SJ, Guttmann CR, Orav EJ, Hohol MJ, Ahn SS, Hsu L, Kikinis R, Mackin GA, Jolesz FA, Weiner HL. Longitudinal MRI in multiple sclerosis: correlation between disability and lesion burden. *Neurology*. 1994;44:2120-2124.

Kidd D, Barkhof F, McConnell R, Algra PR, Allen IV, Revesz T. Cortical lesions in multiple sclerosis. *Brain*. 1999;122:17-26.

Kivisakk P, Tian W, Matusevicius D, Link H, Soderstrom M. Optic neuritis and cytokines: no relation to MRI abnormalities and oligoclonal bands. *Neurology* 1997;50: 217-223.

Koeppen AH. A brief history of brain iron research. *Neurol Sci* 2003; 207:95-97.

Kornek B, Lassmann H. Axonal pathology in multiple sclerosis. A historical note. *Brain Pathol* 1999;9:651-656.

Kriss A, Francis DA, Cuendet F, Halliday AM, Taylor DS, Wilson J, Keast-Butler J, Batchelor JR, McDonald WI. Recovery after optic neuritis in childhood. *J Neurol Neurosurg Psychiatry*. 1988;51:1253-1258.

Kurtzke JF. A new scale for evaluating disability in multiple sclerosis. *Neurology*. 1955;5:580-583.

Kurtzke JF. On the evaluation of disability in multiple sclerosis. *Neurology*.

1961;11:686-694.

Kurtzke JF. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology*. 1983a;33:1444-1452.

Kurtzke JF. Epidemiology of multiple sclerosis. In: Hallpike JF, Adams CWM, Tourtelotte WW (eds) *Multiple Sclerosis, Pathology, Diagnosis and Management*. London: Chapman and Hall, 1983b pp 47-95.

Kurtzke JF. Epidemiologic evidence for multiple sclerosis as an infection/ *Clinical Microbiology Reviews* 1993;6:382-427.

Kurtzke JF, Berlin L. The effects of isoniazid on patients with multiple sclerosis; preliminary report. *Am Rev Tuberc*. 1954;70:577-592.

Lamers KJ, van Engelen BG, Gabreels FJ, Hommes OR, Borm GF, Wevers RA. Cerebrospinal neuron-specific enolase, S-100 and myelin basic protein in neurological disorders. *Acta Neurol Scand*. 1995;92:247-251.

Lampasona V, Franciotta D, Furlan R, Zanaboni S, Fazio R, Bonifacio E, Comi G, Martino G. Similar low frequency of anti-MOG IgG and IgM in MS patients and healthy subjects. *Neurology*. 2004;62:2092-2094.

Laping NJ, Teter B, Nichols NR, Rozovsky I, Finch CE. Glial fibrillary acidic

protein: regulation by hormones, cytokines and growth factors. *Brain Pathol.* 1994;4:259-275.

Leary SM, Miller DH, Stevenson VL, et al. Interferon beta-1a in primary progressive multiple sclerosis: an exploratory randomised controlled trial. *Neurology* 2003;60:44-51.

Leibowitz S. The Immunology of MS. In *Multiple Sclerosis*, ed. by Hallpike JF, Adams CWM and Tourtellotte WW, Chapman and Hall, London, 1983; pp 379-412.

Lepore FE. The origin of pain in optic neuritis. Determinants of pain in 101 eyes with optic neuritis. *Arch Neurol.* 1991;48:748-749.

Leppert D, Waubant E, Burk MR, Oksenberg JR, Hauser SL. Interferon beta-1b inhibits gelatinase secretion and in vitro migration of human T cells: a possible mechanism for treatment efficacy in multiple sclerosis. *Ann Neurol* 1996;40:846-852.

LeVine SM, Lynch SG, Ou CN, Wulser MJ, Tam E, Boo N. Ferritin, transferrin and iron concentrations in the cerebrospinal fluid of multiple sclerosis patients. *Brain Res.* 1999; 821:511-515.

Lim ET, Grant D, Pashenkov M, Keir G, Thompson EJ, Soderstrom M, Giovannoni G. Cerebrospinal fluid levels of brain specific proteins in optic neuritis. *Mult Scler*. 2004;10:261-265.

Litzenburger T, Fassler R, Bauer J, Lassmann H, Linington C, Wekerle H, Iglesias A. B lymphocytes producing demyelinating autoantibodies: development and function in gene-targeted transgenic mice. *J Exp Med*. 1998;188:169-180.

Lou J, Gasche Y, Zheng L, Giroud C, Morel P, Clements J, Ythier A, Grau GE. Interferon- β inhibits activated leukocyte migration through human brain microvascular endothelial cell monolayer. *Lab Invest* 1999;79:1015-1025.

Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology*. 1996;46:907-911.

Lucchinetti C, Bruck W, Parisi J, Scheithauer B, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. *Ann Neurol*. 2000;47:707-717.

Lycke J, Anderson O, Rosengren LE. Neurofilament in CSF: a potential marker

of activity in MS. *Eur J Neurol* 1996;3(Suppl 5):100.

Massaro AR, Michetti F, Laudisio A, Bergonzi P. Myelin basic protein and S-100 antigen in cerebrospinal fluid of patients with multiple sclerosis in the acute phase. *Ital J Neurol Sci* 1985;6:53-56.

Matsuda M, Tsukada N, Miyagi K, Yanagisawa N. Increased levels of soluble vascular cell adhesion molecule-1 (VCAM-1) in the cerebrospinal fluid and sera of patients with multiple sclerosis and human T lymphotropic virus type-1-associated myelopathy. *J Neuroimmunol.* 1995;59:35-40.

Matthews PM, De Stefano N, Narayanan S, Francis GS, Wolinsky JS, Antel JP, Arnold DL. Putting magnetic resonance spectroscopy studies in context: axonal damage and disability in multiple sclerosis. *Semin Neurol* 1998;18:327-336.

McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, McFarland HF, Paty DW, Polman CH, Reingold SC, Sandberg-Wollheim M, Sibley W, Thompson A, van den Noort S, Weinshenker BY, Wolinsky JS. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol.* 2001;50:121-127.

McLean BN, Luxton RW, Thompson EJ. A study of immunoglobulin G in the

cerebrospinal fluid of 1007 patients with suspected neurological disease using isoelectric focusing and the Log IgG-Index. A comparison and diagnostic applications. *Brain* 1990;113:1269-1289.

Medana M, Esiri MM. Axonal damage: a key predictor of outcome in human CNS diseases. *Brain* 2003;126:515-530.

Michetti F, Massaro A, Murazio M. The nervous system-specific S-100 antigen in cerebrospinal fluid of multiple sclerosis patients. *Neurosci Lett*. 1979;11:171-175.

Michetti F, Massaro A, Russo G, Rigon G. The S-100 antigen in cerebrospinal fluid as a possible index of cell injury in the nervous system. *J Neurol Sci* 1980; 44:259-263.

Milligan NM, Newcombe R, Compston DA. A double-blind controlled trial of high dose methylprednisolone in patients with multiple sclerosis: 1. Clinical effects. *J Neurol Neurosurg Psychiatry*. 1987;50:511-516.

Missler U, Wandinger KP, Wiesmann M, Kaps M, Wessel K. Acute exacerbation of multiple sclerosis increases plasma levels of S-100 protein. *Acta Neurol Scand*. 1997; 96:142-144.

Morales DS, Siatkowski RM, Howard CW, Warman R. Optic neuritis in children.

J Pediatr Ophthalmol Strabismus. 2000;37:254-259.

Nabors LB, Songu-Mize E, Mize RR. Quantitative immunocytochemistry using an image analyzer. II. Concentration standards for transmitter immunocytochemistry. J Neurosci Methods. 1988;26:25-34.

Newcombe J, Gahan S, Cuzner ML. Serum antibodies against central nervous system proteins in human demyelinating disease. Clin Exp Immunol. 1985;59:383-390.

Newcombe J, Woodroffe MN, Cuzner ML. Distribution of glial fibrillary acidic protein in gliosed human white matter. J Neurochem. 1986;47:1713-1719.

Nixon RA, Sihag RK. Neurofilament phosphorylation: a new look at regulation and function. Trends Neurosci. 1991;14:501-506.

Norgren N, Rosengren L, Stigbrand T. Elevated neurofilament levels in neurological diseases. Brain Res. 2003; 987:25-31.

Noppe M, Crols R, Andries D, Lowenthal A. Determination in human cerebrospinal fluid of glial fibrillary acidic protein, S-100 and myelin basic protein as indices of non-specific or specific central nervous tissue pathology. Clin Chim Acta. 1986;155:143-150.

Olsson T, Sun JB, Solders G, Xiao BG, Hojeberg B, Ekre HP, Link H. Autoreactive T and B cell responses to myelin antigens after diagnostic sural nerve biopsy. J Neurol Sci. 1993;117:130-139.

Optic Neuritis Study Group. The clinical profile of optic neuritis. Experience of the Optic Neuritis Treatment Trial. Arch Ophthalmol. 1991;109:1673-1678.

O'Riordan JI, Losseff NA, Phatouros C, Thompson AJ, Moseley IF, MacManus DG, McDonald WI, Miller DH. Asymptomatic spinal cord lesions in clinically isolated optic nerve, brain stem, and spinal cord syndromes suggestive of demyelination. J Neurol Neurosurg Psychiatry. 1998a;64:353-357.

O'Riordan JI, Thompson AJ, Kingsley DP, MacManus DG, Kendall BE, Rudge P, McDonald WI, Miller DH. The prognostic value of brain MRI in clinically isolated syndromes of the CNS. A 10-year follow-up. Brain. 1998b;121:495-503.

Ozakbas S, Cagiran I, Ormeci B, Idiman E. Correlations between multiple sclerosis functional composite, expanded disability status scale and health-related quality of life during and after treatment of relapses in patients with multiple sclerosis. J Neurol Sci. 2004;15:3-7.

P

Paterson PY, Day ED, Whitacre CC, et al. Endogenous myelin basic protein-serum factors (MBP-SFs) and anti-MBP antibodies in humans. Occurrence in sera of clinically well subjects and patients with multiple sclerosis. J Neurol Sci. 1981;52:37-51.

Paolillo A, Pozzilli C, Gasperini C, et al. Brain atrophy in relapsing-remitting multiple sclerosis: relationship with 'black holes', disease duration and clinical disability. J Neurol Sci. 2000;174:85-91.

Panitch H, Hirsch R, Schindler J, Johnson K. Treatment of multiple sclerosis with gamma interferon: exacerbations associated with activation of the immune system. Neurology 1987;37:1097-1102.

Pant HC, Veeranna, Grant P. Regulation of axonal neurofilament phosphorylation.

Curr Top Cell Regul. 2000;36:133-150.

Paty DW, Li DK. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group. *Neurology*. 1993;43:662-667.

Paty DW, Oger JJ, Kastrukoff LF, Hashimoto SA, Hooge JP, Eisen AA, Eisen KA, Purves SJ, Low MD, Brandeys V, et al. MRI in the diagnosis of MS: a prospective study with comparison of clinical evaluation, evoked potentials, oligoclonal banding, and CT. *Neurology* 1988;38:180-185.

Pelletier J, Suchet L, Witjas T, Habib M, Guttmann CR, Salamon G, Lyon-Caen O, Cherif AA. A longitudinal study of callosal atrophy and interhemispheric dysfunction in relapsing-remitting multiple sclerosis. *Arch Neurol*. 2001;58:105-111.

Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernandez J, Molinero A, Campbell IL, Hidalgo J. Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. *Exp Neurol*. 2003;181:130-148.

Peterson GL. Review of the folin phenol protein quantitation method of Lowry, Roseborough, Fair and Randall. *Analytical Biochemistry* 1979;100:201-220.

Petzold A, Baker D, Pryce G, Keir G, Thompson EJ, Giovannoni G.

Quantification of neurodegeneration by measurement of brain-specific proteins. *J Neuroimmunol.* 2003b; 138:45-48.

Petzold A, Eikelenboom MJ, Gveric D, et al. Markers for different glial cell responses in multiple sclerosis: clinical and pathological correlations. *Brain* 2002a;125:1462-1473.

Petzold A, Eikelenboom BJ, Keir G, EJ Thompsen, G Giovannoni. Dynamics of axonal pathology in multiple sclerosis. *Multiple Sclerosis.* 2002b;8:Suppl: S25.

Petzold A, Jenkins R, Watt HC, Green AJ, Thompson EJ, Keir G, Fox NC, Rossor MN. Cerebrospinal fluid S100B correlates with brain atrophy in Alzheimer's disease. *Neurosci Lett.* 2003c;336:167-170.

Petzold A, Keir G, Green AJ, et al. A specific ELISA for measuring neurofilament heavy chain phosphoforms. *J Immunol Methods* 2003a;278:179-190.

Pihlaja H, Rantamäki T, Wikström J, Sumelahti ML, Laaksonen M, Ilonen J, Ruutiainen J, Pirttilä T, Elovaara I, Reunanen M, Kuokkanen S, Peltonen L, Koivisto K, Tienari PJ. Linkage disequilibrium between the MBP tetranucleotide repeat and multiple sclerosis is restricted to a geographically defined subpopulation in Finland. *Genes Immun.* 2003;4:138-146.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH, Tourtellotte WW. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983;13:227-231.

Pribyl TM, Campagnoni C, Kampf K, Handley VW, Campagnoni AT. The major myelin protein genes are expressed in the human thymus. *J Neurosci Res*. 1996;15;:812-819.

Prineas JW, McDonald WI, Franklin RJM. Demyelinating diseases. In: Graham DI, Lantos PC, eds. *Greenfield's neuropathology*. Vol.2 &ed. London:Arnold, 2002:471-550.

Prineas JW and Wright RG. Macrophages, lymphocytes, and plasma cells in the perivascular compartment in chronic multiple sclerosis. *Lab Invest*. 1978;38:409-421.

PRISMS Study Group. Randomised double-blind placebo-controlled study of interferon β -1a in relapsing-remitting multiple sclerosis. *Lancet* 1998;352:1498-1504.

Raine CS, Bonetti B, Cannella B. Multiple sclerosis: expression of molecules of

the tumor necrosis factor ligand and receptor families in relationship to the demyelinated plaque. *Rev Neurol (Paris)*. 1998;154:577-585.

Rao MV, Nixon RA. Defective neurofilament transport in mouse models of amyotrophic lateral sclerosis: a review. *Neurochem Res*. 2003;28:1041-1047.

Reindl M, Linington C, Brehm U, Egg R, Dilitz E, Deisenhammer F, Poewe W, Berger T. Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain* 1999;122:2047-2056.

Revel M, Kalinka P. Glycosylated interferon beta in multiple sclerosis. In: Abramsky O, Ovadia H, eds. *Frontiers in multiple sclerosis: clinical research and therapy*. London: M Dunitz, 1997:243-254.

Rizzo JF, Lessell S. Risk of developing multiple sclerosis after uncomplicated optic neuritis: a long-term prospective study. *Neurology* 1988;38:185-190.

Rivers TM, Sprunt DH, Berry GP. Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. *J Exp Med* 1933;58:39-53.

Robertson NP, O'Riordan JI, Chataway J, Kingsley DP, Miller DH, Clayton D, Compston DA. Offspring recurrence rates and clinical characteristics of conjugal

multiple sclerosis. Lancet. 1997;31:1587-1590.

Rodriguez M, Siva A, Cross SA, O'Brien PC, Kurland LT. Optic neuritis: a population-based study in Olmsted County, Minnesota. Neurology. 1995;45:244-250.

Roxburgh RHR, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S, Achiti I, *et al.* Multiple Sclerosis Severity Score: using disability and disease duration to rate disease severity. Neurology. 2005;64:1144-1151.

Rosengren LE, Karlsson JE, Sjogren M, Blennow K, Wallin A. Neurofilament protein levels in CSF are increased in dementia. Neurology. 1999;52:1090-1093.

Rosengren LE, Lycke J, Andersen O. Glial fibrillary acidic protein in CSF of multiple sclerosis patients: relation to neurological deficit. J Neurol Sci. 1995;133:61-65.

Royston MC, McKenzie JE, Gentleman SM, Sheng JG, Mann DM, Griffin WS, Mrak RE. Overexpression of s100beta in Down's syndrome: correlation with patient age and with beta-amyloid deposition. Neuropathol Appl Neurobiol. 1999;25:387-393

Rudick RA, Fisher E, Lee JC, Simon J, Jacobs L. Use of the brain parenchymal fraction to measure whole brain atrophy in relapsing-remitting MS. Multiple Sclerosis Collaborative Research Group. *Neurology* 1999;53:1698-1704.

Sadovnick AD, Baird PA, Ward RH. Multiple sclerosis: updated risks for relatives. *Am J Med Genet.*1988b;29:533-541.

Salmi A, Arnadottir T, Reunanen M, Ilonen J. The significance of virus antibody synthesis in the central nervous system of multiple sclerosis patients. In *Viruses of Demyelinating Diseases*, ed. by Mims CAC, Cuzner ML and Kelly RE, Academic Press, London, 1983; pp 141-152.

Sanchez I, Hassinger L, Sihag RK, Cleveland DW, Mohan P, Nixon RA. Local control of neurofilament accumulation during radial growth of myelinating axons in vivo. Selective role of site-specific phosphorylation. *J Cell Biol.* 2000;151:1013-1024.

Sawcer S., Compston A. The Genetic Analysis of Multiple sclerosis in Europeans: concepts and design. *J of Neuroimmunol*, 2003;143:13-16.

Sawcer S, Jones HB, Feakes R, Gray J, Smaldon N, Chataway J, Robertson N, Clayton D, Goodfellow PN, Compston A. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. *Nat Genet.* 1996;13:464-468.

Schafer BW, Heizmann CW. The S100 family of EF-hand calcium-binding proteins: functions and pathology. *Trends Biochem Sci.* 1996; 21:134-140.

Schmierer K, Scaravilli F, Barker GJ, Altmann DR, Miller DH. Magnetization transfer ratio and myelin content in post-mortem MS brain. *Ann Neurol.* 2004;56:407-415.

Schmidt S. S100B: pathogenetic and pathophysiologic significance in neurology. *Nervenarzt* 1998;69:639

Schumacher FA, Beebe GW, Kibler RF, Kurland LT, Kurtzke JF, McDowell F, Nagler B, Sibley WA, Tourtelotte WW, Willmon TL. Problems of experimental trials of therapy in multiple sclerosis. *Ann NY Acad Sci* 1965;122:552-568.

Sellebjerg F, Christiansen M, Garred P. MBP, anti-MBP and anti-PLP antibodies, and intrathecal complement activation in multiple sclerosis. *Mult Scler.* 1998a;4:127-131.

Sellebjerg F, Christiansen M, Jensen J, Frederiksen JL. Immunological effects of oral high-dose methylprednisolone in acute optic neuritis and multiple sclerosis. *Eur J Neurol.* 2000;7:281-289.

Sellebjerg F, Frederiksen JL, Nielsen PM, Olesen J. Double-blind, randomized, placebo-controlled study of oral, high-dose methylprednisolone in attacks of MS. *Neurology.* 1998b;51:529-534.

Sellebjerg F, Jensen CV, Larsson HB, Frederiksen JL. Gadolinium-enhanced magnetic resonance imaging predicts response to methylprednisolone in multiple sclerosis. *Mult Scler.* 2003;9:102-107.

Sellebjerg F, Nielsen HS, Frederiksen JL, Olesen J. A randomized, controlled trial of oral high-dose methylprednisolone in acute optic neuritis. *Neurology.* 1999;52:1479-1484.

Semra YK, Seidi OA, Sharief MK. Heightened intrathecal release of axonal cytoskeletal proteins in multiple sclerosis is associated with progressive disease and clinical disability. *J Neuroimmunol.* 2002;122:132-139.

Sharrack B, Hughes RA. Clinical scales for multiple sclerosis. *J Neurol Sci.* 1996;135:1-9.

Silber E, Semra YK, Gregson NA, Sharief MK. Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. *Neurology*. 2002;58:1372-1381.

Silberberg DH. Corticosteroids and optic neuritis. *N Engl J Med* 1993;329:1808-1810.

Sindic CJ, Chalon MP, Cambiaso CL, Laterre EC, Masson PL. Assessment of damage to the central nervous system by determination of S-100 protein in the cerebrospinal fluid. *J Neurol Neurosurg Psychiatry*. 1982; 45:1130-1135.

Smith ME, Stone LA, Albert PS, Frank JA, Martin R, Armstrong M, Maloni H, McFarlin DE, McFarland HF. Clinical worsening in multiple sclerosis is associated with increased frequency and area of gadopentetate dimeglumine-enhancing magnetic resonance imaging lesions. *Ann Neurol* 1993;33:480-489.

Söderström M., Ya-Ping J., Hillert J., Link H.. Optic neuritis: prognosis for multiple sclerosis from MRI, CSF and HLA findings. *Neurology* 1998;50:708-714.

Solders G, Correale J, Zhi W, Hojeberg B, Link H, Olsson T. Increased systemic B- and T-lymphocyte responses in hereditary motor and sensory neuropathy (HMSN I). *J Neurol Sci*. 1992;113:62-69.

Stata Corporation. Stata Statistical Software: Release 7.0. College Station, TX: Stata Corporation 2001.

Stefansson K, Marton LS, Dieperink ME, Molnar GK, Schlaepfer WW, Helgason CM. Circulating autoantibodies to the 200,000-dalton protein of neurofilaments in the serum of healthy individuals. *Science*. 1985;228:1117-1119.

Stefflerl A, Brehm U, Linington C. The myelin oligodendrocyte glycoprotein (MOG): a model for antibody-mediated demyelination in experimental autoimmune encephalomyelitis and multiple sclerosis. *J Neural Transm Suppl*. 2000;58:123-133.

Sternberger L., Sternberger N. Monoclonal antibodies distinguish phosphorylated and non-phosphorylated forms of neurofilaments in situ. *Proc. Natl, Acad. Sci. USA*. 1983;82:6126-6130.

Stuve O, Dooley NP, Uhm JH, Antel JP, Francis GS, Williams G, Yong VW. Interferon β -1b decreases the migration of T lymphocytes in vitro: effects on matrix metalloproteinase-9. *Ann Neurol* 1996;40:853-863.

Sun J, Link H, Olsson T, Xiao BG, Andersson G, Ekre HP, Linington C, Diener P. T and B cell responses to myelin-oligodendrocyte glycoprotein in multiple

sclerosis. *J Immunol* 1991;146:1490–1495.

Swingler RJ, Compston DAS. The prevalence of multiple sclerosis in the United Kingdom. *J Neurol Neurosurg Psychiatry*. 1986;49:1115-1124

Tejada-Simon MV, Hong J, Rivera VM, Zhang JZ. Skewed autoantibody reactivity to the extracellular domain of myelin oligodendrocyte glycoprotein in multiple sclerosis. *Immunology*. 2002;107:403-410.

Ter Meulen V and Stephenson JR. The possible role of viral infections in multiple sclerosis and other related demyelinating diseases. In *Multiple Sclerosis*, ed. by Hallpike JF, Adams CWM and Tourtellotte WW, Chapman and Hall, London, first edition, 1983 pp241-274.

The INFB Multiple Sclerosis Study Group Interferon beta-1b is effective in relapsing-remitting multiple sclerosis: Clinical results of a multicenter, randomized, double blind, placebo-controlled trial. *Neurology* 1993;43:655-661.

The Optic Neuritis Study Group. The 5-year risk of MU after optic neuritis: experience of the optic neuritis treatment trial. *Neurology* 1997;49:1404-1413.

Thompson AJ, Brazil J, Feighery C, Hutchinson M, Martin EA. CSF myelin basic protein in multiple sclerosis. *Acta Neurol Scand* 1985;72:577-583.

Thompson EJ, Kaufmann P, Shortman RC, Rudge P, McDonald WI. Oligoclonal immunoglobulins and plasma cells in spinal fluid of patients with multiple sclerosis. *Br Med J*. 1979;1:16-17.

Thompson AJ, Miller D, Youl B, MacManus D, Moore S, Kingsley D, Kendall B, Feinstein A, McDonald WI. Serial gadolinium-enhanced MRI in relapsing-remitting multiple sclerosis of varying disease duration. *Neurology* 1992;42:60-63.

Tintore M, Rovira A, Martinez MJ, Rio J, Diaz-Villoslada P, Brieva L, Borrás C, Grive E, Capellades J, Montalban X. Isolated demyelinating syndromes: comparison of different imaging criteria to predict conversion to clinically definite MS. *Am J Radiology* 2000;21:702-706

Tintore M, Rovira A, Rio J, Nos C, Grive E, Sastre-Garriga J, Pericot I, Sanchez E, Comabella M, Montalban X. New diagnostic criteria for multiple sclerosis: application in first demyelinating episode. *Neurology* 2003;14:27-30.

Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med*. 1998;338:278-285.

Trojano M, Liguori M, Bosco Zimatore G, Bugarini R, Avolio C, Paolicelli D,

Giuliani F, De Robertis F, Marrosu MG, Livrea P. Age-related disability in multiple sclerosis. *Ann Neurol*. 2002;51:475-480

Uhm JH, Dooley NP, Stuve O, Francis GS, Duquette P, Antel JP, Yong VW. Migratory behaviour of lymphocytes isolated from multiple sclerosis: effects of interferon beta-1b therapy. *Ann Neurol* 1999;46:319-324.

Van der Valk V, de Groot CJ. Staging of multiple sclerosis (MS) lesions: pathology of the time frame of MS. *Neuropathol Appl Neurobiol*. 2000;26:2-10.

Van Waesberghe JH, Barkhof F. Magnetization transfer imaging of the spinal cord and the optic nerve in patients with multiple sclerosis. *Neurology*. 1999;53(Suppl 3):S46-48.

Valberg LS, Flanagan PR, Kertesz A, Ebers GC. Abnormalities in iron metabolism in multiple sclerosis. *Can J Neurol Sci*. 1989;16:184-186.

Wallstrom E, Khademi M, Andersson M, Weissert R, Linington C, Olsson T. Increased reactivity to myelin oligodendrocyte glycoprotein peptides and epitope mapping in HLA DR2(15)+ multiple sclerosis. *Eur J Immunol* 1998;28: 3329–3335.

Wandinger K, Jabs W, Siekhaus A, Bubel S, Trillenberger P, Wagner H, Wessel K,

Kirchner H, Hennig H. Association between clinical disease activity and Epstein Barr virus reactivation in MS. *Neurology* 2000;55:178-187.

Wang S, Lees GJ, Rosengren LE, Karlsson JE, Hamberger A, Haglid KG. Proteolysis of filament proteins in glial and neuronal cells after in vivo stimulation of hippocampal NMDA receptors. *Neurochem Res* 1992a;17:1005-1009.

Wang WZ, Olsson T, Kostulas V, Hojeberg B, Ekre HP, Link H. Myelin antigen reactive T cells in cerebrovascular diseases. *Clin Exp Immunol.* 1992b;88:157-162.

Weinshenker BG. Natural history of multiple sclerosis. *Ann Neurol* 1994;36(Suppl):S6-S11.

Weinshenker BG, Bass B, Rice GP, Noseworthy J, Carriere W, Baskerville J, Ebers GC. The natural history of multiple sclerosis: a geographically based study. 2. Predictive value of the early clinical course. *Brain.* 1989;112 (Pt 6):1419-1428.

Wiebe S, Lee DH, Karlik SJ, Hopkins M, Vandervoort MK, Wong CJ, Hewitt L, Rice GP, Ebers GC, Noseworthy JH. Serial cranial and spinal cord magnetic resonance imaging in multiple sclerosis. *Ann Neurol* 1992; 32:643-650.

Wolwijk G. Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells. *J Neurosci* 1998; 18:601-609

Wujek JR, Bjartmar C, Richer E, Ransohoff RM, Yu M, Tuohy VK, Trapp BD. Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis. *J. Neuropathol. Exp. Neurol.* 2002;61;23-32.

Xiao BG, Linington C, Link H. Antibodies to myelin-oligodendrocyte glycoprotein in cerebrospinal fluid from patients with multiple sclerosis and controls. *J Neuroimmunol.* 1991;31:91-96.

Yabe JT, Pimenta A, Shea TB. Kinesin-mediated transport of neurofilament protein oligomers in growing axons. *J Cell Sci.* 1999;112:3799-3814.

Yong VW, Chabot S, Stuve O, Williams G. Interferon beta in the treatment of multiple sclerosis: mechanisms of action. *Neurology.* 1998;51:682-689.

Zhu B, Moore GR, Zwimpfer TJ, et al. Axonal cytoskeleton changes in experimental optic neuritis. *Brain Res* 1999;824:204-217.

