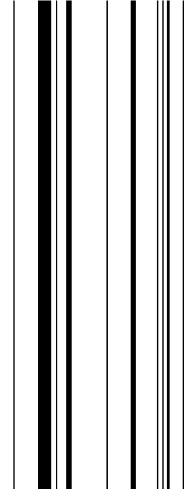

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DOCTORAATSPROEFSCHRIFT

Identification and characterization of novel antibody targets for clinically isolated syndrome and multiple sclerosis

Proefschrift voorgelegd tot het behalen van de graad van doctor in de biomedische wetenschappen, te verdedigen door:

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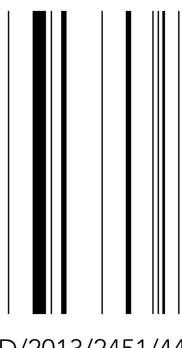

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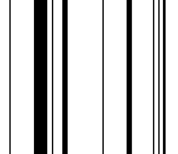

"You never fail until you stop trying."
Albert Einstein

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List of Abbreviations

2-D	2-dimensional
ADP	adenosine diphosphate
AN2	progenitor cell-specific surface glycoprotein
ANCAs	anti-neutrophil cytoplasmic antibodies
BBB	blood-brain barrier
BENEFIT	Betaseron® in Newly Emerging MS for Initial Treatment
BEST1	bestrophin 1
BLAST	basic local alignment search tool
CD58	cluster of differentiation 58
CD86	cluster of differentiation 86
CD-MS	clinically definite multiple sclerosis
cDNA	complementary deoxyribonucleic acid
cfu	colony forming units
CHAMPS	controlled high-risk subjects Avonex® MS prevention study
CIS	clinically isolated syndrome
CLEC16A	C-type lectin-like domain family 16A
CMV	cytomegalovirus
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNPase 1	2′,3′-cyclic-nucleotide 3′-phosphodiesterase 1
CNS	central nervous system
CSF	cerebrospinal fluid
CV	coefficient of variation
CXCL13	C-X-C motif chemokine 13
DAB	3,3′-diaminobenzidine
DAPI	4′, 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EBNA-1	Epstein-Barr nuclear antigen 1
EBV	Epstein-Barr virus
EDDS	expanded disability disease scale
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ETOMS	early treatment of MS
EVI5	ecotropic viral integration site 5 protein
F	female
FDA	Food and Drug Administration
GA	glatiramer acetate
GAGA4	Glc(α1,4)Glc(α)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	germinal center
GFAP	glial fibrillary acidic protein
GPR65	G protein-coupled receptor 65
GTF2H4	general transcription factor IIH, polypeptide 4
GWAS	genome wide association study
HC	healthy control
HCF-1	host cell factor 1

HCFCR1	host cell factor c1 regulator 1
HEK	human embryonic kidney
HERV-W	human endogenous retrovirus-W
HHV-6	human herpesvirus-6
HLA II	human leukocyte antigen class II
Hnrp A1	heterogeneous nuclear ribonucleoprotein A1
Hnrp A2/B1	heterogeneous nuclear ribonucleoprotein A2/B1
HnrpB1	heterogeneous nuclear ribonucleoprotein B1
HRP	Horse radish peroxidase
HSP	heat-shock proteins
HSP60	heat-shock protein 60
HSP70	heat-shock protein 70
HSP90beta	heat-shock protein 90beta
IFNβ	interferon-β
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-10	Interleukin-10
IL-17	Interleukin-17
IL2RA	interleukin-2 receptor alpha
IL7RA	interleukin-7 receptor alpha
IP	immunoprecipitation
KIF1B	kinesin family member 1B
KIRs	killer cell immunoglobulin-like receptors
LB	Luria-Bertani
M	male
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MPBS	skimmed milk powder in phosphate-buffered saline
MRI	magnetic resonance imaging
MS	multiple sclerosis
MTX	mitoxantrone
NA	not applicable/not available
NADH	nicotinamide adenine dinucleotide (reduced form)
NAWM	normal appearing white matter
NCBI	national center for biotechnology information
NF	neurofascin
NF-H	neurofilament-heavy
NF-L	neurofilament-light
NF-M	neurofilament-medium
NIND	non-inflammatory neurological disease
NO	nitric oxide
NWM	normal white matter
OCB	oligoclonal bands
OD	optical density
OIND	other inflammatory neurological disease
PBS	phosphate-buffered saline

PBST	phosphate-buffered saline Tween20
PCR	polymerase chain reaction
PEX6	peroxisomal biogenesis factor 6
pfu	plaque forming units
PGAM	phosphoglycerate mutase
pIII	M13 coat protein 3
PLP	proteolipid protein
PML	progressive multifocal leukoencephalopathy
PP	primary-progressive
PreCISe	early glatiramer acetate treatment in delaying conversion to clinically definite multiple sclerosis in subjects presenting with a clinically isolated syndrome
PVDF	polyvinylidene difluoride
pVI	M13 coat protein 6
pVIII	M13 coat protein 8
RR	relapsing-remitting
RT	room temperature
S1P	sphingosine-1-phosphate
SAS	serological antigen selection
SD	standard deviation
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
SOCS1	suppressors of cytokine signaling 1
SP	secondary-progressive
SPAG16	sperm associated antigen 16
STAT3	signal transducer and activator of transcription 3
SWAP70	switch-associated protein 70
TBS	tris-buffered saline
TMB	tetramethyl-benzidine dihydrochloride
tNAA	N-acetyl-aspartate
TOB1	transducer of ERBB-2,1
TPI	triosephosphate isomerase
TYK2	tyrosine kinase 2
UH-CIS	University Hasselt - clinically isolated syndrome
UH-PPMS	University Hasselt – primary-progressive multiple sclerosis
UH-RRMS	University Hasselt – relapsing-remitting multiple sclerosis
UTR	untranslated region
VCAM1	vascular cell adhesion molecule 1
VZV	varicella zoster virus

1

Introduction and aims

1.1 Multiple sclerosis and clinically isolated syndrome

1.1.1 Clinical presentation

Multiple sclerosis (MS) is a chronic neurological disease of the central nervous system (CNS), characterized by demyelination of axons and the formation of sclerotic plaques. In Europe, the prevalence of MS is 83 per 100,000 individuals [1]. Mainly young adults (20-40) are affected and prevalence is higher among females [2]. A wide variety of clinical symptoms, such as fatigue, weakness, bladder dysfunction, tremor and cognitive impairment have been associated with MS [3]. The majority of MS patients display a relapsing-remitting (RR) disease course, in which relapses are followed by periods of clinical recovery and stability. Of these RR-MS patients, 65% will progress to a secondary-progressive (SP) phase, characterized by a gradual increase in disease disability without periods of remission. In 20% of MS patients, disease has a primary-progressive (PP) disease course from onset [3].

In 85% of MS patients, MS is preceded by a clinically isolated syndrome (CIS), an episode of neurological disturbance which should last at least for 24 hours [4]. Symptoms can be monofocal or multifocal, affecting the optic nerve, spinal cord, brain stem, cerebellum or cerebral hemispheres [5]. In addition, CIS may be accompanied by lesions on magnetic resonance imaging (MRI) [6]. Although CIS can be an early presentation of MS, only 30-70% of CIS patients eventually develop MS [7]. Risk factors for conversion to MS are female sex [8], disease onset at young age [9] or abnormal MRI [10]. However, it has been demonstrated that 20% of CIS patients with abnormal brain MRI do not convert to clinically definite (CD) MS within 20 years [10]. For this reason, much research is currently dedicated to the search for laboratory biomarkers that can predict conversion to MS.

1.1.2 Diagnostic criteria for MS

MS diagnosis is based on clinical presentation, cerebrospinal fluid (CSF) laboratory analysis, visual evoked potential findings and MRI. For many years, the Poser criteria have been used to diagnose MS [11]. To fulfill the Poser criteria for CDMS, patients should have experienced 2 separate clinical attacks caused

by different lesions, which demonstrates dissemination of lesions in time and space. Laboratory findings of the CSF, such as unique oligoclonal bands (OCB) not present in serum can also help to establish a diagnosis of MS. These OCB represent locally produced immunoglobulin G (IgG) and can be found in up to 95% of MS patients [12]. However, OCB are not specific for MS and are also found in other inflammatory neurological diseases. An increased IgG index reflecting an elevated production of intrathecal IgG is another hallmark of MS [13].

In 2001, novel guidelines, also known as the McDonald criteria, were proposed [14]. Using the McDonald criteria, a patient can be diagnosed with MS after one clinical attack and evidence should be present of 2 or more lesions (dissemination in space). In addition, new lesions should be observed on a follow-up MRI performed at least 3 months after the first clinical attack (dissemination in time). When these criteria are fulfilled, but a second clinical attack has not (yet) occurred, a patient will be diagnosed with McDonald MS. Moreover, criteria for PP-MS were proposed, which include abnormal CSF findings, dissemination in space and clinical progression for at least 1 year [14]. The McDonald criteria have been adapted in 2005 and 2010 [5, 15], to simplify and allow a more rapid diagnosis of MS.

1.1.3 Immunopathogenesis

MS is an autoimmune disorder in which defects are present in the immune regulatory system. One of the commonly accepted mechanisms that may initiate MS is molecular mimicry of viral antigens or bacterial superantigens to myelin proteins, which causes activation of autoreactive T cells [16]. Upon activation, the autoreactive T cells upregulate the expression of endothelial adhesion molecules and cross the blood-brain barrier (BBB) [17]. Subsequently, other T cells, macrophages, B and plasma cells are recruited to the CNS [18] (Figure 1.1). These immune cells produce cytokines and other inflammatory factors ($CD4^+$ T cells), directly damage neurons and oligodendrocytes ($CD8^+$ T cells), release inflammatory and toxic molecules (macrophages), initiate the complement cascade and induce antibody-mediated phagocytosis (B and plasma cells), which eventually leads to demyelination and axonal injury [19].

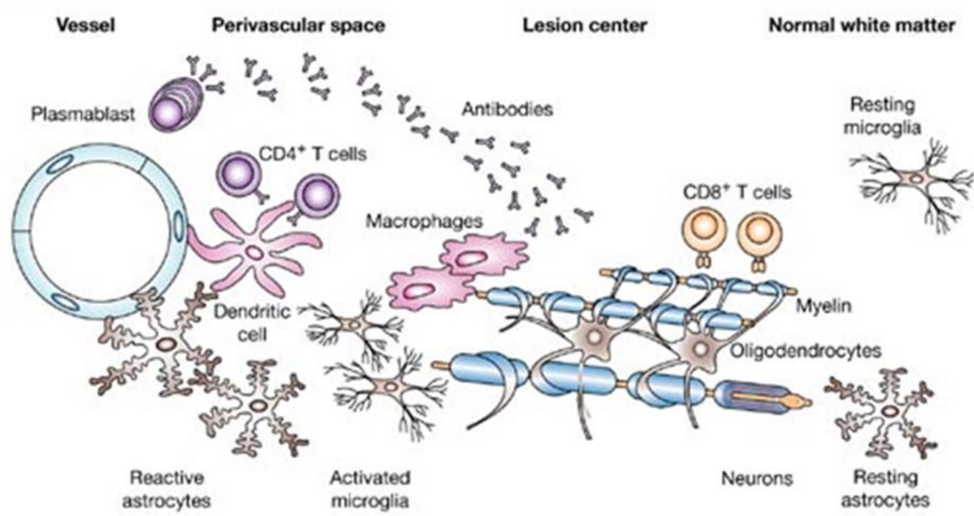

Figure 1.1: Immunopathogenesis of MS. T cells, B cells and macrophages cross the blood-brain barrier and infiltrate the CNS. In the CNS, T cells become reactivated by antigens presented by dendritic and microglial cells, and other T cells, B cells and macrophages are attracted. Damage to the CNS is then caused by the secretion of pro-inflammatory cytokines, other inflammatory mediators and antibodies.
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Other mechanisms are implicated in MS pathogenesis as well. Activated microglia and astrocytes produce nitric oxide (NO) which reacts with O_2^- molecules [20]. This results in the formation of reactive nitrogen species causing damage to proteins. In addition, NO can directly reduce axonal conduction [20]. Moreover, ischemia may cause metabolic impairment of oligodendrocytes [21] and mitochondrial dysfunction may be involved in axonal damage and destruction [22].

The immune system has been shown to play an important role in MS. However, recently, an ‘inside-out’ model has been proposed for MS [23]. This ‘inside-out’ model states that MS is a primary neurodegenerative disorder rather than an autoimmune disease (‘outside-in’ model). According to the ‘inside-out’ model, oligodendrocytes and myelin may initially be affected by degeneration. As a consequence, autoantigenic components such as myelin basic protein (MBP) and myelin lipids are released, which may promote a secondary autoimmune and inflammatory response. However, the severity of this secondary inflammatory

response may differ between individuals and explain the presence of the RR (strong inflammatory response) and the PP phenotype of MS (weak immune response).

1.1.4 Lesion pathology

The pathological hallmark of MS is focal demyelination of regions called plaques or lesions, which harbor variable degrees of inflammation, demyelination, gliosis, and axonal injury [24]. Two lesion types can be distinguished in MS. First, active lesions are characterized by infiltration of macrophages with myelin debris, lymphocytes and astrocytes [24]. Secondly, chronic lesions contain few (inflammatory) cells, although accumulation of macrophages can be found at the lesion edge [24]. Interestingly, the myelin sheath can be restored via remyelination. Such remyelinated areas are also known as shadow plaques. However, the efficiency of remyelination is rather low and further disease progression will be inevitable [25].

Lesions are characterized by several (immune) cell types and 4 distinct demyelination patterns have been described [26]. Pattern I is characterized by T cell infiltration, whereas additional antibody deposition is a hallmark of pattern II lesions. On the other hand, type III and IV demyelination are characterized by oligodendrocyte loss. Furthermore, lesions of individual MS patients were found to be characterized by a single pattern of demyelination. However, in another study several demyelination patterns were detected within a single MS patient [27].

Traditionally, lesions in the white matter of brain and spinal cord have been associated with MS. In recent years, however, gray matter lesions have also become subject of interest. In contrast with white matter lesions, lymphocyte infiltration, complement deposition and BBB disruption are absent in gray matter lesions [28]. Although widespread demyelination of gray matter has been found in the chronic phase of MS [24], other studies demonstrated the occurrence of gray matter lesions in early stages of MS that may even precede the formation of white matter lesions [29].

1.1.5 Genetic factors associated with CIS and MS

It has been recognized for a long time that someone has an increased risk to develop MS when relatives are affected with MS [30]. In addition, the prevalence of MS among monozygotic twins is higher as compared to dizygotic twins [31]. Therefore, a genetic component is likely to be involved in the development of MS.

The human leukocyte antigen class II (HLA II) locus, which resides within the major histocompatibility complex (MHC) region on chromosome 6p21.3 has been associated with an increased risk for MS. Several genetic studies demonstrated a strong association between MS and the HLA allele HLA-DRB1*1501 as well as other HLA alleles [32]. Other candidate genes associated with increased MS susceptibility are interleukin-7 receptor alpha (IL7RA) [33], interleukin-2 receptor alpha (IL2RA) [33], ecotropic viral integration site 5 protein (EVI5) [34], cluster of differentiation 58 (CD58) [35], C-type lectin-like domain family 16A (CLEC16A) [36], kinesin family member 1B (KIF1B) [37], suppressors of cytokine signaling 1 (SOCS1) [38] and tyrosine kinase 2 (TYK2) [39]. These genes are implicated in T cell activation, adhesion, axonal function and cytokine signaling. Recently, several research groups collaborated and performed a large scale genome wide association study (GWAS). In this study, previously reported genetic risk factors could be confirmed and additional novel candidate loci such as vascular cell adhesion molecule 1 (VCAM1), cluster of differentiation 86 (CD86) and G protein-coupled receptor 65 (GPR65) were identified [40]. Interestingly, genetic investigation of CD4+ lymphocytes from 4 discordant MS twin pairs revealed no difference in their genome, mRNA transcriptome and epigenome, which points to a role of other non-genetic factors in MS [41].

Since CIS can represent an early manifestation of MS it is conceivable that CIS and MS patients carry similar genetic risk alleles. In a recent study, Jelcic et al analyzed whether killer cell immunoglobulin-like receptors (KIRs) and HLA class I were associated with MS susceptibility or severity [42]. These KIRs are MHC class I specific receptors expressed on natural killer and T cells and are composed of both activating and inhibitory counterparts. Seventeen KIR genes have been described. However, not all of these KIR genes are found in each individual [43]. A

significant reduction in the frequency of the inhibitory gene KIR2DL3 was observed in both CIS and MS. In another study, an association between progression to MS and the presence of HLA haplotypes HLA-DRB1*1501, DQA1*0102, DQB1*0602 was detected in CIS patients [44].

1.1.6 Environmental factors associated with CIS and MS

Apart from a genetic predisposition, several environmental factors are proposed to increase the risk of developing MS. An example of an environmental factor associated with CIS and MS is infection with the Epstein-Barr virus (EBV), a herpes virus capable of infecting human B cells. Nearly all MS patients have been shown to be EBV seropositive [45], although EBV infection is also widespread in the general population. Moreover, EBV infected B cells have been detected in the MS brain of SP-MS patients [46, 47]. However, the latter finding remains controversial [48, 49, 50].

Other viruses have also been associated with MS. For instance, DNA from the varicella zoster virus (VZV) has been detected in CSF and in peripheral mononuclear blood cells from MS patients [51, 52, 53]. However, this finding could not be replicated in another study [54]. Furthermore, despite the presence of increased antibody levels towards VZV in CSF from MS patients, recombinant antibodies prepared from clonally expanded MS CSF plasma cells did not bind to VZV [54]. Therefore, it is unlikely that VZV is a disease relevant antigen in MS.

In addition, DNA from human herpesvirus-6 (HHV-6) has been detected in brain, serum and CSF of MS patients [55, 56, 57]. Similar to the presence of VZV DNA in MS brain, this finding has been refuted [58, 59]. Interestingly, the HHV-6 virus has been demonstrated to be able to enter the CNS through the olfactory pathway [60]. Constitutive presence of active HHV-6 infection in glial cells in inflamed CNS tissue has been suggested to result in virus-triggered immunopathologies in MS [61, 62]. Furthermore, anti-HHV-6 IgG titers have been shown to correlate with the risk for a subsequent relapse in MS patients [63].

Moreover, other viruses such as cytomegalovirus (CMV) [64], chlamydia pneumonia [65], herpes simplex virus [66], human endogenous retrovirus-W (HERV-

W) [67], measles [68] and the rubella virus [68] have also been associated with MS. Several mechanisms have been proposed how viruses can trigger autoimmune responses in MS, which include the activation of autoreactive immune cells through bystander activation, molecular mimicry or epitope spreading [62].

The prevalence of MS has been shown to increase with increasing geographical latitude [69]. A decrease in vitamin D, which synthesis partly depends on exposure to sunlight, may account for this phenomenon. In MS patients during relapse, decreased serum 25-hydroxyvitamin D levels were detected as compared with patients in remission [70]. In addition, other factors such as smoking [71] and improved hygiene standards [72] have been associated with increased susceptibility to develop MS.

1.1.7 Treatment

Although MS is an incurable disease, various treatment strategies can be applied to reduce clinical symptoms or delay disease progression. The choice of therapy will be dependent on disease phase and progression. The majority of current therapies are suitable to treat RR-MS, whereas limited treatment options are available for patients with progressive MS.

1.1.7.1 First-line treatment for MS

Interferon-β (IFNβ) was the first drug approved by the Food and Drug Administration (FDA) for first-line treatment of RR-MS and is administered via intramuscular (IFNβ-1a) or subcutaneous (IFNβ-1a and IFNβ-1b) injection. Clinical studies demonstrated a reduction in relapses and MRI lesions after treatment with IFNβ [73, 74, 75]. Treatment causes an increase of anti-inflammatory cytokines, whereas the production of pro-inflammatory cytokines is decreased [76]. In addition, IFNβ may cause a reduction in inflammatory cell recruitment across the BBB and may be involved in CNS repair [76].

Glatiramer acetate (GA) has also been approved for first-line treatment of MS. GA is a random polymer of 4 amino acids, mimicking an epitope of the myelin protein MBP. A decrease in the number of relapses and MRI lesions has been demonstrated in RR-MS patients after GA treatment [77, 78]. GA interacts with MHC

molecules on MBP specific antigen-presenting cells, thereby preventing presentation of MBP antigens [79]. In addition, GA causes a shift from a Th1 to a Th2 response and may stimulate neuroprotection [79].

1.1.7.2 Other treatment strategies for MS

When MS patients fail to respond on conventional first-line therapy or display an aggressive disease course, other treatment strategies can be applied. Such alternative treatment may be accompanied by (severe) side effects and/or their clinical efficacy is not yet completely proven.

Several second-line therapeutic agents have been approved by the FDA for the treatment of MS. Mitoxantrone (MTX) is an immunosuppressive drug developed to treat malignancies. MTX is a cytotoxic antineoplastic agent which causes a decrease in proliferation of immune cells, production of pro-inflammatory cytokines and macrophage-mediated myelin degradation [80]. In MS patients, MTX has been shown to reduce neurological disability and the frequency of relapses [80]. Side effects associated with MTX treatment are leukemia or cardiomyopathy [81].

The first approved oral treatment of MS was Fingolimod. Treatment with this compound resulted in a reduction in relapse rate and disease progression in patients with RR-MS [82, 83]. After *in vivo* phosphorylation, Fingolimod binds the sphingosine-1-phosphate (S1P) receptor, which is expressed on lymphocytes. As a result, the S1P receptor is internalized and lymphocytes are sequestered in the lymph nodes [84]. Apart from Fingolimod, oral agents Dimethyl Fumarate [85] and Teriflunomide [86] have recently also been approved for the treatment of MS.

Natalizumab is a humanized monoclonal antibody directed towards the endothelial adhesion molecule α4-integrin. Natalizumab prevents lymphocytes to enter the CNS and has been shown to reduce disability progression and the number of relapses in RR-MS [87]. However, several Natalizumab treated patients developed the severe condition progressive multifocal leukoencephalopathy (PML) [88, 89], which has been linked with prior infection and reactivation of the widespread JC virus [90].

Recently, another monoclonal antibody, Alemtuzumab, directed against CD52 has been approved by the European Medicines Agency (EMA) for the treatment of RR-MS. Its therapeutic mechanism is based on depletion and repopulation of B lymphocytes and T lymphocytes [91].

Furthermore, several clinical trials have been performed with other therapeutics that have not (yet) been approved by the FDA, due to a lack of evidence for clinical efficacy or the occurrence of serious side effects. For instance, Rituximab, a monoclonal antibody that depletes CD20+ B cells, has been shown to reduce inflammatory brain lesions and clinical relapses in RR-MS[92]. In addition, other monoclonal antibodies, such as Ocrelizumab (CD20) [93], Ofatumumab (CD20) [94] and Daclizumab (CD25) [95] have been tested in clinical trials.

The cytotoxic agent Cladribine causes depletion of B and T cells [96]. Oral administration of Cladribine in RR-MS patients resulted in a decrease in relapse frequency, disease disability and MRI disease activity [97]. Finally, Laquinimod was found to suppress the development of new active lesions [98]. Several mechanisms of action for Laquinimod have been proposed, including a shift from a Th1 to a Th2 response and a reduction of leukocyte infiltration into the CNS [98].

1.1.7.3 Clinical trials in CIS

Several studies have been performed to examine the effect of treatment on disease progression in CIS patients. In the CHAMPS (Controlled High-Risk Subjects Avonex® MS prevention study) study, CIS patients were treated with intramuscular injections of IFNβ-1a [99]. After 3 years of follow up, CIS patients who received IFNβ-1a treatment had a significantly lower risk to develop CDMS as compared with placebo treated CIS patients. Similar findings were reported after extension of the study to 5 years [100]. However, after 10 years of follow-up no effect on disability outcomes was observed when the early IFNβ-1a treated CIS patients were compared with a control group in which therapy later was initiated [101].

Another clinical trial, the ETOMS (Early Treatment of MS) study, investigated the effect of weekly subcutaneous injections of IFNβ-1a in CIS patients [102]. IFNβ-1a treatment led to a reduced risk to convert to CDMS and a decrease in progressive loss of brain tissue [103].

In the BENEFIT (Betaseron® in Newly Emerging MS for Initial Treatment) study, CIS patients were treated with subcutaneously injections of IFNβ-1b [104]. Both the development of CDMS and McDonald MS were delayed in the treatment group. In addition, a lower number of MRI lesions were detected in treated CIS patients. After extension of the BENEFIT study, treated CIS patients had still a lower risk to progress to MS, although disability progression was similar when IFNβ-1b treatment was delayed up to 2 years [105, 106].

The other approved first-line treatment of MS, GA, has also been tested in CIS. In the PreCISe (early glatiramer acetate treatment in delaying conversion to clinically definite multiple sclerosis in subjects Presenting with a Clinically Isolated Syndrome) study, CIS patients were treated with GA, which led to a reduction in the risk to develop CDMS and MRI measures [107].

In conclusion, early treatment has been shown to cause a delay in conversion to MS in CIS patients, although the positive effects on disability progression remain controversial. To further investigate the effect of treatment in CIS patients, additional studies with a extensive period of follow-up are mandatory.

1.2 The humoral immune response in CIS and MS

1.2.1 B cells and antibodies

B cells develop in the bone marrow and are derived from pluripotent hematopoetic stem cells [108]. These stem cells differentiate into pro-B cells, pre-B cells and finally into immature B cells, which then migrate to peripheral lymphoid tissue. In the periphery, subsequently, immature B cells develop into mature B cells. This development is characterized by rearrangement of immunoglobulin light and heavy chain gene segments. After encountering their appropriate antigen, mature B cells differentiate into antibody producing plasma cells. B cells function as co-stimulatory and antigen-presenting cells and produce cytokines [109]. However, B cells are primarily known for antibody production.

Several antibody isotypes are produced by plasma cells [108]. Early in the antibody response, immunoglobulin M (IgM) antibodies with relatively low affinity are produced. In subsequent phases of the antibody response, mainly antibodies of the IgG isotype are produced. Antibodies are characterized by different effector functions [108]. First, antibodies are able to bind pathogens, which prevents these pathogens to enter a cell. This process is also known as neutralization. Secondly, bound antibodies can be recognized by macrophages which then phagocytose the antigen–antibody complex (opsonization). Thirdly, the complement system can be activated, which leads to direct destruction of the bound target or acts as a recognition signal for phagocytic cells. Finally, antibody-dependent cellular cytotoxicity causes destruction of antibody-bound cells by natural killer cells.

1.2.2 B cells in CIS and MS

Although T cells have been primary implicated in MS pathogenesis, B cells are likely to be involved in MS pathogenesis as well. This is supported by the positive results achieved with the B cell depleting therapy Rituximab [92]. As shown in Figure 1.2 ii, B cells act as antigen-presenting cells to activate autoreactive T cells. In addition, B cells have regulatory functions, which is reflected by the production of the anti-inflammatory cytokine interleukin-10 (IL-10) (Figure 1.2 iv). Interestingly, B cells from MS patients have been shown to

produce less IL-10 [110]. Furthermore, B cells may activate T cells through non-antigen specific bystander activation [111] (Figure 1.2 v). In brain from SP-MS patients, ectopic B cell germinal centers have been detected [112] (Figure 1.2 iii). In the CSF of CIS and MS patients, memory B cells become activated and differentiate into antibody secreting plasma cells and/or plasma blasts [113]. Antibodies produced by these cells are part of the OCB, which are found in CSF from the majority of CIS and MS patients (Figure 1.2 i) [114]. However, the targets of these (auto)antibodies remain largely elusive.

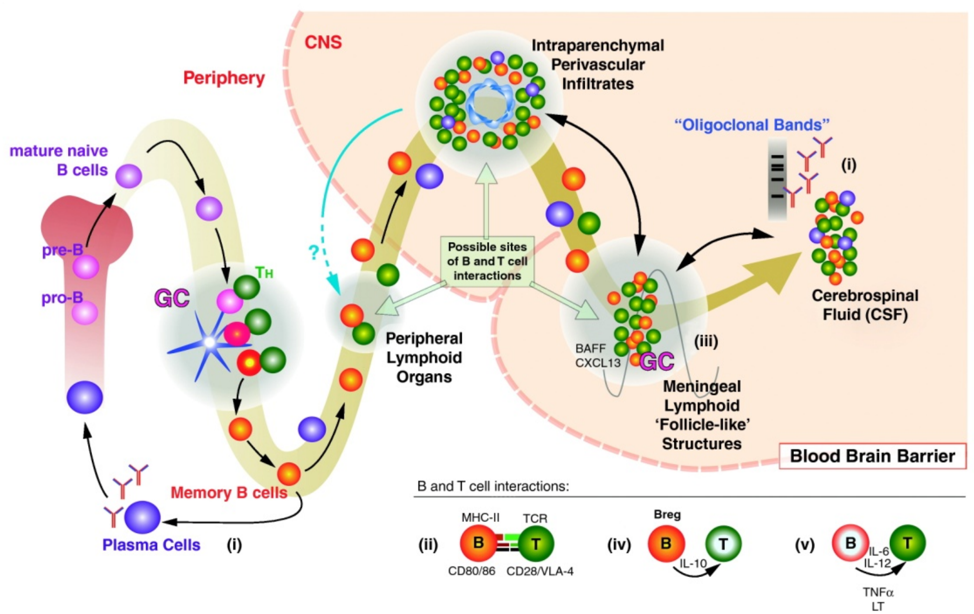

Figure 1.2: Proposed role of B cells in MS. B cells develop in the bone marrow and undergo affinity maturation in germinal centers (GC). Subsequently, B cells differentiate into memory B cells or antibody producing plasma cells. Memory B cells act as antigen-presenting cells, which activate potentially CNS-reactive T cells (ii). After these cells have crossed the blood-brain barrier, perivascular infiltrates and meningeal lymphoid "follicle-like" structures are formed (iii). Plasma cells produce antibodies represented by oligoclonal bands in the cerebrospinal fluid (i). In addition, B cells have regulatory functions (iv) and activate T cells by non-antigen specific bystander B cell activation (v).
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1.2.3 Antibodies and their targets in CIS and MS

To identify (auto)antibody targets in CIS and MS, the CSF and serum antibody repertoire has been studied extensively. Antibody reactivity towards a variety of myelin components, axonal proteins, lipids, heat shock proteins, viruses and ubiquitously expressed proteins has been detected in a subset of CIS and MS patients (Table 1.1). In general, antibody reactivity towards a particular antigen is only detected in a (minor) subset of CIS and MS patients. The sensitivity can be increased when reactivity to several antibody targets is determined. In our group, a panel of MS antibody targets has been identified with a 86% specificity and 45% sensitivity for MS [115]. The potassium channel KIR4.1, a recently identified candidate autoantigen for CIS and MS, may represent an exception since serum antibody reactivity towards KIR4.1 could be detected in 46,9% of CIS and MS patients. On the other hand, serum anti-KIR4.1 antibody reactivity was detected in only 0,9% of neurological controls and was completely absent in healthy controls [116].

It should be noted that the specificity and sensitivity of several antibody targets, including the extensively studied antigen myelin oligodendrocyte glycoprotein (MOG) [117], remains under debate. In addition, antibody reactivity towards CIS and MS antibody targets, for example neurofascin [118], neurofilament light [119] and neurofilament heavy [120] and tau [120], has also been detected in patients with other diseases. Moreover, follow-up studies are currently missing for the majority of the reported antibody targets, including the promising candidate autoantigen KIR4.1.

As shown in Table 1.1, similar antibody targets are found in CIS and MS. The absence of antibody reactivity towards several MS antigens in CIS patients is mainly due to the absence of studies that addressed such issue rather than experimental evidence. Furthermore, in most studies no information concerning MS development in CIS patients was given. Therefore, it is unknown whether antibody reactivity towards a particular target is found in CIS patients irrespective of conversion to MS or is restricted to CIS patients that progressed to MS.

Table 1.1: Antibody targets in CIS and MS

Antigen source	CIS	MS
Myelin components		
Myelin oligodendrocyte glycoprotein (MOG)	121, 122, 123	122, 123, 124, 125, 126, 127
Myelin basic protein (MBP)	121, 128, 129, 130, 131	132, 133, 134
Myelin-associated glycoprotein (MAG)	-	135, 136
Proteolipid protein (PLP)	-	137
Axonal, neuronal and oligodendrocyte proteins		
Neurofascin	138	138
Contactin-2	139	139, 140
Oligodendrocyte specific protein	-	141
Tau	142	142
Neurofilament-light (NF-L)	143	144, 145, 146
Neurofilament-medium (NF-M)	-	147
Neurofilament-heavy (NF-H)	148, 149	148
Tubulin	-	150
Amyloid-beta 1	148	148
2′,3′-Cyclic-nucleotide 3′-Phosphodiesterase 1 (CNPase 1)	-	151
Lipids		
Gangliosides	-	152
Sphingomyelin	-	153
Cerebroside	-	153
Sulphatide	-	153, 154
Galactocerebroside	155	155
CSF114(Glc)	-	156
Viruses		
Epstein-barr virus (EBV)	157, 158, 159	159, 160
Measles	68, 161	161, 162
Rubella	68	162
Varicella zoster virus (VZV)	68	162
Human herpes virus 6 (HHV6)	-	63, 163
Cytomegalovirus (CMV)	-	164, 165
Herpes simplex virus	-	66
Chlamydia pneumonia	-	166
Stress proteins		
αB-crystallin	-	167
Heat shock protein 90beta (HSP90beta)	-	168
Heat shock protein 60 (HSP60)	-	169
Heat shock protein 70 (HSP70)	-	170, 171

Table 1.1: continued

Antigen Source	CIS	MS
Other		
Sperm associated antigen 16 (SPAG16)	-	115
DNA	-	172
Potassium channel KIR 4.1	116	116
Phosphoglycerate mutase 1 (PGAM)	-	173
Transaldolase	-	174, 175
Triosephosphate isomerase (TPI)	176	176
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	176	176
Glc(α1,4)Glc(α) (GAGA4)	177	178, 179
Transketolase	-	151
Heterogeneous nuclear ribonucleoprotein A1 (Hnrp A1)	-	180
Heterogeneous nuclear ribonucleoprotein A2/B1 (Hnrp A2/B1)	-	180
Heterogeneous nuclear ribonucleoprotein B1 (HnrpB1)	-	181
Proteasome	182	182
Nogo-A	-	183
Switch-associated protein 70 (SWAP70)	-	184
Alu-repeats	-	185
Alpha actinin 1	-	186
S-nitrosylated proteins	-	187
Progenitor cell-specific surface glycoprotein (AN2)	-	188

"-" indicates the absence of knowledge for antibody reactivity towards a given target in CIS patients

1.2.4 Functional role of antibodies in CIS and MS

As described in the previous section, antibody reactivity towards a wide variety of targets has been reported in CIS and MS. It is also of interest to investigate whether these antibodies have a functional role in CIS and MS pathogenesis.

Several groups investigated the pathogenicity of antibodies by injecting them into animals with experimental autoimmune encephalomyelitis (EAE), the animal model of MS. Disease exacerbating effects in EAE have been described for antibodies towards MOG [189], neurofilament-light (NF-L) [190], neurofascin (NF) [138], sperm associated antigen 16 (SPAG16) [191] and sulfatide [153]. In addition, IgG antibodies with unknown specificity from a subset of MS patients have been shown to cause complement-dependent antibody mediated demyelination and axonal loss using an *in vitro* myelinating culture system [192]. On the other hand, antibodies may have protective properties as well. IgM antibodies binding to the surface of neural cells promoted polarized axon outgrowth and improved

functional recovery in a mouse model of MS [193]. Alternatively, the presence of antibodies may represent an epiphenomenon caused by dysregulation of the immune system in CIS and MS patients. However, based on the described results, at least a subset of the antibodies in CIS and MS patients are likely to fulfill a functional role.

1.3 Biomarkers

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention [194]. Such biomarkers may be used for MS diagnosis, subtyping and prognosis, give information about disease pathogenesis or can be used for future drug development.

1.3.1 Biomarkers for MS

Body fluids, such as blood, CSF and urine, and (brain) tissue of MS patients have been investigated to identify biomarkers for MS. To this end, high-throughput "omic" techniques can be used. For instance, proteomics allows the study of the proteome of a body fluid or tissue in an unbiased fashion. In this way, MS patients could be differentiated from neurological controls based on their proteome profile present in CSF, serum and brain tissue [195, 196, 197, 198, 199]. Furthermore, MS subtypes as well as treated and untreated MS patients could be distinguished from each other [200, 201, 202, 203]. Transcriptomics revealed differences in gene expression levels in peripheral blood cells of MS patients and controls [204, 205, 206, 207] and in MS subtypes [208], identified differentially expressed genes in MS lesion types [209], discriminated between treated and untreated MS patients [210] and led to the identification of biomarkers that predict the efficacy of IFNβ treatment in MS patients [211]. In addition, several genes that are associated with an increased risk for MS have been identified by the application of genomics (section 1.1.5).

1.3.2 Prognostic biomarkers and risk markers to predict conversion to MS in CIS patients

Eventually, 30 – 70% of the CIS patients develop MS [7]. However, it is difficult to predict which subset of CIS patients will convert to MS. Therefore, many studies have been focused on the identification of biomarkers that could predict whether a CIS patient is likely to develop MS.

For instance, antibody reactivity may be used as a prognostic biomarker. The presence of CSF OCB, increased IgM and IgG antibody levels towards myelin proteins MOG and MBP as well as other antibody specificities have been suggested as biomarkers to predict conversion to MS in CIS patients (Table 1.2). However, the prognostic potential of antibody reactivity towards these myelin proteins remains controversial [128, 129, 130, 131, 212, 213]. In addition, other candidate biomarkers for disease progression in CIS patients have been proposed (Table 1.2). For example, increased levels of chitinase 3-like 1, C-X-C motif chemokine 13 (CXCL13), glial fibrillary acidic protein (GFAP), decreased levels of fetuin-a and certain MRI parameters may predict conversion to MS in CIS patients.

However, in the majority of these studies only a limited number of patients were included, different diagnostic criteria (CDMS versus McDonald MS) were used and for a subset of the identified markers findings could not be confirmed in subsequent studies. Therefore, an ongoing need exists to identify novel markers which are able to identify CIS patients that are at high risk to develop MS.

Table 1.2: Candidate biomarkers and risk markers for conversion to MS in CIS patients

Biomarker/risk marker type	Source	Reference
Antibodies and B cells		
↑ IgM Glc(α1,4)Glc(α) (GAGA4)	serum	[177, 214]
↑ IgM/IgG myelin oligodendrocyte glycoprotein (MOG)	serum	[121, 128, 129, 130, 131, 212, 215]
↑ IgM/IgG myelin basic protein (MBP)	serum	[121, 128, 129, 130, 131, 212]
↑ IgM lipid specific oligoclonal bands (OCB)	CSF	[216, 217]
Total IgG repertoire	serum	[218]
↑ Free kappa chains	CSF	[219]
↑ Epstein-Barr virus-encoded antigen 1	serum	[158]
↑ MRZ response (antibody reactivity towards measles, rubella and varicella zoster virus)	CSF	[68]
↑ OCB	CSF	[220, 221, 222, 223]
↑ CD5+ B cells	blood	[224]
Protein/elements		
↓ Fe	serum	[225]
↑ C-X-C motif chemokine 13 (CXCL13)	CSF	[226, 227]
↑ Chitinase 3-like 1	CSF	[228]
↓ Fetuin-A	CSF	[229]
↑ 14-3-3	CSF	[230, 231]
↑ Tau	CSF	[232]
↑ Glial fibrillary acidic protein (GFAP)	CSF	[232]
↑ Myelin oligodendrocyte glycoprotein (MOG)	CSF	[232]
↓ Transducer of ERBB-2,1 (TOB1)	T cells	[233]
↑ phosphorylated signal transducer and activator of transcription 3 (STAT3)	T cells	[234]
MRI		
↓ N-acetyl-aspartate (tNAA)	brain	[235]
↑ grey matter atrophy	brain	[236]
↑ gadolinium enhancing lesions	brain	[237]
↑ T2 lesions	brain	[237]
Genetic factors		
Human leukocyte antigen (HLA) alleles		[44]
V_H4 and V_H2 repertoire bias		[238]
Other		
↑ Smoking		[239]

Arrows indicate whether decreased or increased levels of a marker enhance the risk for progression to MS in CIS patients

1.4 Strategies to investigate the antibody response in CIS and MS

1.4.1 Approaches for known antibody targets

In the majority of studies, the antibody response towards known (auto)antigens has been analyzed by Western blot analysis or enzyme-linked immunosorbent assay (ELISA). However, depending on the experimental conditions and antigen preparations, the conformation of antigens can be disturbed using these assays. To analyze the antibody response towards proteins expressed in their native conformation, cell based assays can be used. A cell based assay may be in particular suited to detect antibody reactivity towards transmembrane proteins, such as MOG [240]. The above described assays are mainly used for the analysis of the antibody response towards 1 antigen, whereas antigen arrays are used to simultaneously measure antibody reactivity towards multiple antigens. For example, Quintana et al. analyzed the antibody response in MS by means of antigen microarrays spotted with CNS proteins, lipids and heat-shock proteins [241, 242]. Using such arrays, unique antibody reactivity patterns were discerned in serum from MS subtypes as well as different antibody repertoires in paired CSF and serum from RR-MS patients. In addition, arrays probed with lipids and clones derived from expression libraries have been used to study the antibody repertoire of MS patients [153, 154, 160, 243].

1.4.2 Tools to identify novel antibody targets

The major drawback of the above described methods is that they are generally limited to known antibody targets. Other strategies can be applied for the identification of novel antibody targets. For instance, the antibody repertoire of MS patients can be studied using 1-D immunoblot procedures [167, 176, 186]. To this end, myelin or brain lysates are separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Next, proteins are blotted onto membranes (e.g.nitrocellulose or polyvinylidene difluoride (PVDF)), which are then incubated with antibodies of MS patients [167, 176]. In this way, proteins reacting with MS antibodies can be visualized. Apart from the total MS antibody repertoire, such immunoblot procedures can also be used for the identification of novel *in vivo* antibody targets. For example, antibodies towards the synthetic

glycopeptide CSF114(Glc) were found in 30% of RR-MS patients [156, 244]. However, the *in vivo* target of CSF114(Glc) was unknown. Therefore, antibodies towards CSF114(Glc) were purified from positive serum samples and used for immunoblotting on a rat brain homogenate, which led to the identification of alpha actinin-1 as the *in vivo* target for anti-CSF114(Glc) antibodies [186].

However, it must be kept in mind that proteins are denatured during SDS-PAGE and that these denatured epitopes may differ from the corresponding *in vivo* epitopes. Another disadvantage of immunoblot approaches is the absence of direct antigen identification, since proteins cannot be isolated from the blot membranes. Antibody targets can only be identified after performing a protein staining in parallel followed by excision of the corresponding band or spot and subsequent mass spectrometry analysis. This might be a rather difficult procedure, since a single band/spot can contain multiple proteins or it may be difficult to identify the correct corresponding bands/spots. This can be partially avoided by 2-D gel electrophoresis, in which proteins are additionally separated on their isoelectric point [138, 139]. Therefore, alternative approaches which allow direct antigen identification may represent a more suitable strategy. Examples of such alternative techniques are immunoprecipitation and phage display screening technologies.

1.5 Phage display

1.5.1 Basic concepts of phage display

The phage display technology can be used to identify target molecules for ligands and was developed by Smith in 1985 [245]. Phage display is based on the expression of peptides or proteins on the surface of bacteriophage particles, which are viruses capable of infecting bacterial cells [246]. An example of a phage strain that has been used extensively in phage display is the M13 filamentous phage. During infection with M13 filamentous phage and production of novel phage particles, lysis of the infected bacterial cell is prevented. Therefore, the M13 filamentous phage is a so called lysogenic phage. Display of foreign DNA products on the surface of the filamentous phage is enabled by fusion of foreign DNA to filamentous phage coat genes pIII, pVI or pVIII. Expression of the fusion product and its subsequent incorporation into mature phage coat results in the ligand being presented on the phage surface, while its genetic material resides in the phage particle, creating a physical link between genotype and phenotype (Figure 1.3) [247].

Figure 1.3: Principle of phage display. Foreign DNA is genetically fused to a phage coat gene. As a result, the foreign DNA product is expressed as a fusion protein with the phage coat protein on the surface of the phage. In this way, a link between genotype (foreign DNA insert) and phenotype (expressed cDNA product) is created.

Different vector systems are employed in filamentous phage display [248]. Phage vectors enable multivalent expression, which means that all coat proteins display the incorporated DNA product. On the other hand, phagemid vectors give rise to a monovalent expression system in which each phage particle displays only 1 copy of the foreign DNA product. This phagemid vector contains

the gene encoding the coat protein to which the foreign DNA will be fused as well as a plasmid and phage origin of replication. In order to produce phage particles with a phagemid vector, co-infection with a so called helper phage is required. This helper phage contains all wild-type phage genes, but lacks a packaging signal, which prevents replication of the helper phage [248].

The advantage of phage display is that multiple DNA fragments (libraries), instead of 1 DNA product, can be expressed in a population of phage particles. Several types of libraries have been used for phage display. Random peptide libraries are composed of 4 - 36 randomly ordered synthetic amino acids [246]. A drawback of these libraries is that they lead to the identification of mimotopes, peptides that do not necessarily have the same or even a similar amino acid sequence as the natural binding partners, but mimic their binding properties [249] . This may be avoided by the use of cDNA libraries, derived from cDNA repertoires expressed in (diseased) tissue [247]. In addition, antibody libraries have been successfully constructed, which allow the selection of high-affinity antibodies for a given target [250]. Therefore, a large variety of molecules can be expressed through phage display. However, a disadvantage of the phage display technology is that displayed proteins lack post-translational modifications, because proteins are generated via the bacterial protein machinery. Furthermore, some DNA products may contain mutations that inhibit viral replication [251]. On the other hand, DNA products may be highly advantageous in growth rate or viral stability, which may lead to overrepresentation [251].

Apart from M13 filamentous phage, also other phage strains such as lambda phage, T4 and T7 phage have been used for phage display.

1.5.2 Phage display approaches in MS

Several groups applied the phage display technology to identify (novel) antigens recognized by antibodies of MS patients. Cortese and colleagues used a 9-mer (9 randomly ordered amino acids) peptide library fused to protein pVIII to study the antibody repertoire in CSF of MS patients [252]. In this way, several peptides were identified that were recognized by antibodies in the CSF of MS patients. A similar frequency of antibody reactivity towards these peptides was found in

serum from MS patients and healthy controls. In a subsequent study, the surface glycoprotein gB of the herpes simplex virus 1 and a brain-specific protein were found to cross-react with antibodies directed to a peptide identified in the former study [249].

In another study, a 28-mer peptide library fused to pVIII was used for affinity selections on CSF from 1 MS patient, which revealed that antibody reactivity towards identified peptides remained stable in CSF samples taken at different time points [253]. However, antibody reactivity towards the identified targets was absent in CSF samples from 55 other MS patients. To increase the possibility of finding disease-specific antibodies, affinity selections were performed on pooled CSF from 20 MS patients and in parallel on CSF samples from patients with other neurological disorders. A positive antibody response towards peptides identified in selections on MS CSF was rarely observed in CSF from neurological controls, but antibody reactivity was also only detected in CSF samples from 1-2 MS patients [253].

More successful results were obtained by Jolivet-Reynaud et al., who screened a 15-mer peptide library fused to protein pVIII with CSF from 4 MS patients. Antibody reactivity towards 4 identified peptides was detected in 21 out of 60 CSF samples of MS patients and 2 out of 27 CSF samples of patients with other neurological diseases [254]. Moreover, identified peptides displayed homology to multiple-sclerosis associated retrovirus and HERV-W. Homology to viral proteins was also found in other studies in which 6- and 12-mer peptide libraries were used for affinity selection on CSF from MS patients [255, 256].

In 2005, Yu et al generated recombinant antibodies from over-represented IgG sequences expressed from single plasma cells from MS CSF [257]. To identify the corresponding antigens of these recombinant antibodies, a 12-mer peptide phage library was screened. In this way, several antibody-binding peptides were selected and antibody reactivity towards these peptides could be confirmed by the native IgG of the MS CSF.

In addition, cDNA libraries have been used to study the antibody repertoire of MS patients. Owens et al. prepared cDNA lambda expression libraries from chronic MS plaques and screened these libraries with CSF and serum from MS

patients [258]. However, MS-specific antigens were not retrieved. In another study, lambda phage cDNA expression libraries were constructed from an oligodendrocte-precursor cell line and selections were performed on pooled CSF from 54 MS patients [185]. Among 1,000,000 plaques, 6 positive clones were identified. Five out of these 6 clones displayed homology to an *Alu* repeat epitope. Furthermore, antibody reactivity towards these *Alu* repeats could be detected in 44% of serum and CSF samples from MS patients.

1.5.3 Serological antigen selection

Serological antigen selection (SAS) is a high-troughput molecular screening technology based on the expression of a cDNA library fused to the surface exposed C-terminus of phage minor coat protein pVI and allows the interaction and selection of phage particles displaying cDNA products from (diseased) tissue with antibodies in body fluids [247, 259] . The SAS procedure starts with the incubation of pooled serum or CSF containing antibodies with a cDNA phage display library constructed from (disease) relevant tissue (Figure 1.4). This leads to the formation and subsequent capture of antibody/antigen complexes. Next, unbound phage are washed away, followed by elution of bound phage. Eluted phage are infected in bacteria and used as input for a new selection round. In order to enrich phage particles with high affinity to the antibodies, several selection rounds are mandatory.

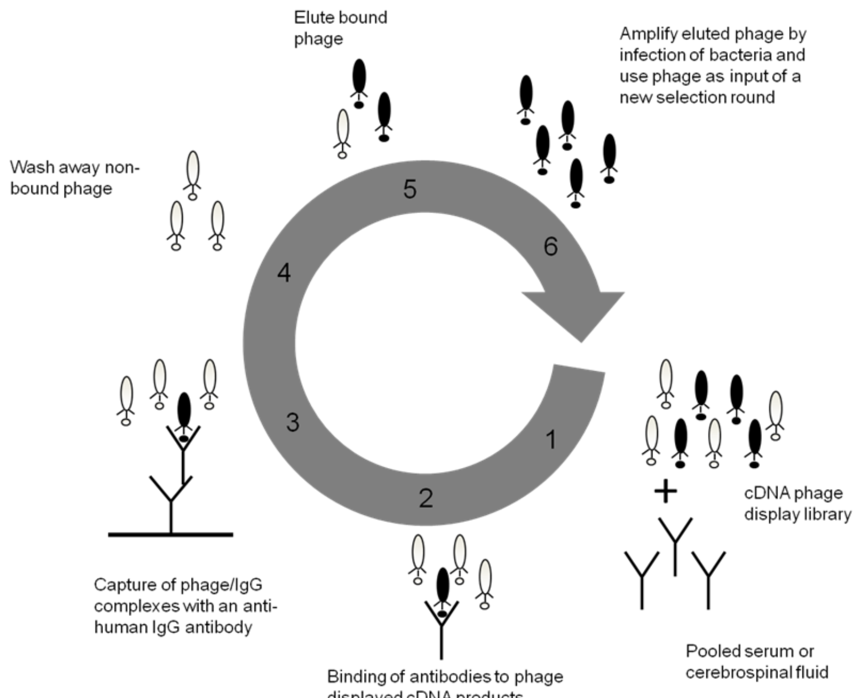

Figure 1.4: Serological antigen selection. Pooled serum or cerebrospinal fluid containing antibodies are incubated with a cDNA phage display library (1). Antigen-antibody complexes are formed between antibodies and their respective target antigens (2). An anti-human immunoglobuling G (IgG) antibody is used to capture phage/IgG complexes (3) and non-bound phage are washed away (4). Next, bound phage are eluted (5) and amplified by infection into bacteria, followed by a new selection round (6).

Three pVI display phagemid vectors have been contructed, pSPVIA, pSPVIB and pSPVIC. These phagemid vectors differ in 1 nucleotide, which allows the expression of the cDNA products in the correct reading frame [247]. The SAS technology has been successfully applied for the identification of antibody targets for colorectal cancer [260], atherosclerosis [261] and rheumatoid arthritis [262].

In addition, SAS has been used in our group to study the CSF antibody repertoire of MS patients. To this end, a normalized cDNA expression library derived from active chronic MS plaques [263, 264] was subcloned into the pVI phage display system and affinity selections were performed on pooled CSF from 10 RR-MS patients [115]. Antibody reactivity towards a panel of 8 candidate targets was further evaluated in CSF samples from 63 other MS patients, 30 patients with other inflammatory neurological diseases and 64 patients with

non-inflammatory neurological disorders, which revealed a 86% specificity and 45% sensitivity for MS. Moreover, 4 out of 8 candidate targets showed exclusive reactivity in CSF from MS patients. In another study, SAS has been used to compare the antibody repertoire in blood and CSF of MS patients [265]. To this end, SAS procedures were performed on paired serum and CSF from a single RR-MS patient and led to the identification of 13 antigenic targets. Five and 2 of these antigenic targets were enriched by serum and CSF antibodies, respectively. Antibody reactivity towards the 6 remaining targets was detected in both CSF and serum. These findings pointed to both common and distinct antibody profiles in CSF and serum of MS patients [265].

1.6 Aims of the study

MS and its possible initial manifestation CIS are characterized by an aberrant (auto)antibody response. Therefore, identification of antibody targets for CIS and MS may provide insight into underlying disease processes and allow early diagnosis of MS. Several antibody targets have been proposed for CIS and MS (Table 1.1). For a subset of these antibody targets, findings could not be confirmed in subsequent studies. In addition, inconclusive results have been reported on antibody reactivity towards several antibody targets and conversion to MS in CIS patients. Therefore, an ongoing need exists for the identification of (novel) antibody targets for CIS and MS with diagnostic properties as well as antibody targets that may predict progression to MS in CIS patients. Moreover, the identified antibody targets may serve as therapeutic targets.

The goal of this study is to identify and characterize novel antibody targets for CIS and MS. To this end, we will use the SAS technology, a powerful high-throughput molecular screening technology based on phage display, to identify antibody targets recognized by antibodies in CSF and serum from CIS and MS patients. In addition, we will investigate the antibody response towards a panel of known MS antibody targets in serum from CIS patients.

Aim 1: Identification of novel antibody targets for CIS

Limited information is available on the antibody response in CIS. Previous studies that investigated the antibody response in CIS primarily focused on antibody targets that were previously identified in MS.

Therefore, we aim to identify novel antibody targets for CIS using the SAS technology (chapter 2). To this end, high-diversity (cDNA) libraries are required. A cDNA library derived from MS plaques was already available in our institute. However, we also aim to construct a cDNA library derived from normal brain. In this way, we are able to investigate whether the antibody repertoire in CIS is primarily targeted at proteins expressed in MS or normal brain.

To identify novel antibody targets for CIS, SAS procedures will be performed in parallel with MS and normal brain libraries on pooled CSF from CIS patients that developed MS. After candidate antibody targets are selected, the antibody response towards these targets will be evaluated in CSF and serum from additional CIS and MS patients, neurological controls and in serum from healthy subjects. An ongoing need exists for prognostic markers that predict conversion to MS in CIS patients. Therefore, we will also investigate the prognostic potential of identified targets in CIS patients.

Aim 2: Investigation and comparison of the antibody response in CSF from RR- and PP-MS patients

RR- and PP-MS are characterized by different clinical presentations and different disease mechanisms may be primarily involved in these MS subtypes. Therefore, the antibody response in RR- and PP-MS may be directed to different antibody targets.

In chapter 3, we aim to identify novel antibody targets for RR- and PP-MS and to compare the antibody repertoire in these MS subtypes. To this end, SAS procedures will be performed in parallel with MS and normal brain cDNA libraries on pooled CSF from RR- and PP-MS patients. After identification of candidate antibody targets, antibody reactivity towards these targets will be examined in CSF and serum samples from patients used in the SAS procedures as well as in CSF samples from additional MS patients and neurological controls.

Aim 3: Characterization of a novel candidate antibody target for CIS and MS

The potential of novel candidate antibody targets needs to be confirmed in subsequent studies. If these antibody targets are identified by phage display, they may encode mimotopes and therefore identification of corresponding *in vivo* antibody targets is warranted. Furthermore, additional characterization of antibody targets may provide more information on their role in CIS and MS pathogenesis.

In chapter 4, we aim to further characterize a novel candidate antibody target for CIS and MS. The first goal of this study is to further study the antibody response towards this target by developing a peptide ELISA. Since this candidate antibody target encodes a mimotope, we also aim to identify and characterize the *in vivo* antibody target.

Aim 4: Analysis of the antibody response towards MS antibody targets in CIS

Several antibody targets for CIS have been reported (Table 1.1). However, the sensitivity and/or specificity of a subset of these antibody targets remains under debate. Moreover, an ongoing need exists for the identification of markers that allow the identification of CIS patients that are at high risk for progression to MS. Additional antibody targets have been identified in MS, but their sensitivity and specificity in CIS is currently unknown.

For this reason we will investigate in chapter 5 the antibody response towards MS antibody targets SPAG16, NF-L and NF in serum from CIS patients and compare this with the antibody response towards a novel candidate antibody target. Moreover, we will examine whether antibody reactivity towards the tested autoantibody targets correlates with clinical parameters and conversion to MS.

2

Novel cerebrospinal fluid and serum autoantibody targets for clinically isolated syndrome

Based on:

Novel cerebrospinal fluid and serum autoantibody targets for clinically isolated syndrome
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Abstract

Limited information is available on the identity of antigens targeted by antibodies present in cerebrospinal fluid (CSF) of patients with clinically isolated syndrome (CIS). The aim of this study was to identify novel antigens for CIS and investigate their prognostic potential to predict conversion to multiple sclerosis (MS). We applied serological antigen selection (SAS) to identify antigens interacting with antibodies present in the pooled CSF from 4 CIS patients, who developed MS. Antibody reactivity towards CIS antigens identified by SAS was tested in CSF and serum from patients with CIS (n=123/n=108), MS (n=65/n=44) and other (inflammatory) neurological diseases (n=75/n=38) as well as in healthy control sera (n=44). Using SAS, a panel of 6 novel CIS candidate antigens was identified. CSF antibody reactivity was detected in both CIS and relapsing-remitting (RR) MS. Serum reactivity was significantly increased in CIS and RR-MS as compared to controls (p=0.03). For 2 antigens, the frequency of antibody positive patients was higher in CIS patients who converted to MS as compared to CIS patients without conversion. We identified novel CIS antigens to which antibody reactivity was primarily detected in CIS and RR-MS as compared to controls. Possible prognostic potential could be demonstrated for 2 antigens.

2.1 Introduction

In the majority of multiple sclerosis (MS) patients, MS is preceded by a condition called clinically isolated syndrome (CIS), which is an acute or sub-acute episode of neurological disturbance due to a single white matter lesion [7]. However, only 30-70% of CIS patients develop MS [7]. An important role of B cells and antibodies has been recognized in CIS and MS. For instance, treatment with the B cell depleting monoclonal antibody Rituximab has shown to reduce inflammatory lesions and clinical relapses in relapsing-remitting (RR) MS [92]. Cerebrospinal fluid (CSF) of both CIS and MS patients is often characterized by the presence of oligoclonal band (OCB) antibodies [266] and it has been demonstrated that CSF B cells produce these antibodies in MS [114]. At this moment, little is known about the identity of the antigens to which antibodies are directed in CIS and if there are antigens associated with progression to MS. Examples of known antigens for antibodies in CIS are myelin components, such as myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP). Moreover, there are reports indicating an association on such antibody reactivity and conversion to MS [121, 128], although this could not be confirmed by others [129].

Previous studies investigating the antibody response in CIS focused mainly on antigens that have been identified in MS or in other diseases [68, 121, 128, 157, 177, 267, 268]. For this reason, we aimed to identify novel antigens for CIS by means of an unbiased technique called serological antigen selection (SAS), which is a high-throughput molecular screening technology based on cDNA phage display [247, 269, 270]. Up till now, this technique has been used successfully to analyze the autoantibody profile in several diseases, such as MS [115, 265, 271], rheumatoid arthritis [262], colorectal cancer [260] and atherosclerosis [261]. To identify novel antigens for CIS, SAS procedures were performed in parallel with 2 cDNA expression libraries, composed of normal white matter (NWM) and MS plaques, to examine whether primarily normal or diseased brain antigens are targeted by the antibody response in CIS. Subsequently, candidate CIS antigens were identified and antibody reactivity towards these antigens was assessed in CSF and serum from CIS and MS patients and in controls. In addition, the prognostic

potential of novel CIS antigens to predict conversion to MS in CIS patients was investigated.

2.2 Materials and methods

2.2.1 Patient material

CSF specimens of 4 CIS patients, all of who developed MS within 2 years after lumbar puncture, were used for SAS procedures (Table 2.1). These CSF samples were pooled and preadsorbed against *E.Coli* and phage extracts, as described previously [271].

Table 2.1: Characteristics of CIS patients selected for SAS procedures

CIS patient	Gender[a]	Age	EDSS[b] score
1	F	27	2.0
2	M	29	1.0
3	F	35	1.0
4	M	17	2.0

[a] F: female; M: male
[b] EDSS, expanded disability status scale

Immunoreactivity directed to antigens identified by SAS was assessed in patient and control samples (Table 2.2). For this reason, paired CSF and serum samples were collected from 105 CIS patients, 30 RR-MS patients, 11 and 8 patients with non-inflammatory neurological disorders (NIND) and other inflammatory neurological diseases (OIND), respectively. In addition, CSF samples were available from 18 other CIS, 25 RR-MS, 10 secondary-progressive (SP) MS, 36 NIND and 20 OIND patients as well as serum specimens from 3 CIS, 14 RR-MS, 13 NIND, 6 OIND patients and 44 healthy controls (HC). MS diagnosis and MS development in CIS patients was established according to McDonald criteria [14, 15]. In general, CIS patients were clinically followed for at least two years after sampling of CSF and serum. This study was approved by the institutional ethics committee and fulfilled guidelines from the declaration of Helsinki. CSF and serum samples were obtained after informed consent.

Table 2.2: Characteristics of patients selected for CSF and serum antibody reactivity screening

Patient group	Gender (M/F[a])	Mean age (years)± SD	Mean disease duration (years)[b] ± SD	EDDS score[c] ± SD
CIS				
CSF (n=123)	27/96	35.9±10.7	0.5±1.4	1.5±1.1
Serum (n=108)	22/86	35.7±10.3	0.5±1.5	1.5±1.1
RR-MS				
CSF (n=55)	10/45	40.8±9.1	8.0±9.0	2.7±1.1
Serum (n=44)	5/39	41.5±8.7	8.9±9.5	2.9±1.1
SP-MS				
CSF (n=10)	2/8	47.2±9.3	14.9±8.1	4.8±1.3
OIND				
CSF (n=28)	15/13	47.4±17.3	NA[d]	NA
Serum (n=14)	8/6	48.9±14.3	NA	NA
NIND				
CSF (n=47)	16/31	47.5±17.8	NA	NA
Serum (n=24)	6/18	44.3±19.1	NA	NA
HC				
Serum (n=44)	15/29	46.1±15.8	NA	NA

[a] M: male, F: female
[b] Data available on 102/99 (CSF/serum) CIS, 49/43 RR-MS and 10 SP-MS patients
[c] EDDS: expanded disability disease scale. Data available on 92/79 (CSF/serum) CIS, 24/25 RR-MS and 8 SP-MS patients
[d] NA: not applicable/not available

2.2.2 cDNA phage libraries for SAS

To identify novel antigens for CIS, 2 cDNA libraries were obtained: a cDNA library derived from active chronic MS plaques, with a primary diversity of 1.0 * 10^6 recombinants (kindly provided by Dr. M. B. Soares [263, 264]) and a NWM cDNA library from multiple areas of white matter from one normal human brain, with a primary diversity of 0.9 * 10^6 recombinants (obtained from Dr. G.P Owens [258]). The cloning procedure of the MS cDNA library into the pVI phage display system has been described previously [271]. The pVI phage display system entails 3 vectors, pSPVIA, pSPVIB and pSPVIC, which allows expression of the cDNA

products in the correct reading frame. An overview of the cloning procedure of the NWM cDNA library is depicted in Figure 1.

2.2.3 Construction of a NWM cDNA library

The NWM cDNA library was directionally cloned into the lambda phage UniZAP vector (Stratagene, La Jolla, USA) (Figure 2.1A). In order to subclone the NWM cDNA inserts into the pVI phage display system, the NWM pBluescript phagemid was mass excised out of the lambda phage UniZAP vector according to manufacturers' instructions (B). Next, NWM pBluescript phage particles were then infected into SOLR bacterial cells and NWM plasmid was isolated (C). Subsequently, NWM cDNA inserts were isolated by restriction digestion and ligated into the pSPVI phage display vectors (D,E).

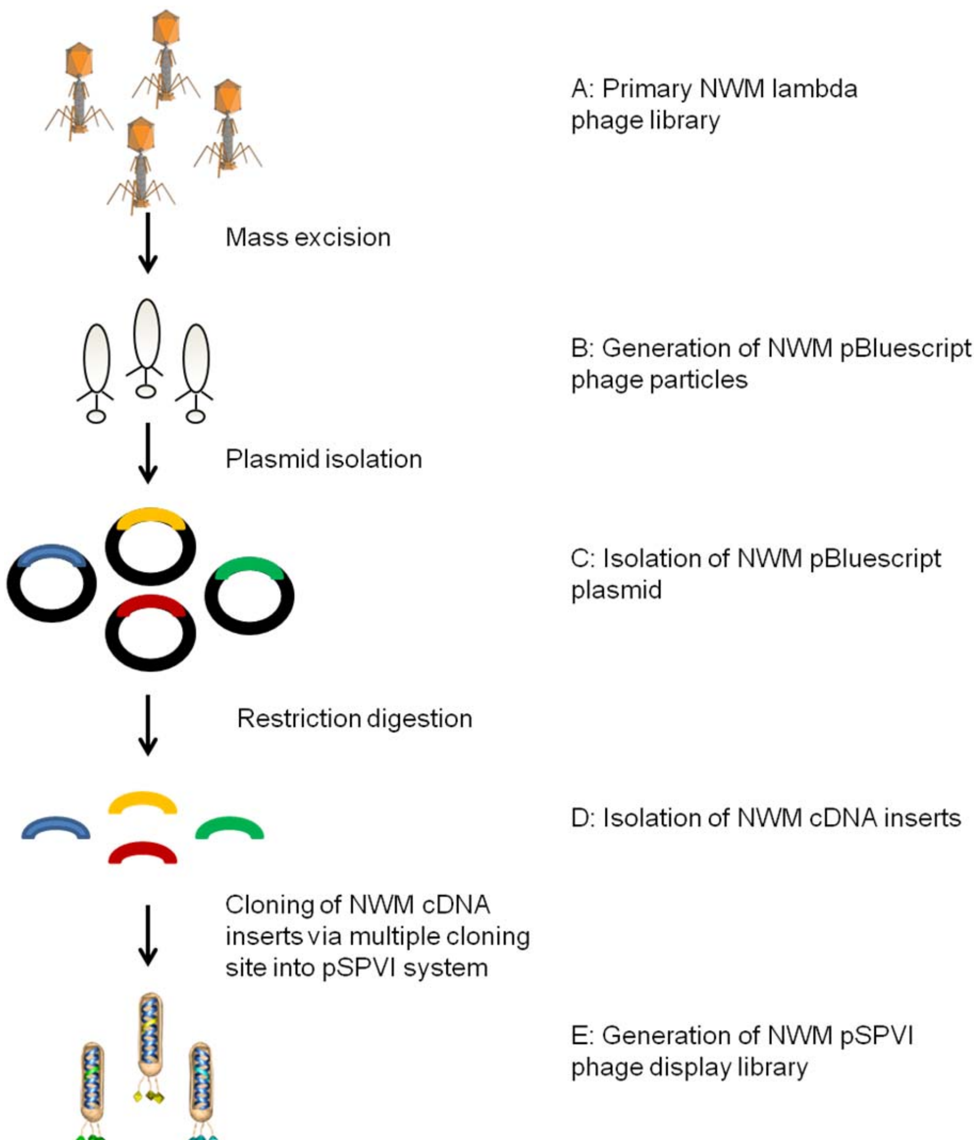

Figure 2.1: Construction of a NWM cDNA phage display library. A NWM library cloned into the lambda phage UniZap vector was obtained (A). A mass excision step was performed to generate filamentous pBluescript phage particles (B). NWM plasmid DNA was isolated (C), followed by restriction digestion to isolate NWM cDNA inserts (D). Subsequently, digested NWM cDNA inserts were ligated into pSPVIA, B and C vectors and a pSPVI phage display library was generated (E).

First, $9*10^7$ plaque forming units (pfu) of lambda NWM phage (100 fold primary diversity of the library) were added to $9*10^8$ XL1-Blue cells (OD_{600} = 1, diluted in 10 mM magnesium sulfate ($MgSO_4$)) and $9*10^9$ pfu of Exassist helper phage. Phage and bacteria were then incubated for 15 minutes at 37°C, which enabled lambda phage to attach to the XL1-Blue cells. Subsequently, 20 ml LB (Luria-Bertani (Life Technologies, Merelbeke, Belgium) medium, supplemented with 200 µl 1 M $MgSO_4$ and 200 µl 20% (w/v) maltose, was added and incubated for 3 hours while shaking at 37°C. In this way, NWM filamentous pBluescript phage particles were generated. Lambda phage particles and bacterial cells were lysed after incubation for 20 minutes at 70°C , which was followed by centrifugation to remove cell debris. The efficiency of the mass excision was determined by infection of NWM pBluescript phage particles into SOLR bacterial cells, which are resistant for infection with lambda or helper phage. For this purpose, dilutions of the supernatant containing the NWM pBluescript phage particles were added to SOLR bacterial cells (OD_{600} = 1, in 10 mM $MgSO_4$) and incubated for 15 minutes at 37°C. Phage infected bacteria were then plated on LB-A agar plates (LB (Life Technologies) supplemented with 100 µg/ml ampicillin (Roche Diagnostics, Vilvoorde Belgium) and incubated overnight at 37°C. The number of colonies was then used to determine the efficiency of the mass excision.

Next, $9 * 10^7$ colony forming units (cfu) pBluescript phage particles (100 fold primary diversity of the library) were infected into SOLR cells (OD_{600} = 1, in 10 mM $MgSO_4$) and plated on LB-A plates. Colonies were scraped and plasmid was isolated using the Qiagen Plasmid Midi Kit (Qiagen, Venlo, the Netherlands), according to manufacturers' instructions.

A restriction digestion with *BamHI* and *KpnI* was performed to isolate NWM cDNA inserts from the pBluescript plasmid. The restriction digestion mixture contained 5 µg NWM pBluescript plasmid DNA, 25 U *BamHI* and 25 U *KpnI* (Promega, Leiden, the Netherlands) and was incubated for 2 hours at 37°C. Subsequently, the restriction digestion product was separated via agarose gel electrophoresis and digested NWM cDNA inserts were isolated and purified from the agarose gel using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE healthcare, Diegem, Belgium). In addition, plasmid DNA from pSPVIA, B and

C vectors was isolated and 2 µg plasmid DNA from each vector was sequentially digested with *Bgl*II and *Kpn*I (Promega).

Individual ligation reactions were performed for each pSPVI vector and NWM cDNA inserts. Ligations reactions were composed of 100 ng *Bgl*II/*Kpn*I digested pSPVI plasmid, 250 ng *BamH*I/*Kpn*I digested NWM cDNA inserts and 2 U T4 DNA ligase (Promega) and incubated overnight at 16°C. Next, ligation mixtures were electroporated into freshly prepared electrocompetent TG1 cells, and plated on TY-AG (TY (BD, Erembodegem, Belgium), 100 µg/ml ampicillin (Roche Diagnostics) and 2% glucose) agar plates. To check whether the original diversity of the library was maintained, dilutions of the electroporation mixture were plated on separate TY-AG plates. In addition, a colony polymerase chain reaction (PCR) with Gene 6 primers (forward: 5'-TTACCCTCTGACTTTGTTCA-3' and reverse: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3')(Eurogentec, Seraing, Belgium) was performed on individual colonies to verify correct cloning sites and diversity in cDNA insert size.

2.2.4 SAS procedures

SAS procedures were performed with NWM and MS cDNA phage display libraries and characterized as described previously [115, 271]. In brief, 4 consecutive selection rounds were performed in parallel with both cDNA libraries on pooled CSF from CIS patients, to selectively enrich for cDNA products with high affinity for CIS immunoglobulin G (IgG). Immunotubes (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µg/ml (first round) or 10 µg/ml (subsequent rounds) rabbit anti-human IgG antibody (Dako, Heverlee, Belgium) in coating buffer (0.1 M sodium hydrogen carbonate pH 9,6). Next, immunotubes were blocked with 2% (w/v) skimmed milk powder in phosphate-buffered saline (MPBS). Meanwhile, 10^{12} – 10^{13} phage particles were pre-incubated with the pooled preadsorbed CSF (diluted 1:15) in 2% MPBS for 1.5 hour at room temperature (RT), while shaking. The pre-incubation mixture was then transferred to the immunotube and incubated for 30 minutes while shaking at RT, followed by 2 hours of stationary incubation. After extensive washing with 0.1% PBS Tween20 (PBST) and PBS, bound phage were eluted with 100 mM triethylamine and neutralized with 1 M Tris-HCl pH 7.4. To characterize the

output from each selection round, eluted phage were used for infection of *E.coli* TG1 bacteria. Subsequently, a colony PCR was performed on phage infected bacteria with Gene 6 primers, followed by restriction digestion of the PCR product. In this way, enriched cDNA products representing identical cDNA clones could be selected and identified by sequencing. The basic local alignment search tool (BLAST) of the national center for biotechnology information (NCBI) was used to compare nucleotide and amino acid sequences of the identified clones with public nucleotide and protein databases (http://blast.ncbi.nlm.nih.gov).

2.2.5 Phage enzyme-linked immunosorbent assay

Phage enzyme-linked immunosorbent assay (ELISA) was used for (1) a pilot screening to select for the most promising enriched candidate targets and for (2) a screening for antibody reactivity in CSF and serum towards these selected antigens.

Ninety-six well flat bottom plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with 10 µg/ml anti-M13 antibody (GE healthcare) in coating buffer. Plates were blocked with 5% MPBS for 2 hours at 37°C, followed by 3 times washing with 0.1% PBS Tween20 and once with PBS. In each well, 100 µl polyethylene glycol purified phage displaying the candidate antigen ($7*10^{11}$ colony forming units/ml) or corresponding empty phage (used as negative control), were added and incubated for 1 hour at 37°C and 30 minutes at RT, while shaking. Washing steps were repeated and 100 µl diluted CSF or serum was added (1 hour at 37°C and 30 minutes shaking at RT). CSF was diluted 1:5 and serum 1:100 with a similar range of IgG concentration for patients and controls (CSF: 1-10 mg/dl, serum: 600–1600 mg/dl). Plates were washed and the amount of bound IgG was detected with a 1:2,000 dilution of horse radish peroxidase labeled goat anti-human IgG antibody preadsorbed against mouse IgG (Life technologies). Colour development was performed by addition of 100 µl 3,3',5,5' tetramethyl-benzidine dihydrochloride (TMB) solution (Perbioscience, Erembodegem, Belgium). The colour reaction was stopped with 50 µl H_2SO_4 and plates were read at 450 nm in a Tecan plate reader (Tecan, Männedorf, Switzerland).

2.2.6 Peptide ELISA and competition assays

Synthetic peptides (GL Biochem, Shanghai, Belgium) corresponding to the peptides displayed on phage were used to confirm immunoreactivity detected with the phage ELISA assay. Based on phage ELISA results, an antibody-positive and an antibody-negative serum sample were selected for phage/peptide competition assays. For the competition assay, a standard phage ELISA was performed, after pre-incubation of antibody-positive and antibody-negative serum samples with increasing amounts of corresponding peptide (0-100 µg/ml).

Antibody reactivity was additionally measured in a solid phase peptide ELISA by coating specific or a random irrelevant peptide (WTKTPDGNFQLGGTEP), as described previously with minor modifications [262]. Briefly, 96-well flat bottom plates (Greiner Bio-One) were coated with specific or irrelevant peptide in coating buffer for 2 hours at 37°C. Plates were blocked with 2% MPBS. Subsequently, 100 µl of a phage ELISA antibody-positive or antibody-negative sample (diluted 1:100) was added and incubated at RT for 2 hours, while shaking. The amount of bound IgG was detected with rabbit anti-human IgG HRP labeled antibody (1:2000 Dako). Colour development was performed by addition of 100 µl TMB and stopped with 50 µl H_2SO_4. Plates were read at 450nm in a Tecan plate reader.

2.2.7 Statistics

Statistical analyses were performed using Graph Pad Prism 5 software (GraphPad software, La Jolla, USA). Phage ELISA data were analysed with the Kruskall Wallis test, followed by Dunn's multiple comparison test. Putative associations between antibody reactivity and disease were analysed using the Fisher's exact test. In addition, associations between antibody and reactivity clinical parameters were examined. The Fisher's exact test was used for categorical variables, while for demographical variables an unpaired two-tailed student t-test or Mann Whitney test was used, when appropriate. For all statistical tests, a p-value lower than 0.05 was considered statistically significant.

2.3 Results

2.3.1 Construction of a NWM cDNA phage display

In order to investigate whether primarily normal or diseased brain antigens are targeted by the antibody response in CIS, a cDNA phage display library derived from NWM from healthy brain was constructed. A MS cDNA library, derived from chronic active plaques was already generated [271].

A NWM cDNA library, directionally cloned into the lambda phage UniZap vector, with a primary diversity of 0.9 $*10^6$ pfu was obtained. NWM cDNA inserts were isolated by restriction digestion and ligated in separate reactions into pSPVIA, B and C vectors (Figure 2.2). The primary diversity of the NWM library was preserved for each reading frame (diversity pSPVIA: 2.2 $* 10^6$ cfu, pSPVIB: 1.3 $*10^6$ cfu and pSPVIC: 1.9 $* 10^6$ cfu). A PCR with Gene 6 primers was performed on 20 randomly selected clones for each pSPVI vector. This revealed the presence of correct cloning sites and a broad range in cDNA insert size (600-1500 bp) (Figure 2.3).

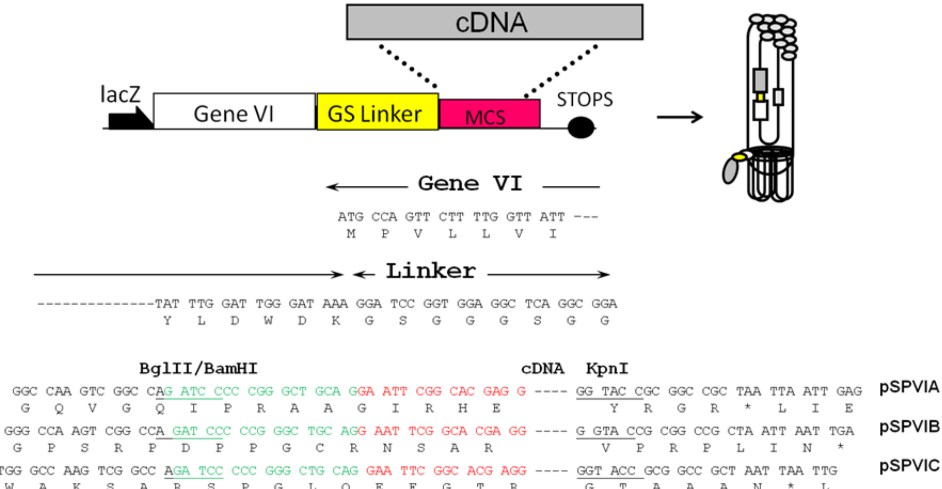

Figure 2.2: Vector sequence of generated NWM pSPVI cDNA library. *BglII*/*BamHI* and *KpnI* restriction sites were used to directionally subclone NWM cDNA inserts into the pSPVIA, B and C vectors. NWM cDNA inserts were originally cloned between *EcoRI* and XhoI sites of the UniZap/pBluescript vector (*EcoRI* and *EcoRI* adaptor sites are depicted in red). Several nucleotides of the UniZap/pBluescript vector are included in the NWM pSPVI cDNA library (depicted in green).

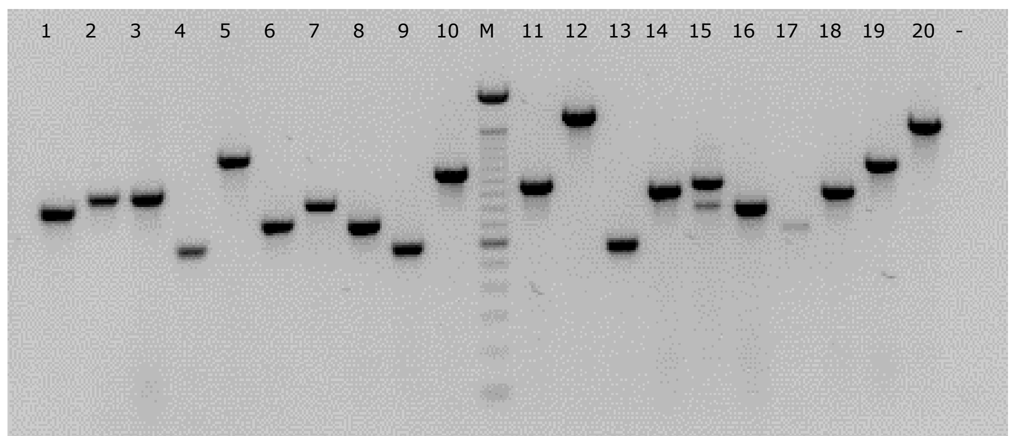

Figure 2.3: Preservation of diversity of the NWM cDNA library after subcloning of NWM cDNA inserts into the pVI phage display system. A PCR with vector primers binding adjacent to the cDNA inserts was performed, which revealed a broad range in insert size. M=100 bp marker, - = negative (no template) control.

2.3.2 Discovery of novel candidate antigens for CIS

To identify CIS associated antigens, 4 consecutive selection rounds were performed in parallel for the MS and NWM cDNA phage display libraries on pooled CSF from 4 CIS patients, who developed MS within two years after lumbar puncture (Table 2.3). As shown in Table 2.3 output titers gradually increased from the second round onwards, indicating enrichment of specific phage.

Table 2.3: Output of SAS procedures

	NWM library		MS library	
Selection round	Input[a]	Output	Input	Output
1	$3.0 * 10^{13}$	$6.6 * 10^{5}$	$3.0 * 10^{12}$	$5.8 * 10^{5}$
2	$1.0 * 10^{13}$	$3.0 * 10^{5}$	$1.0 * 10^{12}$	$3.7 * 10^{5}$
3	$1.0 * 10^{13}$	$8.3 * 10^{5}$	$1.0 * 10^{12}$	$2.3 * 10^{6}$
4	$1.0 * 10^{13}$	$1.9 * 10^{6}$	$1.0 * 10^{12}$	$1.8 * 10^{6}$

[a] Phage input and output is depicted in cfu/ml

PCR and restriction digestion was performed on 200 randomly selected clones from each selection round (2, 3 and 4). Enrichment was demonstrated for 22 clones, all representing novel CIS candidate antigens. Seventeen enriched cDNA clones were obtained from selections on the NWM library, while 5 clones were obtained from selections on the cDNA library from chronic active MS plaques. One candidate clone was identified in selection outputs from both cDNA libraries.

Of the 22 enriched cDNA clones, the most promising cDNA clones were selected based on antibody reactivity in CSF and serum. To this end, a pilot screening was performed on individual CSF and serum samples from CIS patients used for SAS. For 6 out of the 22 clones, named UH-CIS1 to 6 (University Hasselt, CIS, clone number), CSF and serum immunoreactivity in at least 1 out of 4 CIS patients used for the SAS procedure could be detected, confirming specific selection by CIS autoantibodies in CSF and serum. On the other hand, antibody reactivity towards these clones was absent in pooled serum from HC (n=4), NIND (n=5) and OIND (n=5) patients. Apart from antibody reactivity in CIS patients, antibody reactivity towards clone UH-CIS3 could be confirmed in pooled serum from 5 randomly selected MS patients (data not shown). These 6 clones were further used for a large scale screening for antibody reactivity in CSF and serum from patients and controls.

The selected candidate antigens, clones UH-CIS1 to 6, encode for peptides with a size of 6 to 123 amino acids. Subsequently, these peptides were compared to public protein databases using BLAST analysis. One of the candidate antigens, UH-CIS2 encoded part of host cell factor c1 regulator 1, which has not been associated with CIS up till now (Table 2.4). UH-CIS6 showed homology to several proteins including bestrophin 1, peroxisomal biogenesis factor 6 and ring finger protein 157. The other clones, UH-CIS1, UH-CIS3, UH-CIS4 and UH-CIS5, were translated from 3' untranslated regions (UTR) or from non-coding reading frames, but did show partial homology to known proteins (Table 2.4).

Table 2.4: Identity of candidate CIS clones identified from pilot screening

Clone	cDNA library	Translated cDNA product	Size (amino acids)	Homology on protein level (accession number)
UH-CIS1	NWM	ARAKQDSLKKINGE	14	8/10 (80%) ubiquitin carboxyl-terminal hydrolase 8 (P40818.1) 7/8 (88%) myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila) [Homo sapiens] (Q03164.5)
UH-CIS2	NWM	AAFGAKVAGAYRPEPQEP RLPPVSVSQPASLPSLPRE PLRKQFLSEENMATHFSQ LSLHNDHPYCSPPMTFSP ALPPLRSPCSELLLWRYPG SLIPEALRLLRLGDTPSPP YPATPAGDIMEL	123	87/88 (99%) host cell factor C1 regulator 1 (XPO1 dependent) (Q9NWW0.1)
UH-CIS3	NWM	RPWFFSCAHSGLSSSLPI P	19	9/14 (64%) basonuclin 1(Q01954.2) 11/22 (50%) NADH dehydrogenase subunit 4 (ABR57675.1)
UH-CIS4	NWM	LLWASWRQSHLATPFTPT CGVASDGADTQGGTGLS LGIKGTESAHTLSVTKEG TAGPV	58	11/30 (57%) sodium channel, voltage-gated, type V, alpha subunit (Q14524.2) 10/25 (40%) poly (ADP-ribose) polymerase 1(P09874.4)
UH-CIS5	NWM	HIGVWD	6	5/5 (100%) G protein-coupled receptor 124 (NP_116166.9) 5/6 (83%) WD repeat domain 36 (NP_644810.1)
UH-CIS6	NWM	MPVVPATWEAETGESLEP GRRRLQ	24	19/22 (86%) bestrophin 1 (AAC64344.1) 19/24 (79%) peroxisomal biogenesis factor 6 (EAX04126.1) 19/23 (83%) ring finger protein 157 (AAH04231.2)

2.3.3 CSF and serum antibody reactivity in CIS and RR-MS towards UH-CIS1 to 6

To investigate whether antibody reactivity directed to UH-CIS1 to 6 could be detected in a large cohort of CIS and MS patients, a phage ELISA screening was performed on CSF samples from CIS (n=123), RR-MS (n=55), SP-MS (n=10), OIND (n=28) and NIND (n=47) patients (Table 2.5). For all 6 antigens, antibody reactivity could be confirmed in other CSF samples from CIS patients not used for SAS procedures. A trend for an increase in antibody reactivity towards the panel was observed in the CIS and RR-MS group as compared to the OIND and NIND patients, although this was not significant (Table 2.5). For the whole panel of CIS candidate antigens, no reactivity could be detected in CSF from SP-MS patients.

Table 2.5: Antibody reactivity in CSF to UH-CIS 1 to 6

Clone	CIS (n=123)	RR-MS (n=55)	SP-MS (n=10)	OIND (n=28)	NIND (n=47)
UH-CIS1	4/123[a] (3%)	1/55 (2%)	0/10 (0%)	1/28 (4%)	1/47 (2%)
UH-CIS2	3/123 (2%)	1/55 (2%)	0/10 (0%)	0/28 (0%)	2/47 (4%)
UH-CIS3	8/123 (7%)	10/55 (19%)	0/10 (0%)	0/28 (0%)	3/47 (6%)
UH-CIS4	7/120[b] (6%)	1/55 (2%)	0/10 (0%)	0/28 (0%)	2/47 (4%)
UH-CIS5	2/123 (2%)	1/55 (2%)	0/10 (0%)	0/28 (0%)	0/47 (0%)
UH-CIS6	8/123 (7%)	1/55 (2%)	0/10 (0%)	2/28 (7%)	2/47 (4%)
Total	21/123 (17%)	12/55 (22%)	0/10 (0%)	3/28 (11%)	5/47 (11%)

[a] The cut-off for a positive sample was set at 3 times the standard deviation (SD) above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for the NIND and OIND samples
[b] Samples were excluded for analysis when the intra-assay coefficient of variation (CV) >20% for duplicates

An almost equal sensitivity could be obtained by combining ELISA results for UH-CIS3, UH-CIS4 and UH-CIS6 as compared to the whole panel. Therefore, these clones were selected to further study antibody reactivity in serum of CIS (n=108), RR-MS (n=44), OIND (n=14) and NIND (n=24) patients, as well as HC

(n=44). Similar to the CSF antibody screening, reactivity could be detected in serum from CIS patients not used for SAS and from RR-MS patients (Table 2.6).

Table 2.6: Antibody reactivity in serum to UH-CIS3, UH-CIS4 and UH-CIS6

Clone	CIS (n=108)	RR-MS (n=44)	OIND (n=14)	NIND (n=24)	HC (n=44)	Fisher's exact test[b] (p-value)
UH-CIS3	7/108[a] (6%)	4/44 (9%)	0/14 (0%)	0/24 (0%)	4/44 (9%)	0.58
UH-CIS4	4/108 (4%)	1/44 (2%)	0/14 (0%)	1/24 (4%)	1/44 (2%)	1.00
UH-CIS6	25/108 (23%)	7/44 (16%)	0/14 (0%)	3/24 (13%)	3/44 (7%)	0.01
Total	30/108 (28%)	10/44 (23%)	0/14 (0%)	3/24 (13%)	8/44 (16%)	0.03

[a] The cut-off for a positive sample was set at 3 times the SD above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for HC samples
[b] Fisher's exact test was used to investigate a putative association between antibody reactivity and disease (CIS and MS versus OIND, NIND and HC)

Twenty-eight % of the CIS patients and 23% of the RR-MS patients tested positive for the 3 markers, although some serum samples from neurological and healthy controls were also antibody positive. This led to a combined sensitivity of 26% and specificity of 87% for CIS and RR-MS. As shown in Table 2.6, the number of antibody positive CIS and RR-MS patients for the combined panel (26% versus 13%, Fisher's exact test: p=0.03) and UH-CIS6 (21% versus 7%, Fisher's exact test: p=0.01) was significantly increased as compared to controls. In addition, a trend for elevated antibody levels towards UH-CIS6 was detected in both CIS and RR-MS (Figure 2.4).

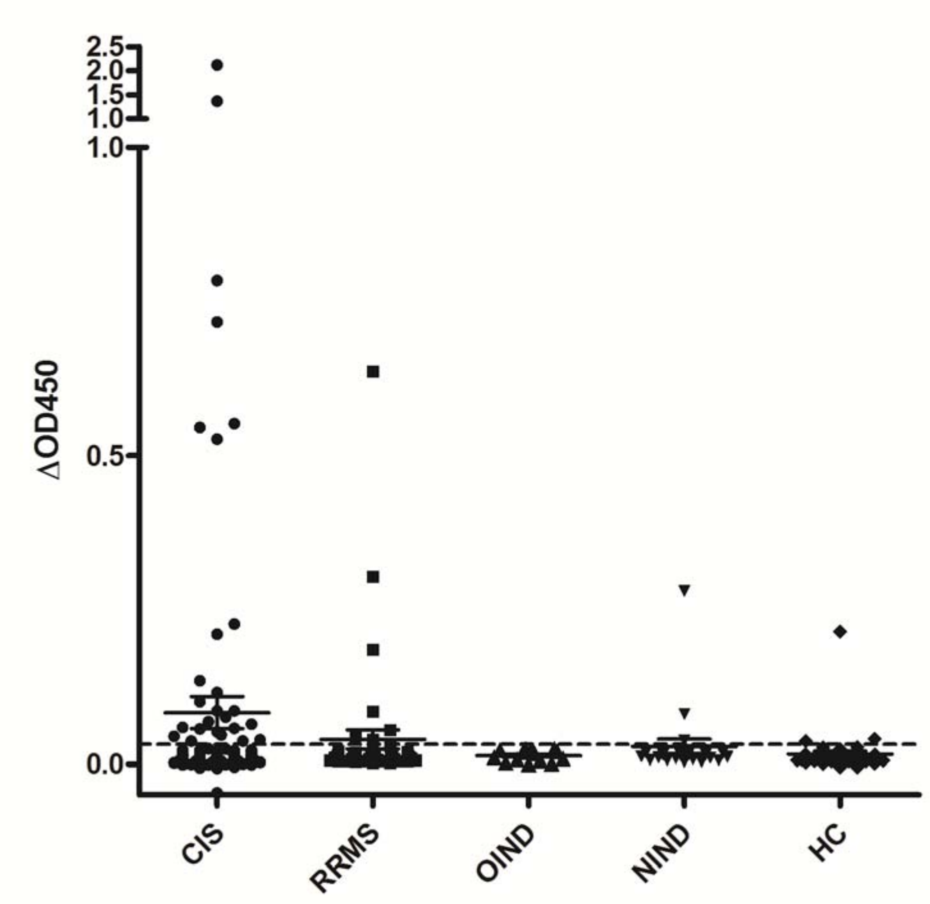

Figure 2.4: Serum antibody reactivity to UH-CIS6. Phage ELISA was used to test serum antibody reactivity to UH-CIS6 in patients with CIS and MS and controls. The displayed results are ΔOD values**.** The cut-off (dash line) for a positive sample was set at 3 times the standard deviation (SD) above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for HC samples.

To evaluate a putative correlation between CSF and serum antibody reactivity, ELISA results for UH-CIS3, UH-CIS4 and UH-CIS6 from 154 paired CSF and serum samples were compared. Out of these 154 CSF samples, 18 CSF samples tested positive for one or more antigens, resulting in a total number of 22 positive ELISA tests. Importantly, 19 out of these 22 positive ELISA tests in CSF could be confirmed in serum (data not shown), implicating the presence of a mostly similar antibody repertoire in CSF and peripheral blood.

For both CSF and serum antibody reactivity towards our panel, no significant correlation could be detected between immunoreactivity and gender, age, disease duration, expanded disability status scale (EDSS) or the presence of unique CSF oligoclonal bands. No information on magnetic resonance imaging (MRI) was available for the tested CIS and MS patients.

Synthetic peptides were generated for UH-CIS3 and UH-CIS6. Antibody reactivity towards phage displayed peptides UH-CIS3 and UH-CIS6 could be confirmed using these synthetic peptides in solid phase peptide ELISA and in phage/peptide competition assays (Figure 2.5).

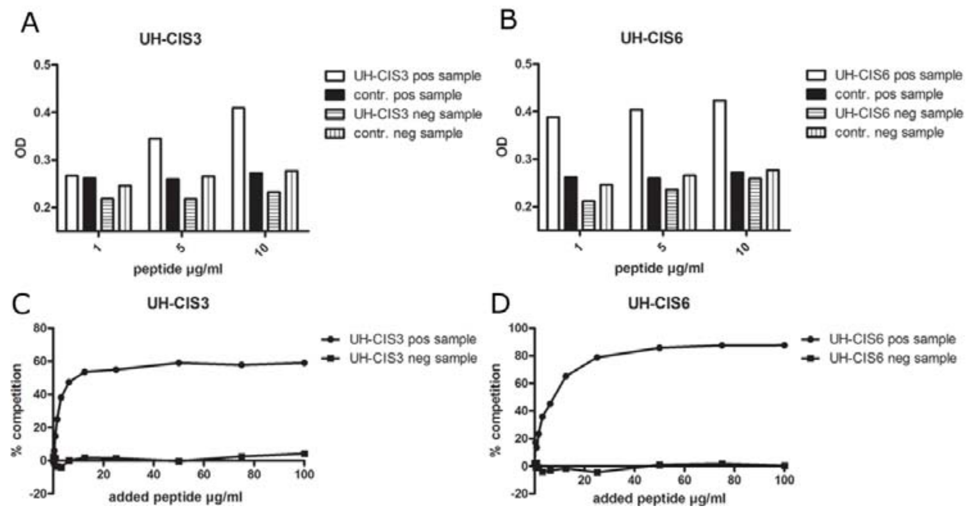

Figure 2.5: Antibody reactivity towards UH-CIS3 and UH-CIS6 synthetic peptides. A,B. Solid phase peptide ELISAs with synthetic peptides corresponding to UH-CIS3 and UH-CIS6 confirmed antibody reactivity in positive serum samples based on phage ELISA. C,D. Increasing amounts of UH-CIS3 and UH-CIS6 synthetic peptides (X-axis) resulted in inhibition (Y-axis) of UH-CIS3 and UH-CIS6 phage ELISA derived signals.

2.3.4 Investigation of prognostic potential of UH-CIS1 to 6 to predict conversion to MS

Antibody reactivity towards the panel of CIS candidate antigens could also be detected in CSF and serum of RR-MS patients. This implies that our panel might have potential prognostic and predictive value for conversion to MS. For this reason, follow-up information concerning MS development in CIS patients was collected. Among the CIS patients tested for CSF and serum antibody reactivity, 59 out of 123 (48%) and 53 out of 108 (49%) CIS patients converted to MS, respectively. As shown in Table 2.7, antibody reactivity towards the panel could be detected in both CSF and serum irrespective of conversion to MS. Although not significant, a trend for an increase in antibody reactivity towards UH-CIS4 and UH-CIS6 was observed in CIS patients with conversion to MS as compared to CIS patients without MS development. This suggests that antibody reactivity towards UH-CIS4 and UH-CIS6 could be used as a prognostic marker. However, additional CIS patients should be tested to further study the prognostic potential of these antigens.

Table 2.7: Antibody reactivity in CSF and serum of CIS patients without and with conversion to MS

	CSF		Serum	
Clone	**CIS (n=64)**	**CIS-MS (n=59)**	**CIS (n=55)**	**CIS-MS (n=53)**
UH-CIS1	2/64[a] (3%)	2/59 (3%)	NA[c]	NA
UH-CIS2	1/64 (2%)	2/59 (3%)	NA	NA
UH-CIS3	6/64 (9%)	2/59 (3%)	5/55 (9%)	2/53 (4%)
UH-CIS4	**2/61[b](3%)**	**5/59 (8%)**	**0/55 (0%)**	**4/53 (8%)**
UH-CIS5	1/64 (2%)	1/59 (2%)	NA	NA
UH-CIS6	**3/64 (5%)**	**5/59 (8%)**	**10/55 (18%)**	**15/53 (28%)**
Total	12/64 (19%)	9/59 (15%)	14/55 (25%)	16/53 (30%)

[a] The cut-off for a positive sample was set at 3 times the SD above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for the NIND and OIND samples (CSF) and HC (serum)
[b] Samples were excluded for analysis when the %CV >20% for duplicates
[c] NA: not applicable

2.4 Discussion

In this study, we used SAS to identify novel CIS antigens. Antibody reactivity towards our panel of 6 novel antigens could be confirmed in CSF from CIS patients used for SAS procedures, as well as in CSF from other CIS and RR-MS patients. In addition, serum antibody reactivity towards 3 antigens, UH-CIS3, UHCIS-4 and UH-CIS 6, was significantly increased in CIS and RR-MS patients as compared to (neurological) controls. Furthermore, possible prognostic potential to predict conversion to MS in CIS patients could be demonstrated for 2 antigens, UH-CIS4 and UH-CIS6.

CIS can be considered either as an isolated event or as an early presentation of MS. Therefore, the antibody response in CIS might be directed to initial antigens involved in pathogenesis. In general, the number of brain lesions and plaques is low and could explain why the antibody response in CIS is preferentially directed towards antigens expressed in the NWM cDNA library. Moreover, another study [264] demonstrated that the MS cDNA library used in our study contained unique transcripts involved in immune activation, which were absent in 2 normal brain cDNA libraries. Thus, the antigen repertoire present in MS lesions is different as compared to normal brain. In addition, no antibody positive CSF samples from SP-MS patients were identified for our panel. This might be explained by a restricted antibody response in the progressive phase of MS, which is characterized by neurodegeneration and to a lesser extent by inflammatory processes [272].

CIS and MS are both considered heterogeneous conditions, supported by the presence of a wide spectrum of symptoms in CIS and MS patients. The heterogeneity of the disease is also reflected by the different types of lesions present in MS brain [26]. Only one of these type lesions, type II, is characterized by the presence of IgG. Therefore, antibodies might play a substantial role in the pathogenesis in only a subset of CIS and MS patients. In addition, due to the fact that phage-displayed proteins are produced by the prokaryotic protein translational machinery, the identification of post-translational modifications is not feasible with phage display systems. Future studies will be performed to

investigate whether the addition of such post-translational modifications can increase the sensitivity of our panel. On the other hand, the phage display system used is based on monovalent expression, which allows high affinity interactions with antibodies.

Zéphir et al. [218] demonstrated that the self-reactive serum IgG repertoire is already distorted in CIS patients and is different from healthy controls. Moreover, they showed that in 80% of CIS patients this IgG repertoire is similar to MS. Therefore, analysis of the antibody profile in CIS patients might have prognostic potential. For instance, IgG antibodies towards measles, rubella and the varicella zoster virus have been proposed as prognostic marker to predict conversion to MS in CIS patients [68]. In addition, similar results have been obtained for the IgG response towards EBV protein Epstein-Barr nuclear antigen 1 (EBNA-1) [268]. On the other hand, conflicting results have been reported for other antibody targets such as MBP, MOG and alpha-glucose based glycans [121, 129, 177, 214]. For our panel, possible prognostic potential could be demonstrated for 2 antigens, UH-CIS4 and UH-CIS6. Although the majority of CIS patients without current conversion were followed for at least 2 years, MS might develop in at least a subset of this group in the future. For example, 5 CIS patients without MS development who tested positive for UH-CIS4 or UH-CIS6 have unique OCB in the CSF, which has been associated with conversion to MS [222]. Therefore, the prognostic value of our panel could increase after a longer period of follow-up. In addition, to further investigate the prognostic potential of our panel, additional CIS patients should be tested.

Although initially identified as CSF antigens, antibody reactivity could be confirmed in the majority of paired serum samples, hereby providing an easily accessible source of antibodies. Whether the antibodies towards our panel are produced in CSF and/or in serum remains elusive and additional studies are needed to investigate their origin. Previously, abnormal antibody production in MS has been described for both serum and CSF. For instance, it has been shown that CSF B cells produce the CSF oligoclonal immunoglobulin bands in MS patients [114]. On the other hand, also an increased number of antibody producing

cells have been demonstrated in the bone marrow and peripheral blood of MS patients as compared to healthy controls [273].

One of the novel CIS candidate antigens was host cell factor c1 regulator 1 (HCFCR1). This nuclear export factor interacts with host cell factor 1 (HCF-1), a ubiquitously expressed transcription factor and HCFCR1 is thought to be implicated in HCF-1 transport into the cytoplasm. Here, HCF-1 binds herpes simplex virus protein, VP16. The HCF-1-VP16 complex is then transported to the nucleus, where it acts as a transcription factor to activate herpes simplex virus immediate early genes in herpes infected cells [274]. Interestingly, an intrathecal antibody response towards the herpes simplex virus has been detected in MS [66]. Other identified CIS antigens from our panel showed homology to proteins that have been linked with MS. For instance, UH-CIS4 showed homology to the sodium channel Nav1.5. Expression of Nav1.5 has been shown to be upregulated in reactive astrocytes in MS lesions and could have compensatory effects [275]. Inhibition of poly (ADP-ribose) polymerase 1, another protein with similarity to UH-CIS4, has been suggested as a therapy to suppress neuroinflammation in MS [276]. UH-CIS6 displayed high similarity to several proteins, including alternatively expressed isoforms of bestrophin 1 (19/22 amino acids, 86%) and peroxisomal biogenesis factor 6 (19/24 amino acids, 79%). In addition, mutations in *BEST1* and *PEX6*, genes encoding UH-CIS6 homologs, have been associated with retinopathies [277] and several neurological diseases [278]. Antibodies towards these described antigens might exert several functions. For example, antibodies towards NaV1.5 could be pathogenic by blocking its proposed compensatory mechanism in MS lesions. On the other hand, antibodies might also have protective roles. For instance, antibodies towards HCFCR1 might lead to a decrease of cytoplasmatic HCF-1, which then indirectly could inhibit viral infection.

As suggested by the BLAST results for UH-CIS6, alternative spliced antigens might be a target for autoimmunity. This is supported by findings reported by Ng et al. [279], who demonstrated that alternative splicing occurred in 100% of 45 randomly selected autoantigens, which was significantly higher than the alternative splicing rate of 42% in 9554 randomly selected gene transcripts. For

this reason, it was suggested that alternative splicing could lead to the generation of untolerized epitopes involved in autoimmunity [279]. Of note, alternative splicing has already been described for several known autoantigens which are associated with MS [280].

Whether antibodies towards our panel are involved in CIS and MS pathogenesis remains to be elucidated. At least a subset of known MS associated autoantibodies has been shown to have pathogenic effects. For instance, oligoclonal IgM against myelin lipids has shown to correlate with disability progression in MS [217]. Co-injection of monoclonal antibodies directed to the axonal antigen neurofascin together with encephalitogenic MOG specific T cells exacerbated disease in experimental autoimmune encephalomyelitis (EAE), the animal model of MS [138]. In addition, human serum with high titers of antibodies binding to native MOG has been to shown to enhance demyelination and axonal damage in EAE [189].

In summary, we used an unbiased high-throughput technology based on phage display, which led to the identification a panel of novel CIS candidate antigens. Both CSF and serum antibody reactivity towards this panel could be primarily detected in CIS and RR-MS and to lesser extent in healthy and neurological controls. In the future, experiments will be performed to further characterize these antigens, explore their prognostic potential into more detail and identify their role in CIS and MS pathogenesis.

3

Exploring the cerebrospinal fluid antibody repertoire in relapsing-remitting and primary-progressive multiple sclerosis

Abstract

Several subtypes of Multiple Sclerosis (MS) have been described; in 80% of the MS patients disease manifests in a relapsing-remitting (RR) fashion, while 20% of the MS patients have a primary-progressive (PP) disease course from onset. Different autoantibody repertoires may be present in these MS subtypes. Therefore, the aim of this study was to identify novel antibody targets for RR- and PP-MS and to compare the antibody repertoire in these MS subtypes.

To identify antibody targets for RR- and PP-MS, serological antigen selection (SAS) procedures were performed on pooled cerebrospinal fluid (CSF) from RR- (n=6) and PP-MS (n=6) patients. Antibody reactivity towards a panel of novel candidate antibody targets was tested in paired serum and CSF samples from patients used in the SAS procedures. In addition, CSF samples were obtained from patients with RR-MS (n=84), PP-MS (n=32), secondary progressive MS (n=9), other inflammatory neurological diseases (OIND) (n=31) and non-inflammatory neurological diseases (NIND) (n=53).

Using SAS procedures, a panel of 9 novel antibody targets for MS was identified (RR-MS n=8, PP-MS n=1). No overlap was present in the SAS output from both CSF pools, which may indicate the presence of distinct antibody repertoires in RR- and PP-MS. Antibody reactivity towards the panel could be confirmed in serum and CSF from patients used for SAS. In addition, CSF samples from 4/51 MS patients and 1/39 neurological controls tested positive for 1 candidate target, UH-RRMS7. In summary, a panel of 9 novel antibody targets for RR- and PP-MS was identified to which antibody reactivity in both CSF and serum of MS patients was detected.

3.1 Introduction

In 80% of multiple sclerosis (MS) patients, disease presents in a relapsing-remitting (RR) fashion followed by a secondary-progressive (SP) phase, while 20% of the MS patients have a primary-progressive (PP) disease course from onset [3]. Apart from a different clinical presentation, the proportion of affected males and average age of disease onset is higher in PP-MS [281]. Furthermore, disease-modifying strategies used for RR-MS are not effective in PP-MS [282]. In addition, neuro-inflammatory processes are more prominent in RR-MS than in PP-MS [282]. Together, these differences may point to the presence of distinct disease mechanisms in RR- and PP-MS.

Since different disease mechanisms may be primarily involved in RR- and PP-MS, it may be hypothesized that these MS subtypes are characterized by different cerebrospinal fluid (CSF) and serum autoantibody repertoires. Quintana and colleagues used antigen arrays to investigate the antibody response towards a set of known autoantibody targets in serum of MS subtypes [241]. RR-MS was characterized by antibody responses towards heat-shock proteins (HSP) 60 and 70, which is consistent with the inflammatory processes associated with RR-MS. On the other hand, HSP antibody reactivity was absent in PP-MS. Both MS subtypes displayed antibody reactivity towards central nervous system (CNS) antigens, such as myelin oligodendrocyte protein (MOG) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP). Although in PP-MS antibody responses were detected towards the same CNS antigens as in RR-MS, a lower number of epitopes were targeted in PP-MS.

Lipids represent another class of antibody targets in MS. Increased antibody reactivity towards the myelin lipid galactocerebroside has been observed in serum from RR-MS patients as compared with PP-MS patients [155]. On the other hand, serum antibody levels towards the lipid ganglioside GM3 were elevated in PP-MS [152]. In addition, the intrathecal and serum antibody response towards the axonal protein neurofilament-light (NF-L) was increased in PP-MS [144, 145].

In general, the majority of these studies focused on serum rather than on CSF antibody responses towards known (MS) antibody targets. In addition, validation studies are lacking or results could not be confirmed in subsequent studies [146]. Therefore, novel MS specific antibody targets need to identified.

The aim of this study was to identify novel antibody targets for RR- and PP-MS and to compare the antibody repertoire in these MS subtypes. To this end, a high-throughput screening technology based on phage display, serological antigen selection (SAS) was applied [259, 269, 270]. Previously, this approach has led to the successful identification of novel antibody targets for MS [115] and clinically isolated syndrome (CIS) [283]. In this study, we prepared CSF pools from RR- (n=6) and PP-MS patients (n=6) and screened both normal and MS brain cDNA phage display libraries to identify RR- and PP-MS antibody targets. Furthermore, we analyzed the antibody response towards identified candidate antibody targets in CSF from additional MS patients and (inflammatory) neurological controls.

3.2 Materials and methods

3.2.1 Patient material

CSF samples of 6 RR- and 6 PP-MS patients were used for SAS procedures (Table 3.1). These CSF samples were pooled and preadsorbed against *E.Coli* and phage extracts, as described previously [271]. Pooled serum from 7 healthy controls (HC) (3 men, 4 women, mean age = 40.7, range 25-55 years) was used for negative selection rounds.

Table 3.1: Characteristics of RR- and PP-MS patients used for SAS procedures

	Patient	Gender[a]	Age	EDSS[b]	Disease duration (years)
Pool 1: RR-MS	RR1	F	26	4.0	0.6
	RR2	F	43	3.0	6.0
	RR3	M	38	5.0	0.1
	RR4	M	62	1.0	0.3
	RR5	F	55	3.5	5.0
	RR6	F	40	2.0	4.0
Pool 2: PP-MS	PP1	F	65	NA[c]	1.0
	PP2	F	30	2.5	2.0
	PP3	F	48	6.5	8.0
	PP4	M	37	2.0	6.0
	PP5	M	55	2.5	10.0
	PP6	M	69	4.5	NA

[a] F: female; M: male
[b] EDDS, expanded disability status scale
[c] NA: not available

In addition, paired serum samples were collected from patients included in the SAS procedures. Serum samples were also obtained from 5 other MS patients, 9 HC and 5 patients with non-inflammatory neurological disorders (NIND) and other inflammatory neurological diseases (OIND), respectively. Furthermore, CSF samples were available from 84 RR-MS, 32 PP-MS, 9 SP-MS, 31 OIND and 53 NIND patients (Table 3.2). MS diagnosis was established according to McDonald criteria [14, 15]. This study was approved by the ethics committee of Hasselt University.

Table 3.2: Characteristics of patients and controls used for antibody reactivity analysis

Patient group	Gender (M/F[a])	Age ± SD	Disease duration (years)[b] ± SD	EDSS[c] ± SD
RR-MS SAS procedures				
Initial cohort				
RR-MS (n=49)	10/39	38.8 ± 9.4	6.1 ± 6.9	2.9 ± 1.2
OIND (n=9)	4/5	34.9 ± 20.1	NA[c]	NA
NIND (n=17)	5/12	47.3 ± 17.6	NA	NA
Second cohort				
RR-MS (n=35)	8/27	38.7±9.5	6.0±7.0	2.7±1.1
PP-MS (n=7)	3/4	47.1±12.9	7.0±5.3	4.5±1.8
SP-MS (n=9)	2/7	46.1±9.5	14.3±9.0	5.0±1.1
OIND (n=15)	7/8	45.3±15.5	NA[d]	NA
NIND (n=24)	8/16	46.4±16.6	NA	NA
PP-MS SAS procedures				
PP-MS (n=25)	11/14	48.3 ± 10.4	8.7±5.9	3.8±1.5
OIND (n=7)	4/3	44.3 ± 24.2	NA	NA
NIND (n=12)	5/7	48.3 ± 16.2	NA	NA

[a] F: female; M: male
[b] Data available for 80 RR-MS and 27 PP-MS patients
[c] EDSS, expanded disability status scale. Data available for 56 RR-MS, 19 PP-MS and 7 SP-MS patients
[d] NA: not applicable/not available

3.2.2 Construction of cDNA phage display libaries

To identify novel antibody targets for RR- and PP-MS, cDNA phage display libraries were constructed from normal white matter (NWM) of healthy brain ($0.9 * 10^6$ primary recombinants) and active chronic MS plaques ($1.0 * 10^6$ primary recombinants). These libraries were subcloned in the pVI phage display vectors, pSPVIA, pSPVIB and pSPVIC, each representing one of the 3 different

reading frames. The subcloning procedure was performed as described previously [271].

3.2.3 SAS procedures

SAS procedures were performed and SAS output was characterized as described previously [271, 283]. In brief, 3-4 consecutive positive selection rounds were performed in parallel with both cDNA libraries on pooled CSF from RR- and PP-MS patients (Figure 3.1A). Immunotubes (Nunc, Roskilde, Denmark) were coated with 50 µg/ml (first round) or 10 µg/ml (subsequent rounds) rabbit anti-human immunoglobulin G (IgG) antibody (Dako, Heverlee, Belgium). Next, NWM or MS phage particles were pre-incubated with the pooled preadsorbed CSF in a glass tube, followed by transfer of the pre-incubation mixture to the immunotube. To remove unbound phage, the immunotube was washed 10 times (first selection round) and 20 times (subsequent rounds) with 0.1% PBS Tween20 (PBST) and PBS, followed by elution of bound phage with 100 mM triethylamine. Output phage were amplified through infection of *E.coli* TG1 bacteria and characterized by polymerase chain reaction (PCR) and restriction digestion. In this way, enrichment of phage clones could be identified. Nucleotide and amino acid sequences of selected clones were compared with public nucleotide and protein databases using the basic local alignment search tool (BLAST) of the national center for biotechnology information (NCBI) (http://blast.ncbi.nlm.nih.gov).

In addition, negative selection procedures were performed to deplete aspecific binding cDNA products (Figure 3.1B) [269, 271]. To this end, output phage from a positive selection procedure were pre-incubated with pooled HC serum for 1.5 hour at room temperature (RT). Next, the pre-incubation mixture was incubated in an immunotube coated with 10 µg/ml of rabbit anti-human IgG antibody (Dako) for 30 minutes while shaking at RT, followed by 2 hours of stationary incubation. Non-bound phage were retained and used for infection of *E.coli* TG1 bacteria. Subsequently, output phage were characterized as described.

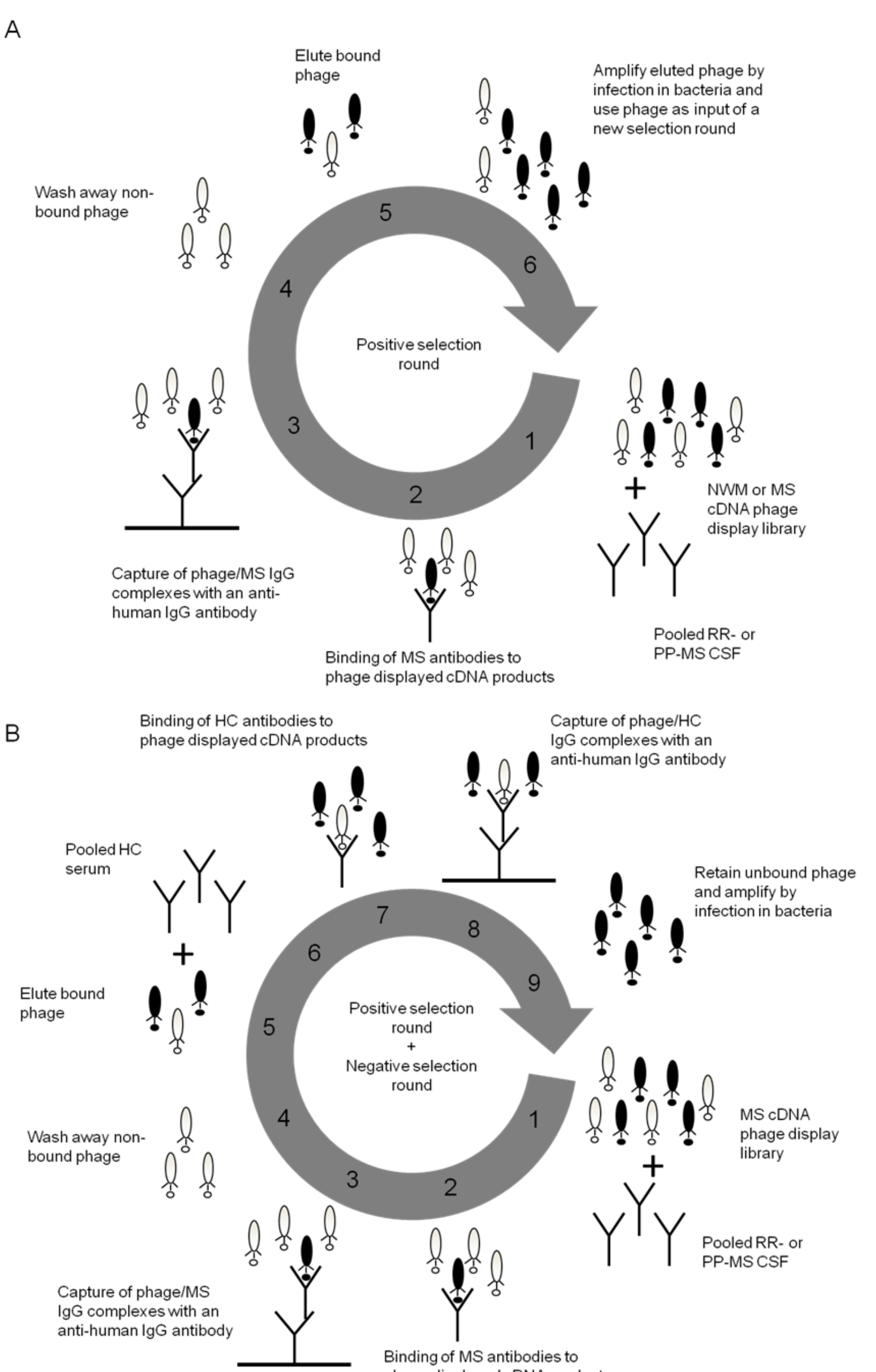
A
Elute bound phage
Amplify eluted phage by infection in bacteria and use phage as input of a new selection round
Wash away non-bound phage
5
6
4
Positive selection round
3
1
2
NWM or MS cDNA phage display library
Capture of phage/MS IgG complexes with an anti-human IgG antibody
Pooled RR- or PP-MS CSF
Binding of MS antibodies to phage displayed cDNA products
B
Binding of HC antibodies to phage displayed cDNA products
Capture of phage/HC IgG complexes with an anti-human IgG antibody
Pooled HC serum
Retain unbound phage and amplify by infection in bacteria
7
8
6
9
Elute bound phage
5
Positive selection round
+
Negative selection round
4
1
3
2
Wash away non-bound phage
MS cDNA phage display library
Pooled RR- or PP-MS CSF
Capture of phage/MS IgG complexes with an anti-human IgG antibody
Binding of MS antibodies to phage displayed cDNA products

Figure 3.1: SAS procedures for the identification of antibody targets for MS. A. Positive selection rounds were initiated with the incubation of MS or NWM phage particles with pooled RR- or PP-MS CSF (1). Complexes were formed consisting of MS antibodies and cDNA products expressed on the surface of the phage (2). Antibody/antigen complexes were captured by an anti-human immunoglobulin G (IgG) antibody (3). Next, unbound phage were washed away (4). This was followed by elution of bound phage (5), which were amplified by infection in bacteria and used as input for a new selection round (6). B. A negative selection round was incorporated in order to deplete aspecific binding cDNA products. First, a positive selection round was performed (1-5). Next, eluted phage of the positive selection round were incubated with pooled serum from healthy controls (HC) (6). HC antibody/antigen complexes were formed (7) and captured (8). Unbound phage were retained and amplified by infection in bacteria, followed by characterization of phage output (9).

3.2.4 Phage ELISA

A phage enzyme-linked immunosorbent assay (ELISA) was performed as described [283]. First, a pilot screening was performed on (paired) serum to select the most promising candidate targets. Next, phage ELISA was used to analyze antibody reactivity towards selected antibody targets in CSF from MS patients and neurological controls.

In brief, 96-well ELISA plates (Greiner Bio-One, Wemmel, Belgium) were coated with 10 µg/ml anti-M13 antibody (GE healthcare, Diegem, Belgium). After ELISA plates were blocked, 100 µl of phage displaying the candidate target ($7*10^{11}$ colony forming units (cfu) /ml) and corresponding empty phage (used as a negative control) were added. Subsequently, 100 µl of diluted serum (1:100) or CSF (1:5 or 1:1.33) was added. Bound human IgG was detected with a 1:2,000 dilution of horse radish peroxidase (HRP) labeled goat anti-human IgG antibody (Life Technologies, Merelbeke, Belgium). Colour development was initiated with the addition of 3,3’,5,5’ tetramethyl-benzidine dihydrochloride (TMB) solution (Perbioscience, Erembodegem, Belgium) and stopped with 50 µl H_2SO_4. Plates were read at 450 nm in a Tecan plate reader (Tecan, Männedorf, Switzerland)

3.3 Results

3.3.1 Affinity selection of NWM and MS cDNA phage display libraries with RR- and PP-MS CSF pools

To investigate and compare the antibody repertoire in CSF from RR- and PP-MS patients, 3-4 consecutive positive selection procedures were performed in parallel with NWM and MS cDNA phage display libraries on pooled RR- and PP-MS CSF (Table 3.3). Using the NWM and MS cDNA phage display libraries, we also examined whether primarily MS or normal brain antigens are targeted by the antibody response in RR- and PP-MS. Due to increased selection stringency (lower coating concentration of rabbit anti-human IgG antibody and more extensive washing), output titers decreased in the second selection round as compared with the first round. However, from the second round onwards, phage output titers increased for all SAS procedures, indicating specific enrichment of phage. Furthermore, output titers were in a similar range for both CSF pools and cDNA libraries. For the MS cDNA library, a separate positive selection round followed by a negative selection procedure was performed to deplete aspecific cDNA products (Table 3.3). This resulted in a 2-3 fold drop of output titers, which points to the depletion of aspecifically binding cDNA products.

Table 3.3: Output of SAS procedures

		RR-MS		PP-MS	
Round	Selection procedure	Input[a]	Output	Input	Output
NWM library					
1	Positive	$3.0 * 10^{13}$	$7.8 * 10^{5}$	$3.0 * 10^{13}$	$1.1 * 10^{6}$
2	Positive	$1.0 * 10^{13}$	$1.5 * 10^{5}$	$1.0 * 10^{13}$	$4.8 * 10^{5}$
3	Positive	$1.0 * 10^{13}$	$7.8 * 10^{5}$	$1.0 * 10^{13}$	$1.6 * 10^{6}$
4	Positive	$1.0 * 10^{13}$	$9.9 * 10^{5}$	$1.0 * 10^{13}$	$4.1 * 10^{6}$
MS library					
1	Positive	$3.0 * 10^{12}$	$5.9 * 10^{5}$	$3.0 * 10^{12}$	$6.4 * 10^{5}$
2A	Positive	$1.0 * 10^{12}$	$4.4 * 10^{5}$	$1.0 * 10^{12}$	$1.7 * 10^{5}$
2B[b]	Positive +	$1.0 * 10^{12}$	$4.6 * 10^{5}$	$1.0 * 10^{12}$	$2.4 * 10^{5}$
	Negative	$4.6 * 10^{5}$	$1.3 * 10^{5}$	$2.4 * 10^{5}$	$1.4 * 10^{5}$
3	Positive	$1.0 * 10^{12}$	$3.3 * 10^{6}$	$1.0 * 10^{12}$	$2.0 * 10^{6}$

[a] Phage input and output is depicted in cfu/ml
[b] Round 2 was repeated (2B) and a positive selection round followed by a negative selection procedure was performed

3.3.2 Characterization of output phage from SAS procedures

The output of the SAS procedures was characterized by PCR and restriction digestion on 200 clones from selection rounds 2, 3 and 4. This led to the selection of 31 clones for further investigation. Fourteen NWM and 3 MS clones were obtained from selections on RR-MS CSF, while for the PP-MS selection procedures 11 NWM and 3 MS clones were selected. Identical clones were absent when output phage from RR- and PP-MS selection rounds were compared, which indicates the presence of different CSF antibody repertoires for these MS subtypes.

Two out of the selected 31 clones (1 clone from each CSF pool) were found to be dominantly enriched in the output from the positive selections with the MS library. These clones were detected in 35-50% of analyzed colonies in the output

of the second positive selection round, while in the third positive round their presence increased up to 75-100%. Negative selection procedures were performed to investigate whether enrichment of these clones was due to specific binding with MS antibodies. Characterization of output phage from negative selection rounds revealed an equal prevalence of the dominantly enriched clones as compared with the output from positive selection rounds. Therefore, the dominant enrichment of these clones was due to specific interaction of antibodies from MS patients.

3.3.3 Identification of novel antibody targets for RR- and PP-MS

To select the most promising antibody targets out of the 31 candidate clones, a pilot screening on paired serum samples from RR-MS and PP-MS patients included in the SAS procedures was performed. For 9 out of the 31 clones, named UH-RRMS1 to 8 and UH-PPMS1 (University Hasselt, RRMS/PPMS, clone number), antibody reactivity was confirmed by phage ELISA in serum from at least 1 RR- or PP-MS patient used for SAS procedures (Table 3.4). Moreover, antibody reactivity towards these candidate targets was absent in serum from individual HC (n=5) and pooled serum from NIND (n=5), OIND (n=5) and other HC (n=4). However, antibody reactivity towards 1 clone, UH-RRMS7, was also observed in pooled serum from other MS patients (n=5), although pooled serum from HC (n=5) tested positive as well. Eight clones were identified in SAS procedures with the NWM cDNA library, while 1 clone was derived from selections with the MS cDNA library. These 9 clones were used for a large scale screening in CSF from patients and controls.

The selected clones encode for peptides with a size of 5 to 32 amino acids (Table 3.5). The amino acids of these peptides were compared to public protein databases using BLAST analysis. UH-RRMS6 corresponded for 21 out of 23 amino acids to hCG2045783. Although other peptides were translated from non-coding reading frames or 3’ untranslated regions, homology could be detected to proteins such as calcyphosine 2 (UH-RRMS1) and kruppel-like factor 14 (UH-RRMS7).

Table 3.4: Antibody reactivity in serum towards selected candidate antibody targets

	RR-MS serum						HC serum					Serum pool			
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**MS**	**HC**	**NIND**	**OIND**
RR-MS SAS procedures															
UH-RRMS1	-	-	+[a]	-	-	-	-	-	-	-	-	-	-	-	-
UH-RRMS2	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
UH-RRMS3	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
UH-RRMS4	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
UH-RRMS5	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
UH-RRMS6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UH-RRMS7	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-
UH-RRMS8	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-

	PP-MS serum						HC serum					Serum pool			
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**MS**	**HC**	**NIND**	**OIND**
PP-MS SAS procedures															
UH-PPMS1	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-

[a] The cut-off for a positive sample was set at a ratio > 1.5 (OD signal candidate target phage/ OD signal corresponding empty phage).

Table 3.5: Identity of candidate RR- and PP-MS clones identified from pilot screening

Clone	cDNA library	Translated cDNA product	Size (amino acids)	Homology on protein level (accession number)
RRMS:				
UH-RRMS1	NWM	SEEKKYTRNPFFLHHSPWQREVTTPLWCGHTDAPP	35	9/26 (35%) calcyphosine 2 (Q9BXY5.2) 13/41(32%)ubiquitin protein ligase E3A (BAD69554.1) 9/23 (39%) chromosome 11 open reading frame 95 (C9JLR9.1)
UH-RRMS2	NWM	LELRDF	6	5/6 (83%) calpain 12 (Q6ZSI9.1) 5/6 (83%) ATP-binding cassette, sub-family A (ABC1), member 13 (Q86UQ4.3)
UH-RRMS3	NWM	-[a]	-	
UH-RRMS4	NWM	AVSPVGHNDVSI	12	6/6 (100%) hCG2038397 (EAX10222.1) 6/9 (67) general transcription factor IIH, polypeptide 4, 52kDa (BAD96327.1)
UH-RRMS5	NWM	DFFDF	5	5/5 (100%) PHD finger protein 20-like 1 (A8MW92.2) 4/5 (80%) zinc finger, DHHC-type containing 17 (EAW97332.1) 4/5 (80%) serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (EAW73763.1)
UH-RRMS6	MS	SFGTFVDVAKEQ	12	7/7 (100%) hCG1804186 (EAX02246.1) 7/8 (88%) aldehyde dehydrogenase 9 family, member A1 (P49189.3) 7/9 (78%) ATPase, class II, type 9B (O43861.4)
UH-RRMS7	NWM	EEEEEEEEDEEDVGHAGAGGGAGSERAAGQPA	32	11/15 (73%) kruppel-like factor 14 (ABF82392.1) 11/13(85%) family with sequence similarity 110, member D (NP_079145.2)
UH-RRMS8	NWM	ESPLISPKSSSASFHPSPDEQCE	23	21/21 (100%) hCG2045783 (EAX08890.1)

Table 3.5 (continued): Identity of candidate RR- and PP-MS clones identified from pilot screening

Clone	cDNA library	Translated cDNA product	Size (amino acids)	Homology on protein level (accession number)
PPMS:				
UH-PPMS1	NWM	VAGRKAPSILLL	12	9/10(90%) hCG1649232 (EAX01317.1) 7/7 (100%) coiled-coil domain containing 60 (AAH40553.1)

[a] Nucleotide sequence of clone UH-RRMS3 started immediately with an A-stretch. Therefore it was impossible to determine the correct reading frame and corresponding size of the cDNA product

3.3.4 Analysis of antibody reactivity towards candidate antibody targets in CSF from MS patients and neurological controls

Next we investigated by phage ELISA whether antibody reactivity towards the panel of 9 candidate targets could be detected in CSF from (additional) MS patients and neurological controls. For all candidate targets, antibody reactivity could be verified in at least 1 CSF sample from patients used for the SAS procedures. Moreover, antibody reactivity could be confirmed in paired serum samples (section 3.3.3), which may indicate a similar antibody repertoire in CSF and serum. However, antibody reactivity towards UH-RRMS1-6 and 8 was absent in CSF from additional RR-MS (n=49), OIND (n=9) and NIND (n=17) patients. In addition, no antibody reactivity towards UH-PPMS1 was detected in CSF from additional PP-MS (n=25), OIND (n=7) and NIND patients (n=12). On the other hand, 2/35 RR-MS, 1/7 PP-MS, 1/9 SP-MS and 1/24 NIND patients tested positive for UH-RRMS7 when the amount of CSF per well was increased from 20 µl (diluted 1:5) to 75 µl (diluted 1:1.33) (Table 3.6). Therefore, UH-RRMS7 may represent a common antibody target for RR- and PP-MS.

As suggested by the described results, the amount of CSF may be an important factor contributing to the identification of a positive CSF sample. In the future, additional phage ELISA assays may be performed for UH-RRMS1-6,8 and UH-PPMS1 using the higher amount of CSF.

Table 3.6: Antibody reactivity in CSF towards UH-RRMS7

	RR-MS	PP-MS	SP-MS	OIND	NIND
# ab pos patients	2/35[a]	1/7	1/9	0/15	1/24
% ab pos patients	6	14	11	0	4

[a] The cut-off for a positive sample was set at 3 times the SD above the mean Δ OD signal (OD specific phage – OD corresponding empty phage) obtained for the NIND and OIND samples.

3.4 Discussion

In this study, we identified a panel consisting of 8 novel candidate antibody targets for RR-MS and 1 candidate target for PP-MS. Antibody reactivity towards this panel could be confirmed in individual serum and CSF samples from patients used for SAS. Moreover, antibody reactivity towards 1 candidate target, UH-RRMS7 was detected in CSF from additional MS patients not used for SAS procedures.

No overlap was observed in the output from SAS procedures of the RR- and PP-MS pools, which may reflect the presence of distinct humoral disease mechanisms in these MS subtypes. On the other hand, immunoreactivity towards UH-RRMS7 was detected in CSF from both RR- and PP-MS patients, which points to common antibody targets for RR- and PP-MS. However, for antibody targets, such as galactocerebroside [155] and NF-L [144, 145], antibody reactivity could be observed in all MS subtypes, although the frequency of antibody reactivity was increased in RR- and PP-MS, respectively. Therefore, the number of RR- and PP-MS patients should be expanded to investigate whether antibody reactivity towards UH-RRMS7 is primarily present in RR-MS.

Antibody reactivity towards the panel of candidate antibody targets was detected in both CSF and serum from patients used for SAS. However, with the exception of UH-RRMS7, no additional positive CSF samples from MS patients not included in the SAS procedures could be identified. This may have a biological cause since MS is a heterogeneous disease and the antibody response may be pathologically involved in a subset of MS patients. In particular in PP-MS, the antibody response may have a less prominent role. For example, type IV lesions, characterized by oligodendrocyte death and without antibody deposition, have been reported only in PP-MS [26]. Quintana and colleagues were able to discriminate PP-MS from healthy subjects based on their antibody profile [241]. However, this was not due to a general increase in antibody levels in PP-MS, but rather to a decrease in antibody reactivity as compared with healthy subjects. In another study, the pathogenic potential of antibodies from MS patients was tested in a myelinating culture system [192]. Although complement-

dependent demyelination caused by IgG of PP-MS patients was observed occasionally, the frequency of a pathogenic antibody response was significantly higher in RR-MS as compared with PP-MS.

Alternatively, technical issues may cause the lack of additional antibody positive samples. When the amount of CSF was increased in the ELISA, additional positive CSF samples from patients not included in the SAS procedures could be identified. However, for the whole panel of 9 antibody targets, antibody reactivity could be detected in at least 1 CSF sample from patients used for SAS. Nevertheless it may be interesting to test whether antibody reactivity towards UH-RRMS1-6,8 and UH-PPMS1 can be detected in additional MS patients when the amount of CSF in the phage ELISA will be increased.

Seven out of 8 selected candidate antibody targets for RR-MS were derived from the NWM cDNA library, whereas 1 clone was derived from selections with the MS library. This may be due to the relative short disease duration of the RR-MS patients included in the SAS procedures (Table 1, average 2.7 years), which may skew the antibody response towards initial antigens involved in disease. Moreover, similar results were obtained in another study, which focused on the antibody repertoire of patients with clinically isolated syndrome (CIS), a possible first manifestation of MS [283].

For both patient groups dominant enrichment of a single clone was present in the output of selections with the MS cDNA library. Enrichment of these clones was not due to aspecific binding, since their prevalence did not decrease after the incorporation of a negative selection round. Importantly, specific antibody reactivity towards these clones could be demonstrated in serum from patients used for SAS. Moreover, due to the high prevalence of these dominantly enriched clones, other enriched candidate targets may be overseen in phage output from the MS library, which may clarify the low number of candidate antibody targets derived from the MS library.

UH-RRMS8 corresponded for 21 out 23 amino acids to hCG2045783. However, no further information for this protein was available. Another candidate antibody

target, UH-RRMS4 displayed homology to general transcription factor IIH, polypeptide 4 (GTF2H4), a protein involved in nucleotide excision repair. Interestingly, a single nucleotide polymorphism (SNP) within intron 11 of GTF2H4 was found to be associated with MS susceptibility [284]. Antibody reactivity towards GTF2H4 may inhibit the DNA repair function of GTF2H4 and lead to an increase in DNA damage, which has been demonstrated in MS plaques [285].

In conclusion, a panel of 9 novel antibody targets was identified for RR and PP-MS. Antibody reactivity was confirmed in both CSF and serum from patients used for the SAS procedures. In the future, further study of these targets is mandatory to validate their potential as antibody targets for MS. Moreover, we provided an initial indication for the presence of different antibody repertoires in RR- and PP-MS. Future studies should be performed to confirm and further investigate the antibody response in MS subtypes.

4

Identification of coronin-1a as a novel antibody target for clinically isolated syndrome and multiple sclerosis

Based on:

Identification of coronin-1a as a novel antibody target for clinically isolated syndrome and multiple sclerosis

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Abstract

Recently, we identified the mimotope UH-CIS6 as a novel candidate antibody target for clinically isolated syndrome (CIS) and relapsing-remitting (RR) multiple sclerosis (MS). The purpose of this study was to further validate UH-CIS6 as an antibody target for CIS and MS and to identify the *in vivo* antibody target of UH-CIS6. First, a UH-CIS6 peptide ELISA was optimized. Next, we investigated the antibody response towards UH-CIS6 in cerebrospinal fluid (CSF) from patients with CIS (n=20), MS (n=43) and other neurological diseases (n=42). Immunoprecipitation of anti-UH-CIS6 antibodies on a normal human brain lysate was performed to identify the *in vivo* antibody target of UH-CIS6. The cellular expression of an *in vivo* candidate target was investigated by immunohistochemistry using MS brain tissue sections. Antibody reactivity towards UH-CIS6 was detected in a significantly increased proportion of CSF samples from CIS and RR-MS patients as compared with neurological controls (p=0.046). We identified and confirmed coronin-1a as the *in vivo* antibody target for UH-CIS6. Furthermore, coronin-1a was expressed by T cells and macrophages in an active MS lesion. Together, these results demonstrate that coronin-1a is a novel antibody target for CIS and MS.

4.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory neurological disorder of the central nervous system (CNS), characterized by inflammation-driven demyelination and neuro-axonal degeneration. In Europe, MS has a prevalence of approximately 0,1% [1]. The majority of MS patients display a relapsing-remitting (RR) disease course, in which disease exacerbations are followed by periods of remission. In 85% of the MS patients, the disease first manifests with a clinically isolated syndrome (CIS), a single episode of neurological disturbance which should last at least for 24 hours [4]. Clinical symptoms and signs in CIS can be caused by a lesion in the optic nerve, spinal cord, brainstem, cerebellum or cerebral hemisphere [4]. Thirty to 70% of CIS patients develop MS by a second clinical relapse or by new lesions on a follow-up MRI [7]. An ongoing need exists for markers which allow early diagnosis.

The presence of antibodies in cerebrospinal fluid (CSF) and in peripheral blood is one of the inflammatory hallmarks of CIS and MS. However, targets of this antibody response are largely unknown. An example of an antibody target shared by CIS and MS is myelin oligodendrocyte glycoprotein (MOG) [117]. However, contradictory results on the anti-MOG antibody response have been obtained, which is reflected by the wide range of antibody positive CIS and MS patients that have been reported [117]. These discrepancies may be caused by the different types of assays, such as Western blot analysis, ELISA and cell based assays, that have been used. In addition, antibody reactivity towards linear and conformational MOG epitopes has been investigated [117]. Recently, the potassium channel KIR4.1 has been identified as a novel antibody target for CIS and MS [116]. Serum antibodies to KIR4.1 were detected in 46.9% of CIS and MS patients, while these antibodies were present in serum from 0.9% of neurological controls and absent in healthy subjects. However, additional studies are needed to confirm these promising findings.

In a recent study, we identified UH-CIS6 as a novel candidate antibody target for CIS and RR-MS by using a phage display approach [283]. Phage ELISA assays confirmed antibody reactivity towards UH-CIS6 in CSF from the CIS discovery

cohort as well as in CSF samples from additional CIS and RR-MS patients. Furthermore, a significantly higher percentage of CIS and RR-MS patients displayed serum antibody reactivity towards UH-CIS6 as compared to neurological and healthy controls (21% versus 7%). A disadvantage of phage display, however, is the identification of mimotopes. These peptides or proteins do not necessarily have the same or even a similar amino acid sequence as the natural epitopes, but mimic their binding properties [249]. UH-CIS6 is an example of a mimotope and therefore the *in vivo* antibody target is unknown.

Since UH-CIS6 represents a promising candidate autoantibody target for CIS and MS, the goal of this study was to further characterize UH-CIS6. First, a UH-CIS6 peptide ELISA was developed to study the antibody response towards UH-CIS6 in CSF from CIS and MS patients. Secondly, since UH-CIS6 encodes a mimotope, we aimed to identify the *in vivo* antibody target and further characterize the expression of a candidate target in MS brain tissue.

4.2 Materials and methods

4.2.1 Patient material

Immunoreactivity towards UH-CIS6 was assessed in CSF samples from 20 CIS, 31 RR-MS, 7 primary-progressive (PP) MS and 5 secondary-progressive (SP) MS patients. In the control group, 26 and 16 CSF samples were included from patients with non-inflammatory neurological disorders (NIND) and other inflammatory neurological diseases (OIND), respectively (Table 4.1). MS diagnosis and MS development in CIS patients was established according to McDonald criteria [14, 15]. In general, CIS patients had a clinical follow-up for 2 years after sampling. This study was approved by the ethics committee of Hasselt University and fulfilled guidelines from the declaration of Helsinki. Samples were obtained after informed consent.

Table 4.1: Characteristics of patients and controls used for anti-UH-CIS6 antibody reactivity analysis in CSF

Patient group	Gender (M/F)[a]	Mean age (years)± SD	Mean disease duration[b] (years) ± SD	EDDS score[c] ± SD
CIS (n=20)	7/13	30.8±9.4	0.4±0.7	1.6±0.7
CIS-CIS (n=5)	2/3	32.0±11.4	0.1±0.2	1.7±0.6
CIS-MS (n=15)	5/10	30.4±9.0	0.5±0.8	1.6±0.7
RR-MS (n=31)	9/22	38.7±9.4	6.3±7.1	2.8±1.2
PP-MS (n=7)	2/5	45.1±13.8	8.2±4.7	4.5±1.4
SP-MS (n=5)	2/3	50.0±9.6	15.8±12.3	4.2±1.3
OIND (n=16)	5/11	51.6±14.7	NA[d]	NA
NIND (n=26)	8/18	48.6±15.1	NA	NA

[a] M: male, F: female
[b] Data not available for 3 CIS (CIS-MS) patients
[c] EDDS: expanded disability disease scale. Data available for 11 CIS (3 CIS-CIS, 8 CIS-MS), 29 RR-MS, 7 PP-MS and 3 SP-MS patients
[d] NA: Not available/not applicable

4.2.2 UH-CIS6 peptide ELISA

Two N-terminal biotinylated synthetic UH-CIS6 peptides (GL Biochem, Shanghai, China) with or without 6 additional amino acids of the phage vector (MPVVPATWEAETGESLEPGRRRLQ and QEFGTSMPVVPATWEAETGESLEPGRRRLQ) were used. An irrelevant peptide (WTKTPDGNFQLGGTEP) was purchased to correct for background reactivity.

To determine human immunoglobulin G (IgG) antibody reactivity towards the biotinylated UH-CIS6 peptide, enzyme-linked immunosorbent assay (ELISA) plates coated with streptavidin (Nunc, Roskilde, Denmark) were used. First, ELISA plates were washed 3 times with 0.05% Tween 20 in phosphate-buffered saline (PBST). Subsequently, plates were coated with 0.5 µg/ml UH-CIS6 peptide in PBST for 1 hour at room temperature (RT), while shaking. Plates were blocked in 5% (w/v) skimmed milk powder in PBS (MPBS) for 2 hours at 37°C, which was followed by washing of the plates. Subsequently, 100 µl 1:100 diluted serum or 1:1.33 diluted CSF was added to each well and incubated for 2 hours at RT, while shaking. After washing steps were repeated, 100 µl of a rabbit anti-human IgG horse radish peroxidase (HRP) labeled antibody (diluted 1:2,000, Dako, Heverlee, Belgium) was added to detect the amount of bound human IgG (1 hour, shaking at RT). Colour development was started after the addition of 100 µl 3,3′,5,5′ tetramethyl-benzidine dihydrochloride (TMB) solution (Perbioscience, Erembodegem, Belgium) and stopped with 50 µl 2N H_2SO_4. Plates were read at 450 nm in a Tecan plate reader (Tecan, Männedorf, Switzerland).

4.2.3 Competition assays

To confirm that UH-CIS6 phage particles and synthetic UH-CIS6 peptides contained identical epitopes, competition ELISA assays were performed as described previously [283]. Briefly, 96-well ELISA plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with 10 µg/ml anti-M13 filamentous phage antibody (GE healthcare, Diegem, Belgium). Plates were blocked for 2 hours at 37 °C with 2% MPBS. Next, 100 µl of polyethylene glycol purified UH-CIS6 phage particles ($7*10^{11}$ colony forming units/ml) were added to each well and incubated for 1 hour at 37 °C, followed by 30 minutes at RT

while shaking. Meanwhile, pre-incubation mixtures were prepared in a separate 96-well round bottom plate (Nunc). These mixtures consisted of 1:100 diluted UH-CIS6 antibody positive or negative serum sample (based on previously reported phage ELISA results [283]) and increasing concentrations of UH-CIS6 or irrelevant peptide (0-10 µg/ml). Serum antibodies and peptides were allowed to interact for 1.5 hour (RT, shaking). Subsequently, 100 µl of each pre-incubation mixture was transferred to the ELISA plate (1 hour 37 °C, 30 minutes RT shaking). The amount of bound IgG was then determined with 100 µl 1:2,000 diluted goat-anti-human IgG HRP labeled antibody preadsorbed against mouse immunoglobulin G (IgG) (Life technologies, Merelbeke, Belgium). The remaining steps were performed as described for the UH-CIS6 peptide ELISA.

4.2.4 Purification of anti-UH-CIS6 antibodies and total IgG

To further study and characterize the target of UH-CIS6, a rabbit polyclonal anti-UH-CIS6 antibody was purchased (Eurogentec, Seraing, Belgium). A Poros-A column (Life technologies) was used to purify total IgG from the pre-immune rabbit serum, which could be used as a negative control. To purify human anti-UH-CIS6 antibodies, UH-CIS6 peptide was coupled to a HiTrap NHS-Activated HP colum (GE healthcare), according to manufacturer's instructions. An Äkta Prime Plus device (GE healthcare) was used for purification. Rabbit and human serum was bound to the resin by addition of binding buffer (0.1 M glycine-sodium hydroxide, 3 M sodium chloride, pH 8.6 or 0.4 M sodium hydrogen carbonate, 1.0 M sodium chloride, pH 8.3), followed by elution of rabbit IgG or human anti-UH-CIS6 antibodies with elution buffer (0.1 M citric acid monohydrate, pH3 or 100 mM glycine, pH3). Positive elution fractions were pooled and rabbit IgG was concentrated using Pierce Protein Concentrators (Thermo Scientific, Erembodegem, Belgium). Subsequently, IgG concentration was determined with the BCA protein assay kit (Thermo Scientific) or with a Nanodrop 2000 (Thermo Scientific).

4.2.5 Preparation of human brain lysate

A human brain lysate was prepared by homogenization of normal human brain tissue with a rotor-stator in RIPA lysis buffer (150 mM sodium chloride, 1% NP-40 (Sigma-Aldrich, Bornem, Belgium), 0.5% sodium dioxycholate, 0.1% sodium

dodecyl sulphate (SDS) 50 mM Tris, pH 8) supplemented with EDTA-free nuclease inhibitors (Roche Diagnostics, Vilvoorde, Belgium). Subsequently, the lysate was centrifuged and the protein concentration of the supernatant was determined with the BCA protein assay kit (Thermo Scientific), following manufacturer's instructions.

4.2.6 Immunoprecipitation

To identify the in vivo antibody target for UH-CIS6, an immunoprecipitation (IP) procedure was performed with a rabbit polyclonal anti-UH-CIS6 antibody on a normal human brain lysate. To this end, the Pierce Crosslink Immunoprecipitation Kit (Thermo Scientific) was used according to manufacturer's instructions with minor modifications. Briefly, 50 µg anti-UH-CIS6 antibody diluted in tris-buffered saline (TBS) (0.025 M Tris, 0.15 M sodium chloride, pH 7.2) was coupled to 20 µl Protein A/G Plus Agarose overnight at 4°C, followed by crosslinking of bound antibody. To remove aspecific binding, human brain lysates were precleared prior to the IP procedure. For this purpose, 2 mg lysate was added to 160 µl control agarose resin (overnight at 4°C). Next, precleared lysate was added to the UH-CIS6 antibody coupled column and incubated overnight at 4°C. The antibody coupled column was washed, followed by 4 sequential elution steps. Elution fractions were neutralized with 1 M Tris (pH 9.5).

4.2.7 Mass spectrometry

IP elution fractions were separated by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) and proteins were stained with the Silverquest Silver Staining Kit, according to manufacturer's recommendation (Life technologies). Protein bands were excised, separately trypsinized and resulting peptides were analyzed by nanoliquid chromatography mass spectrometry as previously described [286]. Thus obtained peptide fragmentation spectra were searched in Proteome Discoverer v1.2 using Sequest v1.2.0.208 and Mascot v2.4 against the International Protein Index Human database (v.3.87; 91464 entries). The output of both search engines was validated with Scaffold v3.6.1.

4.2.8 Western blot

Ten µg of a human coronin-1a overexpressing human embryonic kidney (HEK) lysate (Novus Biologicals, Cambridge, United Kingdom) or 1 µg purified human coronin-1a protein expressed in HEK cells (Origene, Rockville, USA) was separated by SDS-PAGE. Next, proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Brussels, Belgium), followed by a blocking step for 2 hours in 5% MPBS supplemented with 0.1% Tween. The rabbit anti-UH-CIS6 antibody (1:100), pre-immune rabbit IgG (1:100) and a commercial anti-coronin-1a antibody (1:250, Sigma Aldrich) were diluted in blocking buffer and incubated overnight at 4°C. After the PVDF membrane was washed several times with PBST, a swine anti-rabbit IgG HRP labeled antibody (diluted 1:1,000, Dako) was added and incubated for 2 hours at RT. Washing steps were repeated and bound antibody was visualized using the Pierce ECL Plus Western Blotting Substrate kit (Thermo Scientific).

4.2.9 Coronin-1a ELISA

Ninety-six well ELISA plates (Greiner Bio-One) were coated overnight at 4°C with 2 µg purified coronin-1a protein (Origene) in coating buffer (0,1 M sodium hydrogen carbonate pH 9.6). Plates were blocked with 2% MPBS for 2 hours at 37 °C. Next, plates were washed 3 times with 0.1% PBST and once with PBS. Subsequently, the commercial anti-coronin-1a antibody (Sigma Aldrich) was diluted 1:690, the rabbit polyclonal anti-UH-CIS6 antibody 1:20 (stock 1 mg/ml), pre-immune rabbit IgG 1:100 (stock 5 mg/ml), and purified human anti-UH-CIS6 antibodies 1:5 (stock 0.2 mg/ml) and 100 µl diluted antibody was added to each well and incubated for 2 hours at RT while shaking. Plates were washed and 100 µl swine anti-rabbit IgG HRP labeled antibody (diluted 1:1,000, Dako) or rabbit anti-human IgG HRP labeled antibody (diluted 1:2,000, Dako) was added (1 hour, RT, shaking). The remaining steps were performed as described for the UH-CIS6 peptide ELISA.

4.2.10 Immunohistochemistry

Frozen tissue sections from human MS brain and normal white matter from a non-demented neurological control were used to investigate coronin-1a expression. Sections were fixed with aceton and blocked with serum-free protein

block (Dako). Next, sections were incubated with primary antibodies (coronin-1a: 1:1,000 (Sigma Aldrich), proteolipid protein (PLP) : 1:500 (Serotec, Düsseldorf, Germany), major histocompatibility complex class II (MHC II: HLA-DP, DQ, DR) 1:100 (Dako)) for 1.5 hour at RT. To detect binding, the Envision Dual Link System-HRP (Dako) was used, followed by staining with (3,3'-diaminobenzidine) DAB chromogen solution (Dako). Hematoxylin was used as counterstaining. In addition, a double staining was performed for coronin-1a and MHC II. Antibodies were incubated simultaneously overnight at 4°C. As secondary antibodies, a goat anti-rabbit Alexa 488 (1:250, Life Technologies) and a goat anti-mouse Alexa 555 (1:250, Life Technologies) fluorescent labeled antibody were used. A nuclear staining was performed with 4', 6-diamidino-2-phenylindole (DAPI). Stained tissue sections were evaluated with a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and NIS-Elements Basic Research Software (Nikon, Tokyo, Japan).

4.2.11 Statistics

Graph Pad Prism 5 software (GraphPad software, La Jolla, USA) was used for statistical analysis. ELISA data were analyzed with the Kruskall Wallis test, followed by Dunn's multiple comparison test. The cut-off for a positive UH-CIS6 sample was set at 3 times the standard deviation (SD) above the mean OD signal obtained for NIND and OIND samples. Putative associations between antibody reactivity and disease were analyzed using the Fisher's exact test. In addition, associations between antibody reactivity and clinical parameters were examined. An unpaired two-tailed student t-test or Mann Whitney test was used for demographical variables, while the Fisher's exact test was used for categorical variables. A p-value smaller than 0.05 was considered significant.

4.3 Results

4.3.1 Development of a peptide ELISA for UH-CIS6

In order to further validate the potential of UH-CIS6 as an antibody target in CIS and MS, we aimed to screen both CIS and MS patients for anti-UH-CIS6 antibody reactivity in a peptide ELISA format. First, the peptide ELISA was optimized. Two biotinylated UH-CIS6 peptides, with and without linker were tested. This linker consisted of 6 additional amino acids of the phage vector and may further improve the accessibility of the epitope.

As shown in Figure 4.1A, antibody reactivity towards UH-CIS6 could be detected with both UH-CIS6 peptides. A clear increase in the OD signal could be observed for the UH-CIS6 peptide with linker as compared with the UH-CIS6 peptide without linker. Moreover, OD signals increased 2-6 fold for the antibody positive sample with biotinylated UH-CIS6 peptides as compared with unbiotinylated UH-CIS6 peptides with and without linker (data not shown). Competition assays revealed that both UH-CIS6 peptides were able to compete with UH-CIS6 phage particles, which confirmed that the UH-CIS6 peptides and phage particles contain identical epitopes (Figure 3.1B). Based on these results the biotinylated UH-CIS6 peptide with linker was used in further experiments.

To validate the UH-CIS6 peptide ELISA, 52 CSF samples (17 CIS and 17 MS patients, 18 neurological controls) were tested with both peptide and phage ELISA assays (data not shown). Two CIS patients and 2 neurological controls tested positive using the UH-CIS6 phage ELISA. These samples tested also positive when using the UH-CIS6 peptide ELISA. Interestingly, 6 additional UH-CIS6 antibody positive samples (3 CIS and 3 MS patients) were identified using the UH-CIS6 peptide ELISA, demonstrating an increased sensitivity of the UH-CIS6 peptide ELISA.

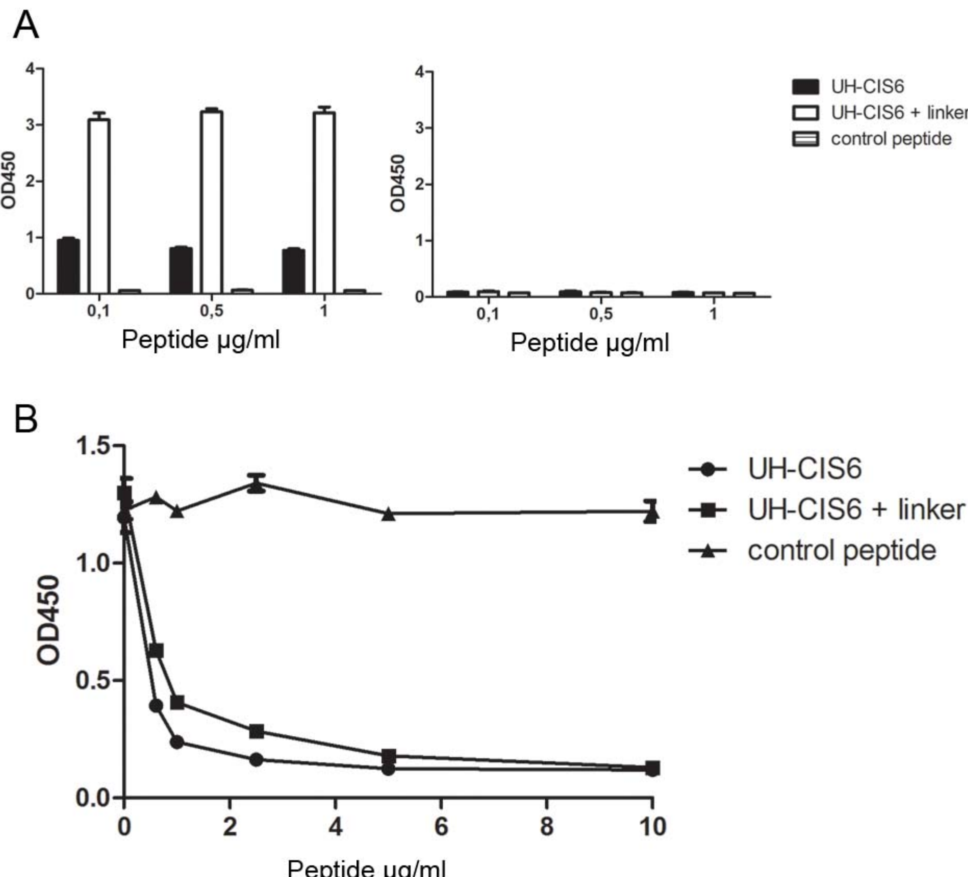

Figure 4.1: Development of the UH-CIS6 peptide ELISA. An antibody positive and negative serum sample were selected based on UH-CIS6 phage ELISA results. A. Antibody reactivity towards the UH-CIS6 peptides could be demonstrated for the antibody positive sample, while no anti-UH-CIS6 antibody reactivity was observed for the antibody negative sample. Addition of the linker markedly increased the observed OD signal for the antibody positive sample. B. Both UH-CIS6 peptides were able to compete with UH-CIS6 phage particles and contained the epitope of the UH-CIS6 phage.

4.3.2 CSF antibody reactivity towards UH-CIS6 in CIS and MS patients

Using the UH-CIS6 peptide ELISA, antibody reactivity towards UH-CIS6 was evaluated in CSF samples from 20 CIS, 31 RR-MS, 7 PP-MS and 5 SP-MS patients. In addition, anti-UH-CIS6 antibody reactivity was analyzed in CSF samples from 26 NIND and 16 OIND patients. This cohort also contained CSF samples that were used for the validation of the UH-CIS6 peptide ELISA. Five CIS patients (25%), 7 RR-MS patients (23%) and 1 SP-MS patient (20%) tested positive for anti-UH-CIS6 antibodies. On the other hand, 3 neurological controls (7%), diagnosed with epilepsy (n=2) and papilla edema (n=1), also displayed antibody reactivity towards UH-CIS6 (Figure 4.2A). As shown in Figure 4.2B, the number of antibody positive CIS and RR-MS patients was significantly increased as compared to controls (24% versus 7%, Fisher's exact test: $p=0.046$). No correlation between antibody reactivity towards UH-CIS6 and clinical parameters was detected (data not shown). In conclusion, antibody reactivity towards UH-CIS6 could be primarily detected in CSF from CIS and RR-MS patients. Thus, these findings confirm that UH-CIS6 is a candidate antibody target for CIS and RR-MS.

Clinical follow-up revealed that 15 out of 20 CIS patients developed MS. Interestingly, all CIS patients that tested positive for antibody reactivity towards UH-CIS6 progressed to MS, which may indicate that UH-CIS6 could serve as a prognostic marker. However, the number of CIS patients should be expanded in the future to further investigate whether antibody reactivity towards UH-CIS6 has prognostic potential to predict conversion to MS.

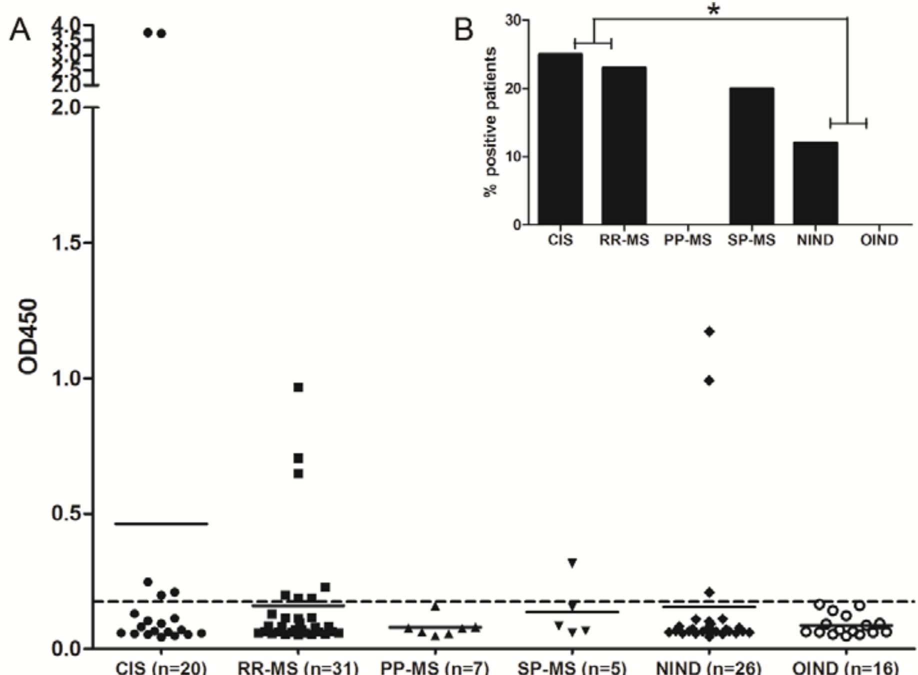

Figure 4.2: CSF antibody reactivity towards UH-CIS6. A. The UH-CIS6 peptide ELISA was used to detect anti-UH-CIS6 antibody reactivity in CSF from patients with CIS, MS and other (inflammatory) neurological diseases (NIND and OIND). Measurements were performed in duplicate and mean values are shown. Lines indicate mean antibody levels. The cut-off for a positive sample (dash line) was set at 3 times the SD above the mean OD signal obtained for NIND and OIND samples. B. CSF samples from a significant larger proportion of CIS and RR-MS patients tested positive for UH-CIS6 as compared with neurological controls. * indicates a p-value<0.05.

4.3.3 Identification of coronin-1a as the antibody target for UH-CIS6

To identify the *in vivo* antibody target corresponding to UH-CIS6, a polyclonal rabbit anti-UH-CIS6 antibody was generated. First, we tested whether this polyclonal rabbit anti-UH-CIS6 antibody bound the same epitope as human anti-UH-CIS6 antibodies. Competition assays revealed that the anti-UH-CIS6 polyclonal rabbit antibody and human anti-UH-CIS6 antibodies indeed bound the same epitope (Figure 4.3). Therefore, the polyclonal rabbit anti-UH-CIS6 antibody was suitable to characterize and identify the *in vivo* antibody target of UH-CIS6.

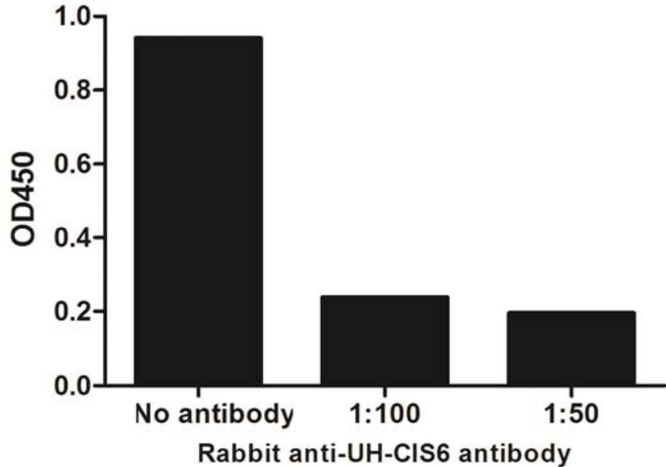

Figure 4.3: Identical epitope for rabbit and human anti-UH-CIS6 antibodies. A human serum sample positive for UH-CIS6 was pre-incubated with increasing amounts of the rabbit anti-UH-CIS6 antibody. The rabbit anti-UH-CIS6 antibody competed with the human anti-UH-CIS6 antibodies as increasing amounts of the rabbit anti-UH-CIS6 antibody led to a strong decrease in bound human anti-UH-CIS6 antibodies. Measurements were performed in duplicate and mean values are shown.

Next, to identify the *in vivo* antibody target for UH-CIS6, a normal brain lysate was immunoprecipitated with the rabbit anti-UH-CIS6 antibody. SDS-PAGE followed by mass spectrometric analysis of obtained precipitates identified the peptide 'AAPEASGTPSSDAVSR' with a peptide probability of 95% corresponding to amino acids 417 - 432 of coronin-1a (Figure 4.4).

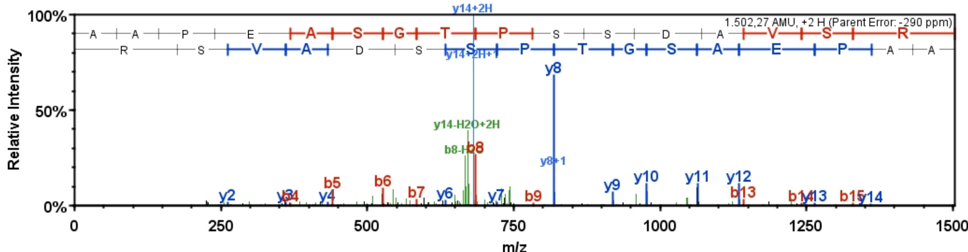

Figure 4.4: Peptide mass spectrum for coronin-1a. Immunoprecipitation output was analyzed by mass spectrometry. N-terminal b and C-terminal y fragment ions are indicated as well as corresponding amino acids. This revealed the identification of the peptide "AAPEASGTPSSDAVSR" identical to amino acid 417- 432 of coronin-1a.

4.3.4 Confirmation of coronin-1a as an antibody target in CIS and MS

To confirm coronin-1a as the *in vivo* antibody target for UH-CIS6, an *in silico* alignment of the amino acid sequence of UH-CIS6 and coronin-1a was performed (http://blast.ncbi.nlm.nih.gov/). This led to the identification of a shared protein motif, which could represent the UH-CIS6/coronin-1a epitope (Figure 4.5A). Furthermore, binding of the rabbit anti-UH-CIS6 antibody to coronin-1a could be demonstrated by Western blot analysis on purified coronin-1a protein and a coronin-1a overexpressing lysate (Figure 4.5B,C). In addition, these findings could be confirmed by ELISA (Figure 4.5D). To investigate whether human anti-UH-CIS6 antibodies also displayed reactivity towards coronin-1a, anti-UH-CIS6 antibodies were purified from a UH-CIS6 positive human serum sample and tested by ELISA. As shown in Figure 4.5D, the human anti-UH-CIS6 antibodies were also positive for coronin-1a. In conclusion, UH-CIS6 was found to correspond to coronin-1a, which represents a novel antibody target for CIS and MS. In the future, the antibody response towards coronin-1a should be analyzed into more detail by analysis of anti-coronin-1a antibody reactivity in serum and CSF samples from patients with CIS and MS.

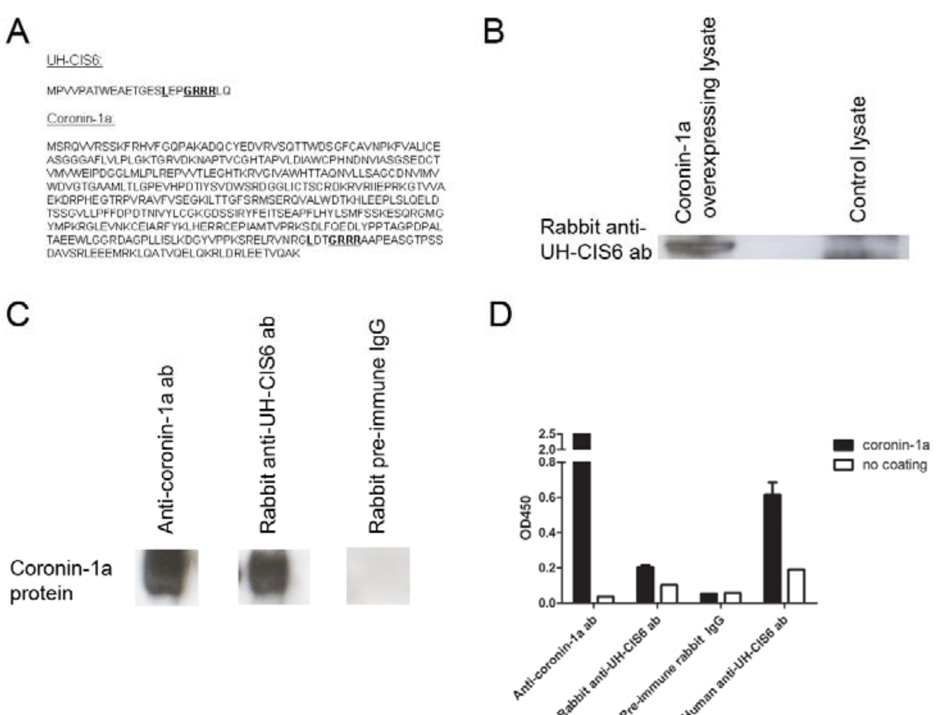

Figure 4.5: Confirmation of anti-UH-CIS6 antibody binding towards coronin-1a.
A. Alignment of UH-CIS6 and coronin-1a protein sequences (Swiss prot accession number P31146) led to the identification of a shared protein motif (indicated in bold). B. Western blot analysis with the rabbit anti-UH-CIS6 antibody on a human coronin-1a overexpressing lysate revealed the presence of a band with a molecular weight similar to coronin-1a. This band was absent in Western blot analysis with the anti-UH-CIS6 antibody on a control lysate without coronin-1a overexpression. C. Binding of the rabbit anti-UH-CIS6 antibody to coronin-1a could be confirmed by Western blot analysis on purified human coronin-1a protein. A commercial anti-coronin-1a antibody was used as a positive control. Pre-immune IgG from the rabbit used for UH-CIS6 immunization was used as a negative control D. Binding to coronin-1a and anti-UH-CIS6 antibodies could also be demonstrated by ELISA. Moreover, purified human anti-UH-CIS6 antibodies were also positive for coronin-1a.

4.3.5 Expression of coronin-1a in MS brain

Immunohistochemistry was performed to investigate the expression pattern of coronin-1a in human brain samples. An active MS lesion, characterized by reduced PLP and increased MHC II expression (Figure 4.6A,B), contained many coronin-1a immunopositive cells (Figure 4.6C). Coronin-1a expression was markedly increased inside the demyelinated MS lesion as compared with surrounding normal appearing white matter (NAWM). In NAWM, microglia were decorated with anti-coronin-1a expression (Figure 4.6D), which was similar to normal white matter from a non-demented neurological control (data not shown). Within the active MS lesion, coronin-1a expression was restricted to macrophages and T cells (Figure 4.6E). Moreover, double positive coronin-1a and MHC II cells were identified (Figure 4.6F). These results demonstrate that coronin-1a immunoreactivity is enhanced and predominantly expressed by infiltrated immune cells in active MS lesions.

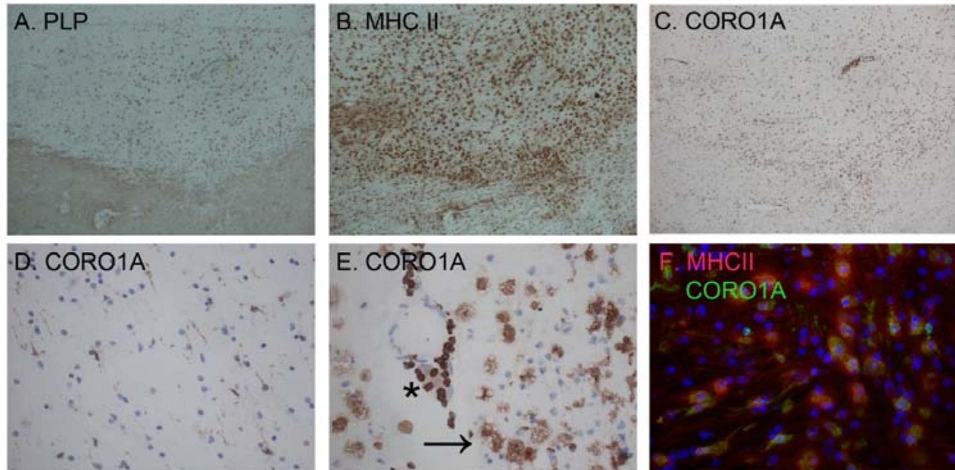

Figure 4.6: Expression of coronin-1a in MS brain. An active MS lesion is shown, reflected by reduced PLP expression (A) and a strong increase in MHC II immunoreactivity (B). Coronin-1a expression was increased inside the active lesion as compared to the surrounding NAWM (C). When the primary antibody was omitted no staining was visible (data not shown). Resting microglia in NAWM were positive for coronin-1a (D), whereas inside the lesion coronin-1a expression was found in macrophages (arrow) and T cells (asterisk) (E). Furthermore, double positive coronin-1a (green) and MHC II (red) cells were identified (F). Representative images are shown. Images are 40X (A-C) and 400X (D-F) magnified.

4.4 Discussion

In a previous study, we identified UH-CIS6 as a novel candidate antibody target for CIS and MS. Therefore, the goal of this study was to further characterize UH-CIS6. First, we developed a sensitive peptide ELISA to detect antibody reactivity towards UH-CIS6. Using this ELISA, anti-UH-CIS6 antibody reactivity could be detected in CSF samples from a significantly increased proportion of CIS and RR-MS patients as compared with neurological controls. Since UH-CIS6 encodes a mimotope, we also aimed to identify the *in vivo* antibody target for UH-CIS6. To this end, immunoprecipitation of a rabbit anti-UH-CIS6 antibody on a normal human brain lysate was performed, which led to the identification of coronin-1a as the *in vivo* antibody target for UH-CIS6. Binding of the rabbit anti-UH-CIS6 antibody to coronin-1a could be confirmed by Western blot analysis and ELISA. Moreover, purified human anti-UH-CIS6 antibodies also bound coronin-1a. In addition, coronin-1a positive T cells and macrophages were identified inside an active MS lesion which demonstrates the presence of coronin-1a expression in MS brain. Therefore, coronin-1a represents a novel antibody target for CIS and MS.

In this study, a sensitive UH-CIS6 peptide ELISA was developed. The improved sensitivity of the peptide ELISA as compared with the phage ELISA was demonstrated by the identification of a higher number of UH-CIS6 antibody positive samples (10 versus 4). This may also clarify why the percentage of UH-CIS6 antibody positive CSF samples from CIS and RR-MS patients was increased as compared to findings reported in our previous study (25% vs. 7% and 23% vs. 2%) [283].

Antibody reactivity towards UH-CIS6 was detected in a significantly larger proportion of CSF samples from CIS and RR-MS patients as compared with neurological controls. Interestingly, all CIS patients that tested positive for antibody reactivity towards UH-CIS6 progressed to MS. These findings further support the possible prognostic potential of UH-CIS6 to predict conversion to MS as was demonstrated in our previous study [283]. However, the number of CIS patients should be expanded in the future, to further investigate the prognostic

potential of anti-UH-CIS6 antibody reactivity. In this way, it would be relevant to investigate whether antibody reactivity towards UH-CIS6 associates with disease activity and if a higher anti-UH-CIS6 antibody response correlates with a more rapid conversion to MS.

Coronin-1a was identified as the *in vivo* antibody target for UH-CIS6. Coronin-1a, primarily expressed in hematopoietic cells, has been proposed to be an actin regulatory protein, which functions as a bridging protein between plasma membrane domains and the dynamic actin cytoskeleton of leukocytes and may allow for the remodeling of the cytoskeleton in response to outside signals transmitted into leukocytes [287]. However, coronin-1a may also have actin-independent functions [288]. In T cells, a role for coronin-1a has been recognized in survival through promotion of Ca^{2+} mobilization form intracellular stores [288] and in T cell receptor induced immunological synapse formation and signaling [289]. In addition, coronin-1a is expressed in macrophages, where it has been associated with survival of mycobacteria within phagosomes [290], Ca^{2+} dependent signaling processes [291] and with lipoprotein uptake and degradation [292]. Finally, coronin-1a has been implicated in phagocytosis in neutrophils [293]. Mutations in coronin-1a have been described in an individual with severe combined immunodeficiency [294, 295] and in 3 siblings with immunodeficiency and EBV-associated B-cell lymphoproliferation [296], which suggest a key role for coronin-1a in the immune system. Furthermore, coronin-1a deficient mice did not develop experimental autoimmune encephalomyelitis (EAE), the animal model of MS after immunization with MOG_{35-55} [297], although after re-immunization coronin-1a deficient mice exhibited enhanced EAE signs that correlated with increased number of interleukin 17 (IL-17) producing CD4+ cells in the CNS [298].

Up till now, coronin-1a has not been associated with antibody reactivity in humans. On the other hand, antibody reactivity towards coronin-1a has been observed after T-cell vaccination in mice [299]. To our knowledge, antibody reactivity towards proteins which are primarily expressed in immune cells, such as coronin-1a, has not been described yet in CIS and MS. In other diseases, however, antibody reactivity towards such antibody targets has been reported. For example anti-neutrophil cytoplasmic antibodies (ANCAs) directed towards

antigens expressed in neutrophils and monocytes, have been described in diseases such as vasculitis, rheumatoid arthritis and inflammatory bowel disease [300, 301, 302]. Once ANCAs have bound their target antigen, intracellular molecules are recruited to activate the neutrophil, which will lead for example to degranulation, super-oxide generation and cytokine production [303]. Apart from these pathogenic effects, antibodies may have protective effects as well. For instance, immunoglobulin M (IgM) antibodies towards neurons have been shown to promote axonal outgrowth and improve functional recovery in a mouse model of MS [193]. Whether anti-coronin-1a antibodies have pathogenic effects and mediate activation of immune cells or have protective properties by blocking the inflammatory response, needs to be examined in future studies. Alternatively, the presence of anti-coronin-1a antibodies may represent an epiphenomenon caused by the infiltration of coronin-1a expressing immune cells in MS brain. To this end, anti-coronin-1a antibodies may be injected into animals with EAE. It may be also interesting to investigate how antibodies can be generated towards an intracellular protein such as coronin-1a. Several hypotheses on the generation of an antibody response towards intracellular proteins have been proposed [304]. Such antibodies may be generated by dysregulation of the immune system, which causes expansion of polyreactive natural antibodies. Apoptosis may result in exposure of intracellular antigens on the cell surface or release into the extracellular environment. Furthermore, alterations in epigenetic modifications, such as DNA methylation, histon acetylation and microRNA expression, as well as molecular mimicry to extracellular or foreign antibody targets may cause an antibody response towards intracellular targets. Moreover, antibody reactivity towards other intracellular targets has already been described in MS [144, 150, 151, 176].

In summary, antibody reactivity towards UH-CIS6 was detected in CSF from a significantly increased proportion of CIS and RR-MS patients as compared with neurological controls. Coronin-1a was identified as the *in vivo* antibody target for UH-CIS6, which represents a novel antibody target for CIS and MS. Future research is needed to fully explore the role of an anti-coronin-1a antibody response in the pathogenesis of CIS and MS. In addition, studies should be

performed to investigate the prognostic potential of anti-coronin-1a antibody reactivity to predict conversion to MS in CIS patients.

5

Comparison of a novel candidate CIS marker to a panel of MS antibody targets in serum from CIS patients

Based on:

Comparison of a novel candidate CIS marker to a panel of MS antibody targets in serum from MS patients
Myrthe Rouwette, et *al*
In preparation

Abstract

Clinically isolated syndrome (CIS) is a possible first presentation of multiple sclerosis (MS). Autoantibody reactivity has been detected in cerebrospinal fluid and serum from CIS patients. However, the antibody response towards several MS autoantibody targets is unknown in CIS. The aim of this study was to further investigate the antibody response towards candidate CIS antibody target UH-CIS6 in serum from CIS patients and compare this with the antibody response towards MS antibody targets sperm associated antigen 16 (SPAG16), neurofilament-light (NF-L) and neurofascin (NF). Antibody reactivity towards the panel of 4 antibody targets was measured in serum from CIS (n=92) patients and healthy subjects (n=77) by ELISA. Fourteen CIS patients tested positive for UH-CIS6 (15%), 12 patients for SPAG16 (13%), 6 patients for NF-L (7%) and 7 patients for NF (8%), while a specificity of 90-95% was obtained (SPAG16:95%, UH-CIS6:90%, NF-L: 94% and NF: 95%). Furthermore, although not significant, a trend for mean increased antibody levels towards UH-CIS6 was observed (p=0.08) in CIS patients as compared with healthy controls. Anti-UH-CIS6 antibody reactivity correlated with an increase in IgG concentration of paired cerebrospinal fluid (p=0.012). In conclusion, UH-CIS6 and SPAG16 may represent candidate autoantibody targets for CIS.

5.1 Introduction

Clinically isolated syndrome (CIS) is an episode of neurological disturbance due to a single white matter lesion and can be an early manifestation of multiple sclerosis (MS) [7]. In CIS patients the optic nerve, brainstem, cerebellum, spinal cord or cerebral hemispheres may be affected [4]. Similar to MS, the (auto)antibody response may play an important role in CIS. For instance, oligoclonal band (OCB) antibodies have been detected in the cerebrospinal fluid (CSF) of CIS patients and antibody reactivity towards myelin and other viral proteins has been observed [68, 116, 121, 177, 305]. Additional antibody targets have been identified in MS, although their sensitivity and specificity in CIS is currently unknown.

In a recent study, we identified UH-CIS6 as a novel candidate antibody target for CIS and MS by application of a phage display approach [283]. Elevated antibody levels towards UH-CIS6 were detected in both cerebrospinal fluid (CSF) and serum from a subset of CIS and RR-MS patients [283]. UH-CIS6 encodes for a 24 amino acid mimotope and we recently identified and confirmed coronin-1a as the *in vivo* antibody target for UH-CIS6.

Previously, our group also identified sperm associated antigen 16 (SPAG16) isoform 2 as an antibody target in MS. Elevated antibody levels towards SPAG16 were detected in serum and CSF samples from MS patients [115, 191]. In addition, disease exacerbating effects have been observed after adoptive transfer of anti-SPAG16 antibodies in mice with experimental autoimmune encephalomyelitis, the animal model of MS [191]. However, in CIS patients the antibody response towards SPAG16 is unknown.

Neurofilament-light (NF-L) and neurofascin (NF) are other examples of antibody targets to which increased antibody reactivity has been demonstrated in MS [138, 143, 144, 145]. Although antibody reactivity towards these targets has been identified in several patients with CIS or possible MS, confirmation is lacking [138, 143]. Therefore, detailed investigation onto the antibody reactivity towards NF-L and NF in CIS is mandatory.

Accurate assays are a prerequisite to measure the antibody response towards a particular antibody target in human samples. Non-human proteins used in a test should display a large degree of homology to the tested human antibody targets and the natural conformation of a protein should be preserved. For instance, different results were obtained by enzyme-linked immunosorbent assay (ELISA) and cell based assays used for detection of antibody reactivity towards myelin oligodendrocyte glycoprotein (MOG) [240], another antibody target in MS.

The aim of this study was to further explore the antibody response towards UH-CIS6 in serum from CIS patients and healthy controls (HC) by using a UH-CIS6 peptide ELISA. Furthermore, the antibody response towards MS antibody targets SPAG16, NF-L and NF was examined in the same cohort in order to compare antibody reactivity. Moreover, we analyzed putative correlations between clinical parameters and antibody reactivity as well as whether antibody reactivity could predict conversion to MS.

5.2 Materials and methods

5.2.1 Patient material

Serum samples from 92 CIS patients and 77 HC were collected to evaluate the antibody response towards UH-CIS6, SPAG16, NF-L and NF (Table 5.1). A subset of this HC cohort, consisting of 47 healthy subjects was used for the NF screening. CIS and MS diagnosis was established according to McDonald criteria [14, 15]. In general, CIS patients were clinically followed for 2 years. This study was approved by the ethics committee of Hasselt University.

Table 5.1: Characteristics of CIS patients and healthy subjects used for ELISA screening

Diagnosis	Gender (M/F)[a]	Mean age (years)± SD	Mean disease duration[b] (years)± SD	EDDS score[c] ± SD	IgG serum[d] (mg/dl)	IgG paired CSF[e] (mg/dl)	Unique OCB in CSF[f] (%)
CIS (n=92)	19/73	34.9±10.2	0.4±0.9	1.5±1.1	1096.1±200.4	3.8±2.8	52 (58%)
CIS-CIS (n=45)	8/37	37.9±11.3	0.5±1.2	1.3±1.2	1142.5±205.0	3.9±3.2	12 (28%)
CIS-MS (n=47)	11/36	32.1±8.2	0.3±0.5	1.7±1.0	1049.8±186.8	3.8±2.5	40 (87%)
HC (n=77)	27/50	39.3±15.9	NA[g]	NA	NA	NA	NA

[a] M: male, F: female
[b] Data available for 86 CIS patients (CIS-CIS n=42, CIS-MS n=44)
[c] EDDS: expanded disability disease scale. Data available for 71 CIS patients (CIS-CIS n=33, CIS-MS n=38)
[d,e] Data available for 82 CIS patients (CIS-CIS n=41, CIS-MS n=41)
[f] OCB: oligoclonal bands, information available for 89 CIS patients (CIS-CIS n=43 CIS-MS n=46)
[g] NA: not available/not applicable

5.2.2 Recombinant SPAG16 production

Recombinantly expressed full-length SPAG16 isoform 2 was produced as a fusion protein to thioredoxin using the pBAD/TOPO ThioFusion Expression Kit (Life Technologies, Merelbeke, Belgium) as described [306]. In brief, the cDNA sequence corresponding to human full length SPAG16 isoform 2 was cloned into the pBAD/TOPO ThioFusion vector and transformed into TOP10 *E.coli* bacteria. Next, the SPAG16 construct was transformed into LMG194 *E.coli* bacteria. LMG bacterial cells were grown to an OD_{600}=0,5 and recombinant SPAG16 expression was induced by the addition of 0,2% arabinose. Bacterial cells were lysed using a guanidium chloride containing lysis buffer (6 M guanidium chloride, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). Next, recombinant SPAG16 protein was purified with Ni-NTA sepharose beads (IBA, Leusden, the Netherlands). In addition, thioredoxin was produced by transformation of the empty pBAD/TOPOThioFusion vector into LMG194 bacteria. In this way, a compensation could be performed for aspecific reactivity.

5.2.3 ELISA assays to detect antibody reactivity towards SPAG16, NF-L, NF and UH-CIS6

Ninety-six well ELISA plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with protein in coating buffer (0.1 M sodium hydrogen carbonate pH 9.6). To this end, 1 µg/ml SPAG16/thioredoxin, 2.5 µg/ml bovine NF-L (Progen, Heidelberg, Germany) or 2.5 µg/ml rat NF (R&D systems, Abingdon, United Kingdom) was used. Plates were washed twice with PBS and blocked with skimmed milk powder in phosphate-buffered saline (MPBS) (2% (w/v) SPAG16, 5% NF-L and NF) for 2 hours at 37 °C while shaking. Next, plates were washed 3 times with 0.1% PBS Tween20 (PBST) and once with PBS. Serum samples were diluted 1:100 (SPAG16 and NF-L) or 1:200 (NF) in MPBS and 100 µl was added to each well (2 hours at room temperature (RT), shaking). Subsequently, plates were washed and 100 µl rabbit anti-human immunoglobulin G (IgG) horse radish peroxidase (HRP) labeled antibody (diluted 1:2,000, Dako, Heverlee, Belgium) was added (1 hour, RT, shaking). Washing steps were repeated and the colour reaction was started by the addition of 100 µl of 3,3′,5,5′ tetramethyl-benzidine dihydrochloride (TMB) solution (Perbioscience, Erembodegem, Belgium). Subsequently, the colour reaction was

stopped with 50 µl 2N H_2SO_4. Plates were read at 450 nm in a Tecan plate reader (Tecan, Männedorf, Switzerland).

A biotinylated synthetic peptide corresponding to UH-CIS6 was generated (GL Biochem, Shanghai, Belgium). Prior to coating of the UH-CIS6 peptide, 96-well ELISA plates coated with streptavidin (Nunc, Roskilde, Denmark) were washed 3 times with 0.05% PBST. Next, plates were coated with 0.5 µg/ml UH-CIS6 peptide (GL Biochem, Shanghai, China) in 0.05% PBST for 1 hour at RT while shaking. Subsequently, washing steps were repeated. Serum samples were diluted 1:100 and 100 µl was added to each well (2 hours at RT, shaking). The remaining steps were performed as described for the other ELISA assays.

5.2.4 Statistics

Graphpad prism 5 software (GraphPad software, La Jolla, USA) was applied for statistical analysis. The cut-off for a positive sample was set at 3 times the standard deviation (SD) above the mean OD signal obtained for HC samples. Mean antibody levels between CIS patients and healthy controls were compared with the Mann-Whitney test. To investigate differences in clinical parameters between CIS patients with and without conversion to MS, the Fisher's exact test was used. Correlations between antibody reactivity of the tested antibody targets was investigated by a Spearman's rank test. The IgG index was calculated by dividing the quotient of CSF IgG and serum IgG by the quotient of CSF albumin and serum albumin. An IgG index >0.7 was considered elevated and indicative of antibody production.

In addition, SAS 9.2 (SAS Institute Inc, Cary, USA) was applied to study putative correlations between antibody reactivity and clinical parameters. Correlations between antibody reactivity towards the tested antibody targets in CIS patients and their clinical parameters were measured by an univariate statistical model. In addition, linear mixed models were built to study the relationship between combined results for the antibody targets and clinical parameters. Whether antibody reactivity towards the tested antibody targets predicted conversion to MS was investigated via multiple logistic regression models. A p-value smaller than 0.05 was considered significant.

5.3 Results

5.3.1 The serum antibody response towards SPAG16, UH-CIS6, NF-L and NF in CIS

The antibody response towards UH-CIS6, SPAG16, NF-L and NF was investigated in serum from 92 CIS patients and 77 HC (47 HC were included in the NF screening). Fourteen CIS patients tested positive for UH-CIS6 (15%), 12 patients for SPAG16 (13%), 6 patients for NF-L (7%) and 7 patients for NF (8%). A specificity of 90-95% was obtained (UH-CIS6:90%, SPAG16:95%, NF-L: 94% and NF: 95%). The sensitivity could be further increased to 26% by combining UH-CIS6 and SPAG16, while a specificity of 84% was maintained.
Although not significant, a trend for an increase in mean anti-UH-CIS6 antibody levels was present in CIS patients as compared with healthy subjects (p=0.08). Although a subset of CIS patients clearly demonstrated increased antibody reactivity towards SPAG16, mean anti-SPAG16 antibody levels did not differ between CIS patients and controls (p=0.56). For NF-L and NF, antibody levels were similar among CIS patients and healthy controls (p=0.87 and p=0.93).

As shown in Table 5.2 , no correlation between antibody reactivity towards the tested antibody targets was detected. Furthermore, individual CIS patients and healthy subjects displayed only occasionally antibody reactivity towards multiple antibody targets.

Table 5.2: Correlation between antibody reactivity towards SPAG16, UH-CIS6, NF-L and NF in CIS patients

	SPAG16	UH-CIS6	NF-L	NF
SPAG16	-	0.67	0.57	0.19
UH-CIS6	0.67[a]	-	0.78	0.21
NF-L	0.57	0.78	-	0.21
NF	0.19	0.21	0.21	-

[a]Displayed values represent p-values of the Spearman's rank test

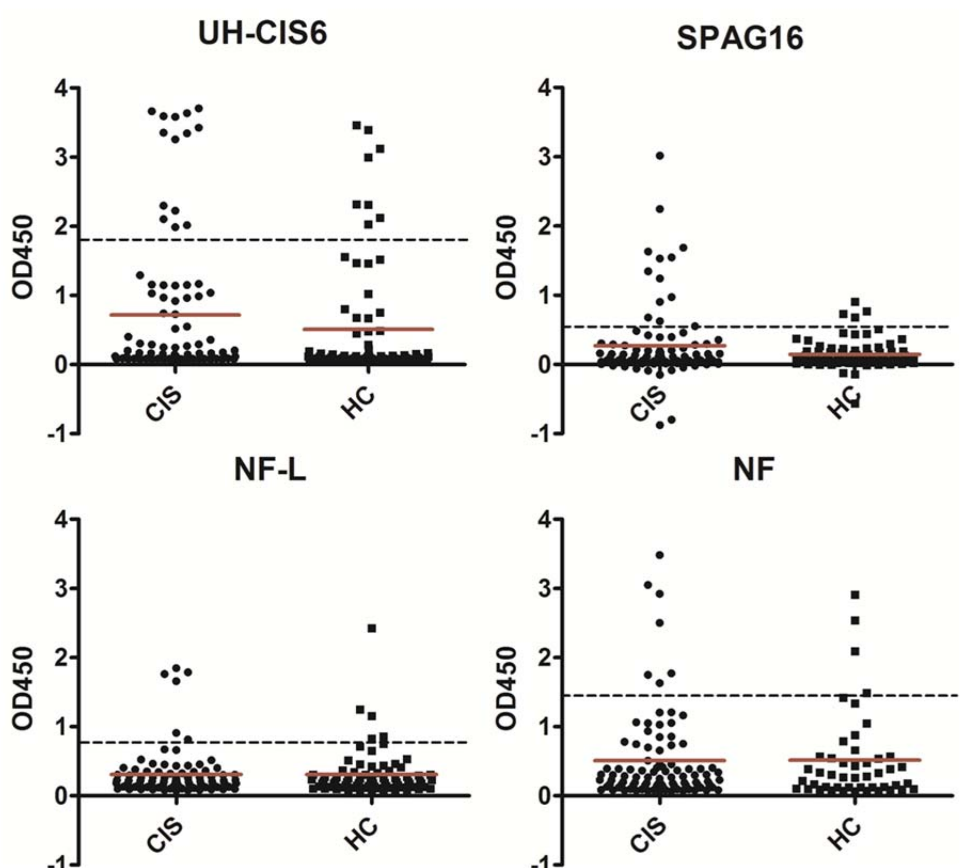

Figure 5.1: Serum antibody reactivity towards candidate autoantibody targets UH-CIS6, SPAG16, NF-L and NF in CIS patients and HC. The cut-off for a positive sample was set at 3 times the standard deviation (SD) above the mean OD signal obtained for HC samples (dash line). Displayed OD450 values for SPAG16 are mean OD450 SPAG16 signal – OD450 Thio signals, while for UH-CIS6, NF-L and NF mean OD450 values are shown. Red lines indicate mean OD450 value for a given group. Samples were excluded for analysis when the intra-assay coefficient of variation (CV) was >20% for duplicates.

5.3.2 Correlations between antibody reactivity towards UH-CIS6, SPAG16, NF-L and NF and clinical parameters

To investigate whether a correlation was present between antibody reactivity towards individual antibody targets and clinical parameters, statistical models were built. Anti-UH-CIS6 antibody reactivity correlated with an increase in IgG levels of paired CSF samples (p=0.01), which may imply an intrathecal antibody production in the CSF. Data on the IgG index were available for 4 CIS patients.

One of these CIS patients tested positive for antibodies towards UH-CIS6 and displayed an elevated IgG index of 2.1, which supports the presence of intrathecal antibody production. However, it should be kept in mind that this calculation is based on the total IgG repertoire.

Despite the observation that a group of CIS patients clearly displayed increased antibody reactivity towards SPAG16, no correlation was found between antibody reactivity and any of the tested clinical parameters (gender, age, disease duration, expanded disability disease scale score, serum IgG levels, paired CSF IgG levels and the presence of unique CSF OCB). In addition, correlations were absent for clinical parameters and antibody reactivity towards NF-L, NF or combined results for the 4 antibody targets.

Since there is an ongoing need to identify markers that can predict progression to MS, we investigated whether antibody reactivity towards the tested antibody targets correlated with MS development in CIS patients. Clinical follow-up information revealed that 47/92 (51%) of the CIS patients developed MS. The percentage of CSF samples with unique OCB was significantly increased in the CIS group with conversion to MS as compared with the CIS patients without disease progression ($p<0.0001$, Fisher's exact test) (Table 1). However, no association between conversion to MS and antibody reactivity towards UH-CIS6, SPAG16, NF-L and/or NF was detected.

5.4 Discussion

In this study, we investigated the antibody response towards UH-CIS6 in serum from CIS patients and compared this with the antibody response towards MS autoantibody targets SPAG16, NF-L and NF. For UH-CIS6 a sensitivity of 15% and a sensitivity of 90% was obtained. Thirteen % of the CIS patients displayed antibody reactivity towards SPAG16 (specificity 95%), 7% towards NF-L (specificity 94%) and 8% towards NF (specificity 95%). Furthermore, although not significant, a trend for an increase in mean anti-UH-CIS6 antibody levels was observed in CIS patients as compared with healthy controls. Moreover, the anti-UH-CIS6 antibody response in serum correlated with an increase in IgG of paired CSF samples.

A trend for increased antibody levels towards UH-CIS6 was observed in serum from CIS patients, which is consistent with previous results [283]. Elevated anti-UH-CIS6 antibody levels correlated with increased IgG levels in paired CSF samples, which suggests a local CSF production of anti-UH-CIS6 antibodies in CSF. This is further supported by our previous study described in chapter 4 in which we demonstrated increased anti-UH-CIS6 antibody levels in CSF from CIS and RR-MS patients. However in the future, the antibody index should be calculated to further investigate whether anti-UH-CIS6 antibodies are produced intrathecally. A local antibody response in the CSF has also been demonstrated for other antibody targets in CIS and MS, such as the Epstein-Barr virus [160] and the human herpesvirus 6 [307].

Increased antibody reactivity towards SPAG16 could be demonstrated in a subset of serum from CIS patients, which is in line with previous finding on the SPAG16 antibody response in serum from MS patients [191]. Even though clearly elevated antibody levels were present in a subgroup of CIS patients, a correlation between clinical parameters and the SPAG16 antibody response was absent. Two isoforms of SPAG16 have been described and experimentally validated; isoform 2 represents a shorter variant of isoform 1. SPAG16 isoform 2 was initially identified as a candidate antibody target for MS, but antibody reactivity to SPAG16 isoform 1 could also be confirmed (unpublished

observations). Little is known about the function of SPAG16 isoform 2, while SPAG16 isoform 1 has been demonstrated to be an essential component of the axoneme, a microtubular complex, which can be found in cilia and flagella and is involved fertility and spermatogenesis [308, 309]. The role of SPAG16 in MS is currently investigated, but it can be hypothesized that SPAG16 is part of the microtubule system in cells present in brain. Moreover, other cytoskeletal components such as neurofilament proteins and tubulin have already been recognized as antibody targets in MS [146, 147, 150].

The serum antibody response towards NF-L was similar among CIS patients and healthy controls. These results are in agreement with findings described by Bartos and colleagues [143]. Antibody reactivity towards NF-L in CSF may be the consequence of an increase in NF-L protein levels in CSF, which is suggested to be the result of axonal damage. NF-L antibody reactivity may be restricted to the CSF compartment and only be additionally present in serum when a certain threshold of axonal damage is reached. Indeed, elevated anti-NF-L antibody levels in serum have been reported in patients with progressive MS [145], which has been associated with neurodegeneration. However, other researchers were not able to replicate these findings [146]. Importantly, anti-NF-L antibody reactivity is not restricted to CIS and MS and has been reported in amyotrophic lateral sclerosis and stroke [119, 310]. Similar to NF-L, serum antibody reactivity towards NF did not discriminate between CIS patients and HC. In addition, other reports demonstrated elevated anti-NF antibody levels in serum from patients with Guillain-Barré syndrome and other peripheral neuropathies [118, 311].

Reliable and accurate assays are required to determine the antibody reactivity towards a particular antibody target. Compared with our previous results, which were based on the use of a UH-CIS6 phage ELISA assay, antibody reactivity towards UH-CIS6 was observed more frequently in serum from healthy controls [283]. This might be attributable to the improved sensitivity of the UH-CIS6 peptide ELISA (chapter 4) as well as to the inclusion of additional healthy controls that were not tested in the UH-CIS6 phage ELISA screening. In this study, we used rat NF protein to study the antibody response towards NF [138, 311]. Although this rat NF is highly similar to human NF, Ng and colleagues recently reported that

this rat NF protein may not be suitable to detect antibody reactivity towards NF [118]. They demonstrated that antibody reactivity was directed to antigens from the myeloma cell line in which the rat NF was produced rather than to the rat NF protein itself [118].

Antibody reactivity towards the tested antibody targets did not correlate with each other and antibody reactivity towards multiple antibody targets was observed occiasionally. This may represent the heterogeneity of CIS, which is also reflected by the wide spectrum of clinical presentations and affected central nervous areas [4].

Serum antibody reactivity towards UHCIS6, SPAG16, NF-L and NF did not predict conversion to MS in CIS patients. This is in contrast with our previous findings in which we observed a trend for increased UH-CIS6 antibody levels in serum from CIS patients who converted to MS as compared with CIS patients without progression. However, as described in chapter 4 and other publications, anti-UH-CIS6 and anti-NF-L antibody reactivity in CSF may have prognostic potential [143, 283]. For this reason, it may be worthwhile to further investigate the antibody response towards the tested antibody targets in CSF samples from CIS patients. Furthermore, 28% of the CIS patients without current conversion to MS included in this study displayed unique OCB in CSF, which has been recognized as a risk factor for conversion to MS [222]. A longer period of clinical follow up may therefore improve the prognostic potential of the tested antibody targets in serum. On the other hand, antibody reactivity towards the tested antibody targets may represent general (neuro)inflammatory processes or axonal damage which may clarify why increased antibody levels are observed in CIS patients irrespective of conversion to MS.

In summary, UH-CIS6 and SPAG16 may represent candidate serum autoantibody targets for CIS. In the future, more CIS patients should be tested to validate UH-CIS6 and SPAG16 as autoantibody targets in CIS. Moreover, studies should be performed to elucidate the role of the antibody response towards these targets in CIS.

6

Summary, discussion and future perspectives

Summary

An important role of B cells and antibodies has been recognized in clinically isolated syndrome (CIS) and multiple sclerosis (MS). For instance, treatment with the B cell depleting antibody Rituximab has been shown to reduce inflammatory lesions and clinical relapses in relapsing-remitting (RR) MS [92]. In addition, oligoclonal band (OCB) antibodies have been detected in CSF samples from the majority of CIS and MS patients [266]. However, the targets of the antibody response in CIS and MS are largely unknown. Identification of antibody targets for CIS and MS may help to establish a rapid diagnosis and allows early initiation of therapy. In addition, this may provide additional information on pathogenic mechanisms involved in CIS and MS.

In this thesis, we aimed to identify novel candidate antibody targets for CIS and MS by using serological antigen selection (SAS), a powerful high-throughput screening technology based on phage display. One of the identified candidate targets, UH-CIS6, was further characterized and we aimed to identify the *in vivo* antibody target for UH-CIS6. Moreover, we examined the antibody response towards UH-CIS6 in serum from CIS patients and compared this with 3 known MS antibody targets, which were analyzed in the same cohort. In this chapter, the main results of this study are described and discussed.

Aim 1: Identification of novel antibody targets for CIS

Several studies have been performed in which the antibody response in CIS is investigated. However, these studies were mainly restricted to known MS antibody targets. Therefore, we aimed to identify novel CIS antigens by using the SAS technology (**chapter 2**). In addition, we investigated whether the antibody repertoire in CIS was primarily directed to normal or MS brain antibody targets. Furthermore, we examined whether antibody reactivity towards the identified targets had prognostic potential to predict conversion to MS in CIS patients.

A MS cDNA phage display library was already available in our institute, however a normal brain cDNA phage display library was lacking. Therefore, we also constructed a normal white matter (NWM) cDNA phage display library derived

from healthy brain. SAS procedures were performed in parallel with NWM and MS cDNA phage display libraries on pooled cerebrospinal fluid (CSF) from 4 CIS patients, who developed MS within 2 years after lumbar puncture. This led to the identification of a panel of 6 novel antibody targets, UH-CIS1 to 6, which were all derived from the NWM library. Interestingly, all candidate clones were shown to represent novel antibody targets for CIS and MS. For example, UH-CIS2 encoded part of host cell factor c1 regulator 1, a protein involved in herpes simplex virus infection [274], whereas UH-CIS6 displayed homology to several proteins including bestrophin 1, peroxisomal biogenesis factor 6 and ring finger protein 157. Antibody reactivity towards UH-CIS1 to 6 could be confirmed in CSF and serum from at least 1 CIS patient included in the SAS procedures, by using a phage enzyme-linked immunosorbent assay (ELISA). Furthermore, antibody reactivity towards this panel was identified in CSF samples from additional CIS patients not used for SAS as well as in CSF from RR-MS patients. The antibody response towards UH-CIS3, UH-CIS4 and UH-CIS6 was also evaluated in (paired) serum samples. Antibody reactivity towards these targets was detected in a significantly increased proportion of CIS and RR-MS patients as compared with neurological and healthy controls ($p=0.03$). Interestingly, antibody reactivity towards UH-CIS3, UH-CIS4 and UH-CIS6 in CSF could be confirmed in the majority of paired serum samples, which demonstrates an overlap in the antibody repertoire in CSF and serum. Since progression to MS is restricted to a subset of CIS patients, we also investigated the prognostic potential of our identified panel. Although not significant, an increased frequency of antibody reactivity was observed in CIS patients who converted to MS as compared with CIS patients without disease progression for 2 targets, UH-CIS4 and UH-CIS6. This implies that antibody reactivity towards these targets may serve as a prognostic marker.

Aim 2: Investigation and comparison of the antibody response in CSF from MS subtypes

MS is characterized by several subtypes: 80% of the MS patients display a disease course reflected by disease exacerbations and periods of remission (RR), while in 20% of the MS patients disease is progressive (PP) from onset. These

MS subtypes may be characterized by different antibody repertoires. Therefore, we aimed to identify novel antibody targets for RR- and PP-MS and to compare the antibody repertoire in these MS subtypes (**chapter 3**).

SAS procedures were performed with NWM and MS cDNA phage display libraries on pooled CSF from RR- (n=6) and PP-MS (n=6) patients. Characterization of output phage revealed the absence of common clones for RR- and PP-MS, which indicates the presence of distinct antibody repertoires in RR- and PP-MS. A panel of 9 novel candidate clones was selected for further investigation. Eight clones were derived from SAS procedures on CSF from RR-MS patients (UH-RRMS1 to 8), while 1 clone was selected from PP-MS selection procedures (UH-PPMS1). These clones displayed homology to proteins, which had not yet been associated with antibody reactivity in MS. For example, UH-RRMS6 showed homology to the putative protein hCG2045783. In addition, UH-RRMS4 displayed homology to general transcription factor IIH, polypeptide 4 (GTF2H4), a protein involved in nucleotide excision repair. Furthermore, a single nucleotide polymorphism (SNP) within intron 11 of GTF2H4 was found to be associated with MS susceptibility [284]. Interestingly, an association between autoantibody reactivity and a SNP within the autoantibody target SPAG16 has been described in rheumatoid arthritis [312]. Antibody reactivity towards the panel could be confirmed in serum and CSF from patients used for SAS. For UH-RRMS7, antibody reactivity could be identified in CSF samples in 4 additional MS patients (8%) and 1 neurological control (3%). In conclusion, a panel of 9 novel antibody targets for MS was identified. Moreover, this study provides an initial indication for the existence of distinct antibody repertoires in RR- and PP-MS.

Aim 3: Characterization of a novel candidate antibody target for CIS and MS

A drawback of phage display is the identification of mimotopes [249]. To decrease the identification of these mimotopes, we used cDNA phage display libraries in the SAS procedures. However, a number of identified CIS candidate antibody targets in **chapter 2** still represent a mimotope. UH-CIS6 is an example of such a target. By using phage ELISA, antibody reactivity towards UH-CIS6 was

identified in CSF from CIS and MS patients as well as in serum from a significantly increased proportion of CIS and RR-MS patients as compared with controls. Therefore, we aimed to further characterize UH-CIS6 **(chapter 4)**.

First we developed a UH-CIS6 peptide ELISA to further study the anti-UH-CIS6 antibody response. This UH-CIS6 peptide ELISA was shown to have an increased sensitivity as compared with the phage ELISA since more anti-UH-CIS6 antibody positive CSF samples were identified when the UH-CIS6 peptide ELISA was used. Next, we investigated the antibody response towards UH-CIS6 in CSF from patients with CIS, MS and other (inflammatory) neurological diseases. The frequency of antibody reactivity towards UH-CIS6 was significantly increased in CIS and RR-MS as compared to neurological controls ($p=0.046$). Moreover, antibody reactivity towards UH-CIS6 was restricted to CIS patients who converted to MS, which further confirms the prognostic potential of UH-CIS6. A second aim of this study was to identify the *in vivo* antibody target of UH-CIS6. We used a polyclonal rabbit anti-UH-CIS6 antibody and we demonstrated that this antibody bound the same epitope as human anti-UH-CIS6 antibodies. Next, the rabbit anti-UH-CIS6 antibody was used for immunoprecipitation on a human brain lysate. Analysis of the immunoprecipitation output revealed coronin-1a as a candidate target for anti-UH-CIS6 antibodies. Moreover, binding of rabbit and human anti-UH-CIS6 antibodies to coronin-1a could be confirmed. In addition, coronin-1a expressing T cells and macrophages were identified inside a MS brain lesion. In summary, we identified in this study coronin-1a as a novel candidate antibody target for CIS and MS.

Aim 4: Analysis of the antibody response towards MS antibody targets in CIS

In a previous study (**chapter 2**), we identified UH-CIS6 as a novel candidate antibody target for CIS and RR-MS and we successfully developed a sensitive UH-CIS6 peptide ELISA to detect antibody reactivity towards UH-CIS6 (**chapter 4**). Apart from the antibody response towards UH-CIS6 in CSF, we were also interested to analyze anti-UH-CIS6 antibody reactivity in serum from CIS patients. Since CIS can represent a putative first presentation of MS it is

conceivable that the antibody response in CIS and MS is directed to a similar antigen repertoire. Indeed, for several antibody targets, such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and the potassium channel KIR 4.1, antibody reactivity has been demonstrated in both CIS and MS (total overview shown in Table 1.1). However, for several antibody targets in MS the antibody response in CIS is unknown. Examples of such antibody targets are sperm associated antigen 16 (SPAG16), neurofilament-light (NF-L) and neurofascin (NF). Therefore, we investigated in **chapter 5** the antibody response towards MS antibody targets SPAG16, NF-L and NF in serum from CIS patients and compared this with the antibody response directed to UH-CIS6.

We measured the antibody response towards the panel of 4 antibody targets in serum from CIS patients and healthy subjects. For UH-CIS6 a sensitivity of 15% and a sensitivity of 90% was obtained. Furthermore, although not significant, a trend for an increase in mean antibody levels towards UH-CIS6 was observed in CIS patients as compared with HC (p=0.08). Moreover, anti-UH-CIS6 antibody levels correlated with an increase in immunoglobulin G (IgG) concentration of paired CSF (p=0.012), which may point to the production of anti-UH-CIS6 antibodies in CSF. Thirteen % of the CIS patients displayed antibody reactivity towards SPAG16 (specificity 95%), 7% towards NF-L (specificity 94%) and 8% towards NF (specificity 95%). In addition, antibody reactivity towards multiple antibody targets was observed only occasionally in CIS patients. The findings described in this study indicate that UH-CIS6 and SPAG16 are candidate antibody targets in CIS.

General discussion and future perspectives

Are antibody targets in CIS and MS expressed in normal or in MS brain?

In **chapter 2** and **3**, SAS procedures were performed with 2 cDNA libraries, derived from normal white matter (NWM) from healthy brain and MS brain, respectively. In this way, we aimed to examine whether antibody targets in CIS and MS are primarily expressed in diseased or normal brain tissue. The MS cDNA library was composed of active chronic plaques with varying degrees of demyelination and inflammatory activity and was derived from a patient with an 11 year history of MS that was progressive from onset [264]. In a previous study, Becker et *al*. performed a sequence analysis on the MS cDNA library and showed that the MS cDNA library used in this study contained unique transcripts involved in immune activation and expression of genes encoding autoantigens, which were absent in 2 normal brain cDNA libraries [264]. Therefore, normal and MS brain contain distinct antigen repertoires.

In **chapter 2**, we investigated the antibody repertoire in CSF from CIS patients. Selected CIS candidate antibody targets were all derived from the NWM cDNA library. This may be due to the relative short disease duration of these CIS patients, and it is likely that the antibody repertoire in these patients is directed to antigens involved in early disease, which may be primarily expressed in normal brain and are not present in MS brain from long established disease as was used for the construction of the MS cDNA library. In addition, 7 out of 8 antibody targets for RR-MS that were identified in **chapter 3** were also derived from the NWM library. Although established disease was present in these RR-MS patients, the average disease duration was still relatively short (average 2.7 years). Moreover, the expanded disability status scale (EDSS) score of the RR-MS patients included in the SAS procedures was also rather low (average EDSS score 3.1). Therefore, it may be hypothesized that in patients with a longer disease duration, the antibody repertoire may also be directed to antigens whose expression is restricted to MS brain. Although we performed SAS procedures with CSF samples from PP-MS patients with longer disease (average 5.4 years), only 1 PP-MS candidate antibody target was identified. This may be clarified by the underlying disease mechanisms in PP-MS. Less inflammation has

been observed in PP-MS as compared with RR-MS and neuro-axonal degeneration has been suggested to underlie PP-MS [282]. Therefore, these findings may point to a less prominent role of the antibody response in PP-MS. In order to further investigate whether the antibody repertoire changes during the disease course, SAS procedures may be performed in the future with CSF samples from RR-MS patients with long established disease.

Which proteins are targeted by the antibody reponse in CIS and MS?

In this study, several novel antibody targets for CIS and MS were identified. For example, host cell factor c1 regulator 1, (HCFCR1) a protein involved in herpes simplex virus infection was identified [274]. Furthermore, the herpes simplex virus has already been recognized as an antibody target in MS [66]. Although we used cDNA libraries in the SAS procedures, a number of identified candidate clones were not expressed in the correct reading frame or were derived from 3' untranslated regions (UTR). Interestingly, homology analysis revealed that several of these clones displayed homology to proteins that were associated with MS. For example, UH-CIS4 showed similarity to the sodium channel Nav1.5, whose expression has been shown to be upregulated in reactive astrocytes in MS lesions [275]. In addition, UH-CIS4 displayed homology to poly (ADP-ribose) polymerase 1, which has been suggested as a therapeutic target to suppress neuroinflammation in MS [276]. Another candidate target, UH-RRMS4 was similar to general transcription factor IIH, polypeptide 4 (GTF2H4), a protein involved in nucleotide excision repair, and a SNP within intron 11 of GTF2H4 was found to be associated with MS susceptibility [284]. Importantly, although these proteins were already associated with MS, antibody reactivity towards these targets has not been investigated and thus reported in CIS or MS. Antibodies directed to these proteins may exert several functions. For instance, antibodies may have pathogenic properties. For instance, anti-Nav1.5 antibodies may be pathogenic by blocking its proposed compensatory mechanism in MS lesions [275] and anti-GTF2H4 antibodies may inhibit the DNA repair function of GTF2H4. On the other hand, antibodies might also have protective roles as antibodies towards HCFCR1 may indirectly inhibit viral infection.

A subset of the clones encoding non-coding reading frames may represent alternative spliced forms that have not yet been experimentally described. Interestingly, alternative splicing may lead to the generation of untolerized epitopes involved in autoimmunity [279]. Moreover, alternative splicing has been implicated in genes associated with MS, including several known autoantigens, such as MBP and MOG [280]. In addition, alternative splicing has been implicated in other autoimmune diseases [280, 313].

Are the antibody targets different in CIS, RR-MS and PP-MS?

For all antibody targets identified in **chapter 2**, UH-CIS1 to 6, antibody reactivity could be identified in both CIS and RR-MS patients. These findings point to similar antibody reactivity in CIS and RR-MS and this is in line with other known antibody targets such as MOG, MBP and the Epstein-Barr virus (summarized in Table 1.1). Interestingly, antibody reactivity towards UH-CIS1 to 6 was absent in CSF from patients with secondary-progressive (SP) MS, which may be due to a less prominent role of the inflammatory response in the progressive phase of MS. On the other hand, antibody reactivity towards UH-RRMS7 was detected in CSF samples from both RR- and PP-MS patients (**chapter 3**). In general, antibody reactivity towards a given target is observed in all MS subtypes. However, for several antibody targets, such as galactocerebroside [155], ganglioside GM3 [152] and NF-L [144, 145], antibody reactivity is primarily observed in either RR- or PP-MS. It should be noted that a relatively low number of progressive MS patients were included in **chapter 2** and **chapter 3**. Therefore, in order to investigate whether antibody reactivity towards UH-CIS1 to 6 and UH-RRMS7 is restricted or not to CIS and/or certain MS subtypes, the number of PP and SP-MS patients should be expanded in the future.

Which assays are suitable to investigate the antibody response?

Initially, we measured antibody reactivity using phage particles displaying the cDNA products of interest. In **chapter 4**, we describe the optimization of a UH-CIS6 peptide ELISA. Moreover, we demonstrated that anti-UH-CIS6 antibodies bound purified coronin-1a, produced in human embryonic kidney (HEK) cells, under reducing (Western blot) and native conditions (ELISA). In this case, linear and conformational epitopes display a large similarity. However, it should be

kept in mind that this is not always the case and that different antigen preparations and assay types may lead to contradictory results. This may be of particular importance for transmembrane proteins such as MOG. Initially, the presence of antibodies towards MOG was identified in a large proportion of CIS and MS patients [117]. However, in these studies antibody reactivity was measured towards incorrectly folded or denatured MOG. On the other hand, in subsequent studies, cell based assays in which antibody reactivity was measured towards native MOG were used and revealed the presence of anti-MOG antibody reactivity in a minor subset of MS patients [117]. In **chapter 5**, we investigated the antibody response towards NF in serum from CIS patients. We used recombinant expressed NF that was successfully used in other studies [138, 311]. Serum antibody levels towards NF were similar in CIS and HC. However, recently, Ng et *al*. stated that the recombinant NF used in our study is not suitable to measure anti-NF antibody [118]. Instead of measuring antibody reactivity towards NF, antibody reactivity was directed to antigens from the myeloma cell line in which the rat NF was produced rather than to the rat NF protein itself. Therefore, additional studies are mandatory to further investigate the antibody response towards NF in CIS.

Do patients display antibody reactivity towards multiple targets ?

To further investigate whether patients displayed antibody reactivity towards multiple antibody targets, we examined the presence of putative correlations in serum antibody reactivity towards SPAG16, UH-CIS6 (peptide ELISA), NF-L, NF, UH-CIS3, UH-CIS4 and UH-CIS6 (phage ELISA) in a cohort of 92 CIS patients (Table 6.1). A significant correlation ($p<0.01$) was detected for antibody reactivity towards UH-CIS4 and UH-CIS6 (phage ($r_s=0.36$) and peptide ELISA ($r_s=0.29$)). Interestingly as shown in Figure 6.1, similar to UH-CIS6, a shared protein motif could be identified for UH-CIS4 and coronin-1a (http://blast.ncbi.nlm.nih.gov/). This may explain the correlation of antibody reactivity towards UH-CIS4 and UH-CIS6. Therefore, it would be relevant to test whether anti-UH-CIS4 antibodies are also able to bind coronin-1a. Moreover, UH-CIS6 peptide and UH-CIS6 phage ELISA results were shown to correlate with each other ($p<0.0001$; $r_s=0.57$). This finding further supports that the UH-CIS6 peptide ELISA is a well suited alternative for the UH-CIS6 phage ELISA.

Additional correlations between antibody reactivity towards the tested CIS serum antibody targets were absent.

Table 6.1: Correlation between reactivity of the tested CIS serum antibody targets in a cohort of CIS patients

	SPAG16	UH-CIS6[a]	NF-L	NF	UH-CIS3	UH-CIS4	UH-CIS6[b]
SPAG16	-	0.67	0.57	0.19	0.11	0.72	0.51
UH-CIS6[a]	0.67[c]	-	0.78	0.21	0.42	**$p<0.01$**	**$p<0.0001$**
NF-L	0.57	0.78	-	0.21	0.28	0.74	0.54
NF	0.19	0.21	0.21	-	0.28	0.58	0.57
UH-CIS3	0.11	0.42	0.28	0.28	-	0.62	0.05
UH-CIS4	0.72	**$p<0.01$**	0.74	0.58	0.62	-	**$p<0.01$**
UH-CIS6[b]	0.51	**$p<0.0001$**	0.54	0.57	0.05	**$p<0.01$**	-

[a] UH-CIS6 peptide ELISA
[b] UH-CIS6 phage ELISA
[c] Displayed values represent p-values of the Spearman's rank test

UH-CIS4

LLW**A**S**W**RQS**HL**AT**P**FTPTCGVASDGADTQGGTGLSLGIKGTESAHTLSVTKEGTAGPV

Coronin-1a

MSRQVVRSSKFRHVFGQPAKADQCYEDVRVSQTTWDSGFCAVNPKFVALICEASGGGAFL
VLPLGKTGRV DKNAPTVCGHTAPVLDIAWCPHNDNVIASGSEDCTVMVWEIPDGGLMLPL
REPVVTLEGHTKRVGIVAWHTTAQNVLLSAGCDNVIMVWDVGTGAAMLTLGPEVHPDTIYS
VDWSRDGGLICTSCRDKRVRIIEPRKGTVVAEKDRPHEGTRPVRAVFVSEGKILTTGFSRM
SERQV**A**L**W**DTK**HL**EE**P**LSLQELDTSSGVLLPFFDPDTNIVYLCGKGDSSIRYFEITSEAPFLH
YLSMFSSKESQRGMGYMPKRGLEVNKCEIARFYKLHERRCEPIAMTVPRKSDLFQEDLYPP
TAGPDPALTAEEWLGGRDAGPLLISLKDGYVPPKSRELRVNRGLDTGRRRAAPEASGTPSS
DAVSRLEEEMRKLQATVQELQKRLDRLEETVQAK

Figure 6.1: Putative shared epitope of UH-CIS4 and coronin-1a. Alignment of UH-CIS4 and coronin-1a protein sequences (Swiss prot accession number P31146) led to the identification of a shared protein motif (indicated in bold).

Is the antibody response towards our targets different in CSF and serum?

The central nervous system (CNS) is affected in CIS and MS and therefore it is plausible that disease relevant antibodies are present in the CSF, which is in close proximity of the CNS. However, a lumbar puncture is an invasive procedure and serum would be an easily available alternative. Therefore, we also investigated whether antibody reactivity towards antibody targets initially identified in CSF was also present in (paired) serum from CIS and MS patients (**chapter 2** and **chapter 3**). Antibody reactivity towards our CSF antibody targets could be confirmed in the majority of paired serum samples, which points to the existence of similar antibody repertoires in CSF and serum. This is further supported by a study performed by Larman et al [314], in which an unbiased proteomic technology, "phage immunoprecipitation sequencing", was used to investigate the antibody repertoire in matched serum and CSF samples from MS patients. Indeed, similar to our findings, the majority of enriched peptides was found to be recognized by antibodies present in CSF and serum. In addition, an exchange of IgG expressing B cells between the CNS and peripheral blood has been demonstrated [315]. Moreover, the SAS procedure has been used to study the antibody response in paired CSF and serum from a single RR-MS patient [265]. This revealed the presence of common, but also to distinct antibody profiles in paired CSF and serum. The presence of distinct antibody repertoires in paired CSF and serum samples has also been reported by Quintana et al, [242]. Interestingly, the authors reported that the antibody response in a CSF sample was more similar to that of other CSF samples than to a paired serum sample from the same individual. However, in the study of Quintana *et al.*, analysis of antibody reactivity was restricted to a set of known (auto)antigens and the targets included in our study were not tested [242]. Therefore, antibody reactivity towards a subset of targets may be present in both CSF and serum, while other antibodies are restricted to the CSF compartment.

Are the antibodies towards the studied targets produced in CSF or in serum?

Antibodies in CIS and MS may be produced in CSF, since unique OCB not present in serum are found in CSF samples from CIS and MS patients. These

OCB were found to be produced by CSF B cells, by comparing the Ig proteomes of CSF samples and Ig transcriptomes of CSF B cells [114]. Moreover, by using the same experimental approach a strong overlap of the IgG repertoires in brain lesions and in CSF could be demonstrated [316]. It should be noted that the identity of the targets reacting with the OCB are (largely) unknown. Therefore, it remains elusive whether these antibodies are relevant in the pathogenesis of CIS and MS. Antibodies may be produced in the CSF and transported to the peripheral blood by passage through the venous sinuses. This may in particular explain the presence of antibodies towards brain proteins in serum. However, antibodies in CIS and MS patients may also be produced in the periphery, since an increased number of antibody producing cells has been demonstrated in the bone marrow and peripheral blood of MS patients as compared to healthy controls [273]. In the future, the antibody index may be used to investigate whether antibodies towards a particular target are intrathecally produced. In this formula, the quotient of the antibody response towards a target in CSF and serum is calculated, divided by the quotient of total CSF and serum immunoglobulin G (IgG) [13]. For several antibody targets in MS an intrathecal antibody production has been demonstrated [127, 160]. However, the absence of an intrathecal production does not automatically imply that a particular antibody target may not be relevant in CIS and MS. For instance, antibodies towards KIR4.1 were shown not to be intrathecally produced in the majority of MS patients [116]. Nevertheless, serum antibody reactivity towards KIR4.1 could be identified in 46.9% of CIS and MS patients, while anti-KIR4.1 antibodies where nearly absent in serum from healthy and neurological controls. In addition, injection of anti-KIR4.1 antibodies in cisternae magnae of mice led to a loss of KIR4.1 expression, altered expression of glial fibrillary acidic protein in astrocytes and activation of the complement cascade.

How can antibodies towards the identified targets be generated?

Proteins with an extracellular domain (MOG) or that are secreted (viral particles) are well accessible for antibodies. However, a subset of the targets identified in our study displayed homology to intracellularly expressed proteins, which may be at first sight less accessible for antibodies. Nevertheless, several mechanisms leading to antibody reactivity towards intracellular antigens have been proposed

[304]. First, dysregulation of the immune system may cause expansion of existing polyreactive natural antibodies. Secondly, defective clearance of apoptotic bodies, whose membranes may express intracellular antigens, may trigger an autoimmune disorder. In addition, epigenetic modifications and molecular mimicry causing cross-reactivity with foreign or extracellular proteins may induce antibody reactivity towards intracellular antigens. Importantly, it has been demonstrated that antibodies towards intracellular antigens can enter the cell by membrane Fc receptor mediated intracellular uptake [317], electrostatic interactions of arginine residues in the complementarity determining region of antibodies with negatively charged sulfated polysaccharides on the cell surface [318] or by endocytosis [319]. Of note, antibody reactivity towards intracellular targets, such as DNA [172], the proteasome [182] as well as enzymes triosephosphate isomerase (TPI) [176] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [176], has already been detected in CIS and/or MS.

Does antibody reactivity towards the investigated targets have prognostic potential to predict progression to MS in CIS patients?

Possible prognostic potential to predict conversion to MS in CIS patients could be demonstrated for 2 antibody targets, UH-CIS4 and UH-CIS6, that were identified in this study (**chapter 2** and **chapter 4**). A higher frequency of antibody reactivity towards these targets was observed in CIS patients who converted to MS, although antibody reactivity was also present in CIS patients who did not (yet) display progression to MS. Therefore, further studies are needed to investigate the prognostic potential of these antibody targets. To this end, the number of CIS patients should be increased. Although, we generally followed CIS patients for 2 years after lumbar puncture, it may take additional time to develop MS. Therefore, the length of clinical follow-up may also be expanded. In this way, it would also be possible to investigate whether an association exists between antibody reactivity towards UH-CIS4 and UH-CIS6 and time to progress to MS. Moreover, it may be interesting to investigate whether the prognostic potential of our antibody targets can be increased when other prognostic markers (shown in Table 1.2) are added.

Does antibody reactivity correlate with clinical parameters?

Associations between antibody reactivity and other clinical and demographical parameters were absent. However, the majority of the CIS and MS patients included in this study were in an early disease phase. Therefore, it may be difficult to detect an association between disease duration and antibody reactivity. Antibody reactivity towards the targets may be measured in CSF/serum samples from a single patient taken at different time points, to investigate whether the antibody response towards a target changes during the disease course. In this way, also correlations between antibody reactivity and disease activity, for instance relapse and remission, can be examined.

The majority of the patients included in our study were untreated. It may be of interest to investigate CIS and MS patients treated with different treatment strategies and investigate whether the antibody response is affected as compared with non-treated patients. Several studies have been reported which suggest an effect of treatment on the antibody response. For instance, treatment with the steroid methylprednisolone, which is used to treat MS exacerbations, led to a decrease in antibody reactivity, epitope spreading and intrathecal autoantibody synthesis [242]. However, the treated group consisted of a different cohort of MS patients as compared with the untreated group, which may hamper the comparison between treated and untreated patients. In another study, Natalizumab treatment has been shown to lead to a decrease in immunoglobulin M (IgM) and IgG levels in patients with MS [320]. A reduction in myelin-specific autoantibody titers was observed in RR-MS patients after treatment with a DNA vaccine encoding human myelin basic protein [321]. Moreover, plasma exchange led to a rapid reduction in demyelinating activity of IgG [192]. Therefore, treatment may indeed affect the antibody response in MS.

How can the pathogenicity of antibodies be studied?

In this study, we successfully identified novel antibody targets for CIS and MS (**chapter 2** and **3**). Antibody reactivity towards these targets may be used for diagnosis of CIS and MS, since antibody reactivity was increased in CIS and MS as compared with neurological and healthy controls. However, it is also of interest to investigate whether the antibody response directed towards these

targets can mediate pathogenic effects. To this end several experiments can be performed. First, antibodies may be injected in animals with experimental autoimmune encephalomyelitis (EAE), the animal model of MS. In this way, the effect of the antibodies on the disease course can be evaluated. In similar experiments, disease exacerbating effects have been observed for other MS antibody targets, such as NF [138] and SPAG16 [191]. Secondly, the pathogenic effect of antibodies can be studied *in vitro*. Elliott and colleagues [192] used a myelinating culture system and developed a sensitive and reproducible assay to detect and quantify the ability of patient-derived Ig to mediate demyelination and axonal injury in vitro. Using this assay, complement-dependent demyelinating IgG responses, occasionally accompanied by complement-dependent antibody mediated axonal loss, were observed in 30% of MS patients. Interestingly, pathogenic IgG antibody responses were absent in patients with other neurological diseases or in healthy controls.

What is the role of coronin-1a and the antibody response towards coronin-1a in CIS and MS?

UH-CIS6 is a CIS candidate antibody target identified in **chapter 2** and is an example of a mimotope. In **chapter 4** we were able to demonstrate that coronin-1a is the *in vivo* antibody target for anti-UH-CIS6 antibodies. Coronin-1a is a protein composed of 461 amino acids and contains 7 WD repeat domains. The WD domain has been implicated in a wide variety of cellular and biochemical functions, including a role in the cytoskeleton [322]. Indeed, coronin-1a may play a role in actin binding [323]. Coronin-1a is primarily expressed in hematopoietic cells and may function as an actin-regulatory protein, responsible for the remodeling of the cytoskeleton in response to external signals transmitted into leukocytes [287]. However, coronin-1a may also have actin-independent functions [288]. In T cells, coronin-1a is involved in survival [288], activation [324] and is required for chemokine-mediated migration [325]. In addition, in macrophages, coronin-1a may be involved in survival of mycobacteria within phagosomes [290] and may promote and accelerate phagocytosis [326]. On the other hand, coronin-1a has been shown to be a negative regulator of endosomal delivery, degradation of modified lipoprotein and cholesterol deposition in macrophages [292]. Finally, coronin-1a has been implicated in phagocytosis in neutrophils [293]. Coronin-1a may also be

of relevance in MS, since mice deficient for coronin-1a did not develop EAE, which implicates that coronin-1a is crucial for EAE induction [297]. However, after re-immunization of coronin-1a deficient mice, disease was even more severe as compared with wild-type mice and coronin-1a has been demonstrated to downregulate interleukin-17 and interferon-γ expression in Th17 cells [298]. The majority of described functions for coronin-1a suggest a role for coronin-1a in maintaining or even promoting the inflammatory response in CIS and MS. Alternatively, coronin-1a may have anti-inflammatory properties.

Known antibody targets in CIS and MS constitute proteins expressed in myelin, axons, oligodendrocytes, lipids, viruses or are expressed ubiquitously (Table 1.1). Interestingly, to our knowledge, antibody reactivity towards an antigen primarily expressed in immune cells has not been described yet in CIS and MS. Antibodies towards coronin-1a may be responsible for (1) the activation of coronin-1a expressing immune cells (Figure 6.1). This is supported by the function of anti-neutrophil cytoplasmic antibodies (ANCAs) directed towards antigens expressed in immune cells such as neutrophils and monocytes, which have been shown to recruit and activate neutrophils [303]. Interestingly, these ANCAs have been described in other autoimmune diseases such as vasculitis, rheumatoid arthritis and inflammatory bowel disease [300, 301, 302]. Alternatively, (2) it may be that the generation of an antibody response towards coronin-1a is the consequence of the infiltration of coronin-1a expressing immune cells in MS brain and may have putative protective functions by blocking this inflammatory response or (3) may represent an epiphenomenon (Figure 6.1).

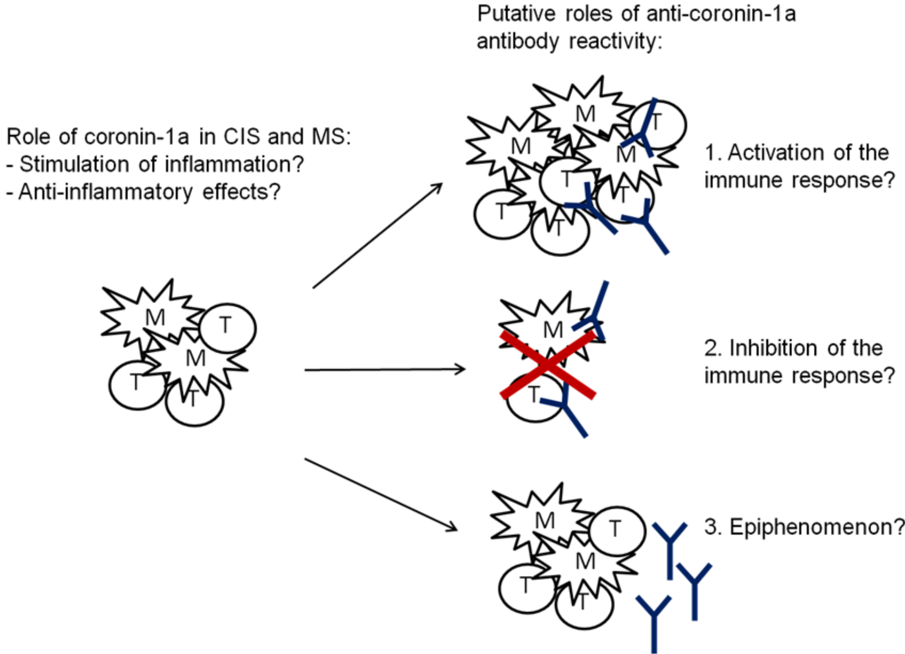

Figure 6.2: Role of coronin-1a and antibody reactivity towards coronin-1a in CIS and MS. Coronin-1a may stimulate or inhibit the immune response in CIS and MS. Anti-coronin-1a antibody reactivity may have several effects. First, the anti-coronin-1a antibody response may activate immune cells. Secondly, anti-coronin-1a antibodies may inhibit the immune response. Finally, the presence of antibodies directed towards coronin-1a may represent an epiphenomenon caused by the infiltration of immune cells expressing coronin-1a in brain of CIS and MS patients.

In the future, the frequency and specificity of the anti-coronin-1a antibody response should be further investigated. To this end, CSF and serum samples from patients with CIS, MS and other (inflammatory) neurological diseases as well as healthy controls should be tested for antibody reactivity towards coronin-1a. In addition, future studies should be aimed at the elucidation of the role of coronin-1a protein and the anti-coronin-1a antibody response in CIS and MS.

Final conclusions

In this thesis, we successfully identified novel antibody targets for CIS and MS by using the powerful SAS technology. For the majority of identified antibody targets, antibody reactivity could be detected in CSF and/or serum from a subset of tested CIS and MS patients, which indicates that the identified targets may represent common antibody targets in CIS and MS. Furthermore, we successfully identified coronin-1a as the corresponding *in vivo* antibody target for 1 of our identified targets, UH-CIS6. With the obtained results, future studies can be undertaken to further investigate the antibody response towards coronin-1a and other targets identified in this thesis and elucidate their role in the pathogenesis of CIS and MS.

7

Nederlandse samenvatting

Multiple sclerose (MS) is een chronische inflammatoire, neurodegeneratieve ziekte van het centrale zenuwstelsel. MS wordt gekarakteriseerd door demyelinisatie van axonen en het ontstaan van zogenaamde sclerotische plaques. Een breed scala aan symptomen wordt geassocieerd met MS, zoals moeheid, spierzwakte en cognitieve problemen [3]. De ziekte manifesteert zich op relatief jonge leeftijd (20-40 jaar) en vrouwen lopen een groter risico op het ontwikkelen van MS [2]. Het grootste gedeelte (80%) van de MS patiënten heeft een zogenaamde "relapsing-remitting" (RR) vorm, waarbij ziekte opstoten worden afgewisseld door perioden van herstel. Vijfenzestig % van deze RR-MS patiënten zal uiteindelijk in een secundair-progressieve (SP) fase terechtkomen, welke is gekenmerkt door een aanhoudend progressief verloop. In 20% van de MS-patiënten is de ziekte progressief vanaf het begin, dit wordt ook wel "primair-progressieve" (PP) MS genoemd [3].

In 85% procent van de MS-patiënten wordt de ziekte vooraf gegaan door een zogenaamd "clinically isolated syndrome" (CIS), een periode van neurologisch disfunctioneren die ten minste 24 uur moet aanhouden [4]. CIS-patiënten kunnen MS ontwikkelen door een tweede klinische opstoot of door het ontstaan van nieuwe laesies op een "magnetic resonance imaging" (MRI) scan. Belangrijk is dat slechts 30 - 70% van de CIS-patiënten uiteindelijk MS zal ontwikkelen en op dit moment is moeilijk te beoordelen welke CIS-patiënten tot de risicogroep behoren [7]. Om deze reden wordt momenteel veel onderzoek gedaan naar specifieke prognostische (laboratorium) merkers, die mogelijk kunnen bijdragen tot de opsporing van deze CIS-patiënten.

MS is een auto-immuun ziekte, waarbij het immuunsysteem ontregeld is en lichaamseigen eiwitten worden aangevallen. Onderdelen van dit immuunsysteem, B cellen en antilichamen, blijken een belangrijke rol in CIS en MS te spelen. Dit wordt ondermeer ondersteund door de positieve resultaten die werden verkregen wanneer MS-patiënten werden behandeld met het antilichaam Rituximab, dat er voor zorgt dat B-cellen worden uitgeschakeld [92]. Tevens worden er vaak zogenaamde oligoklonale band antilichamen waargenomen in cerebrospinaal vocht (CSV) van CIS- en MS-patiënten [266]. Echter de doelwitten van de antilichaamrespons in CIS en MS zijn grotendeels onbekend. De

identificatie van deze antilichaam targets kan mogelijk bijdragen aan een snelle diagnose en maakt vroege initiatie van behandeling mogelijk. Bovendien kan de identiteit van de antilichaam targets extra informatie geven over de pathogene mechanismen betrokken in CIS en MS.

Het doel van deze thesis was om nieuwe antilichaam targets te identificeren voor CIS en MS door gebruik te maken van serologische antigen selectie (SAS), een krachtige technologie gebaseerd op faag display. Eén van de geïdentificeerde kandidaat targets in deze thesis, UH-CIS6, werd tevens verder gekarakteriseerd. Ook probeerden we de identiteit van het *in vivo* antilichaam target voor UH-CIS6 te achterhalen. Verder onderzochten we de antilichaamrespons tegen UH-CIS6 in serum van CIS-patiënten en vergeleken dit met de antilichaamrespons tegen 3 bekende MS antilichaam targets.

Doelstelling 1: Identificatie van nieuwe antilichaam targets voor CIS

In het verleden werden meerdere studies uitgevoerd, die gericht waren op de analyse van de antilichaamrespons in CIS. Echter werd in deze studies met name de antilichaamrespons tegen bekende MS antigenen onderzocht. Daarentegen was het doel van onze studie om nieuwe CIS antigenen te identificeren door gebruik te maken van de SAS technologie (**hoofdstuk 2**). Daarnaast hebben we ook onderzocht of de antilichaam respons in CIS primair gericht is op eiwitten die tot expressie komen in normaal of in MS hersenweefsel. Ook onderzochten we of antilichaam reactiviteit tegen onze targets mogelijk prognostisch potentieel had om conversie naar MS in CIS patiënten te voorspellen.

Een MS cDNA faag display bibliotheek was al beschikbaar binnen ons instituut, maar dit gold niet voor een cDNA faag display bibliotheek gemaakt van normaal hersenweefsel. Om deze reden construeerden we een cDNA faag display bibliotheek van normale witte stof (NWS) afkomstig van gezond hersenweefsel. SAS procedures werden in parallel uitgevoerd met de NWS en MS cDNA faag display bibliotheken op gepoold CSV van 4 CIS-patiënten, die binnen 2 jaar na CSV afname MS ontwikkelden. Uiteindelijk werd een panel bestaande uit 6

nieuwe antilichaam targets (UH-CIS1 tot en met 6) geïdentificeerd. Deze targets waren allemaal afkomstig uit de selecties met de NWS bibliotheek. Dit kan mogelijk verklaard worden door het vroege ziektestadium waarin de CIS-patiënten verkeren. Een van deze targets, UH-CIS2 bleek te coderen voor een gedeelte van *host cell factor c1 regulator 1*, een eiwit betrokken bij de infectie van het herpes simplex virus [274]. Opmerkelijk is dat in het verleden antilichaam reactiviteit tegen het herpes simplex virus al werd aangetoond in MS [66]. Een ander kandidaat target, UH-CIS6, vertoonde overeenkomsten met meerdere eiwitten, waaronder isovormen van *bestrophin 1* en *peroxisomal biogenesis factor 6* die via alternatieve splicing waren gegenereerd. Alternatieve splicing werd reeds geassocieerd met MS en andere auto-immuunziekten en werd tevens gededecteerd in bekende autoantigenen zoals *myelin basic protein* (MBP) en *myelin oligodendrocyte glycoprotein* (MOG) [280].

Antilichaam reactiviteit tegen UH-CIS1 tot en met 6 kon worden bevestigd in CSV en serum van tenminste 1 van de 4 CIS-patiënten, die waren geïncludeerd in de SAS procedures. Bovendien konden we ook antilichaam reactiviteit tegen ons panel detecteren in CSV van andere CIS-patiënten en in RR-MS patiënten, wat er op wijst dat de antilichaam reactiviteit tegen deze targets vaker voorkomt in CIS en MS. Deze bevindingen zijn in overeenkomst met antilichaam reactiviteit tegen bekende antilichaam targets in CIS en MS (Tabel 1.1).

Omdat een lumbaal punctie een invasieve procedure is, onderzochten we ook of antilichaam reactiviteit kon worden gedetecteerd in (gepaarde) serum samples. Aangezien de combinatie van CSV antilichaam reactiviteit tegen UH-CIS3, UH-CIS4 en UH-CIS6 leidde tot een vergelijkbare sensitiviteit en specificiteit ten opzichte van het gehele panel, werden deze targets geselecteerd voor analyse van antilichaam reactiviteit in serum. Serum antilichaam reactiviteit tegen deze 3 targets werd in significant meer CIS en RR-MS patiënten waargenomen, wanneer dit werd vergeleken met neurologische en gezonde controles ($p=0.03$). Interessant is het feit dat antilichaam reactiviteit gericht tegen UH-CIS3, UH-CIS4 en UH-CIS6 in CSV kon worden bevestigd in het merendeel van de gepaarde serum samples, wat mogelijk kan duiden op een vergelijkbaar antilichaam repertoire in CSV en serum. Dit is in overeenkomst met bestaande studies, echter in een klein aantal publicaties werd gesuggereerd dat het

antilichaam repertoire verschillend is in CSV en serum. Toekomstige studies moeten uitwijzen of antilichamen tegen UH-CIS3, UH-CIS4 en UH-CIS6 worden geproduceerd in het CSV en/of serum.

Aangezien progressie naar MS slechts beperkt is tot een gedeelte van de CIS-patiënten, onderzochten we ook het prognostisch potentieel van ons panel. Voor 2 targets, UH-CIS4 en UH-CIS6, was de frequentie van antilichaam reactiviteit hoger, hoewel niet significant, in CIS-patiënten die MS ontwikkelden vergeleken met CIS-patiënten zonder ziekte progressie. Dit impliceert dat antilichaam reactiviteit tegen deze targets mogelijk gebruikt kan worden als een prognostische merker. Echter verdere studies zijn nodig om het prognostisch potentieel van UH-CIS4 en UH-CIS6 verder te onderzoeken.

Doelstelling 2: Analyse en vergelijking van de antilichaam respons in CSF van MS subtypes

Verscheidene subtypes van MS kunnen worden onderscheiden. Tachtig procent van de MS-patiënten vertoont een ziekteverloop dat gekarakteriseerd is door ziekte opstoten en perioden van remissie (RR), terwijl in 20% van de MS-patiënten de ziekte een progressief verloop vanaf het begin heeft (PP). Deze MS subtypes kunnen mogelijk gekarakteriseerd zijn door verschillende antilichaam repertoires. Het doel van deze studie was om nieuwe antilichaam targets te identificeren voor RR- en PP-MS en om het antilichaam repertoire te vergelijken in RR- en PP-MS (**hoofdstuk 3**).

SAS procedures werden uitgevoerd met NWS en MS cDNA faag display bibliotheken op gepoold CSV van RR- (n=6) en PP-MS (n=6) patiënten. Karakterisatie van de SAS output wees op de afwezigheid van gemeenschappelijke klonen voor RR- en PP-MS, wat mogelijk kan wijzen op de aanwezigheid van verschillende antilichaam repertoires in RR- en PP-MS. Een panel bestaande uit 9 nieuwe antilichaam targets werd geselecteerd voor verdere analyse. Acht kandidaat targets waren afkomstig van SAS procedures uitgevoerd op CSV van RR-MS patiënten (UH-RRMS1 tot en met 8), terwijl 1 target was geselecteerd uit de PP-MS selectie procedures (UH-PPMS1). Het feit dat slechts 1 target werd geselecteerd voor PP-MS kan mogelijk te wijten zijn

aan een minder belangrijke rol van neuro-inflammatoire processen in de progressieve fase van MS. De geidentificeerde kandidaat targets vertoonden homologie met eiwitten die tot nu toe nog niet waren geassocieerd met antilichaam reactiviteit in MS. Bijvoorbeeld, UH-RRMS6 was homoloog aan het hypothetische eiwit *hCG2045783*. Bovendien vertoonde UH-RRMS4 gelijkenis met *general transcription factor IIH, polypeptide 4* (GTF2H4), een eiwit betrokken bij DNA herstelmechanismen. In het verleden werd reeds een *single nucleotide polymorfisme* (SNP) in intron 11 van GTF2H4 geïdentificeerd dat werd geassocieerd met MS [284]. Antilichaam reactiviteit tegen het panel kon worden bevestigd in serum en CSV van patiënten die gebruikt waren in de SAS procedures, wat er mogelijk op duidt dat het antilichaam repertoire overeenkomstig is in CSV en serum . Verder werd antilichaam reactiviteit tegen UH-RRMS7 gedetecteerd in CSV van 4 bijkomende MS patiënten (8%) en 1 neurologische controle (3%). Samenvattend, in deze studie slaagden we er in om nieuwe antilichaam targets te identificeren voor RR- and PP-MS. Verder kregen we een indicatie dat het antilichaam repertoire mogelijk verschillend is binnen RR- en PP-MS. Echter is het noodzakelijk dat deze bevindingen in de toekomst verder onderzocht worden.

Doelstelling 3: Karakterisatie van een nieuw kandidaat antilichaam target voor CIS en MS

Een nadeel van faag display is de identificatie van mimotopen. Deze mimotopen lijken op hun natuurlijke targets, maar dit wordt niet perse weerspiegeld in een vergelijkbare aminozuur compositie. Om de identificatie van deze mimotopen te verminderen, hebben we in de SAS procedures gebruik gemaakt van cDNA faag display bibliotheken. Echter, een aantal van de geïdentificeerde kandidaat antilichaam targets in **hoofdstuk 2**, waaronder kandidaat target UH-CIS6, bleek toch een mimotoop te zijn. Antilichaam reactiviteit tegen UH-CIS6 werd gedetecteerd in CSV van CIS- en MS-patiënten alsmede ook in serum van een significant verhoogd aantal CIS- en RR-MS patiënten vergeleken met gezonde en neurologische controles.

Naar aanleiding van deze resultaten was het doel van deze studie om UH-CIS6 verder te karakteriseren (**hoofdstuk 4**). Ten eerste ontwikkelden we een UH-CIS6 peptide "enzyme-linked immunosorbent assay" (ELISA) om de UH-CIS6 antilichaamrespons verder te bestuderen. Deze UH-CIS6 peptide ELISA bleek een verhoogde sensitiviteit te hebben ten opzichte van de in **hoofdstuk 2** gebruikte faag ELISA, aangezien meer anti-UH-CIS6 antilichaam positieve CSV samples konden worden geïdentificeerd wanneer de UH-CIS6 peptide ELISA werd gebruikt. Hierna onderzochten we de antilichaamrespons tegen UH-CIS6 in CSV van patiënten met CIS, MS en andere (ontstekingsgerelateerde) neurologische ziekten. Vergelijkbaar met de resultaten in **hoofdstuk 2**, was de frequentie van antilichaam reactiviteit gericht tegen UH-CIS6 significant verhoogd in CIS en RR-MS vergeleken met neurologische controles ($p=0.046$). Bovendien werd antilichaam reactiviteit tegen UH-CIS6 alleen gedetecteerd in CIS-patiënten die converteerden naar MS en kon niet worden waargenomen in CIS-patiënten zonder ziekte progressie. Deze bevindingen ondersteunen verder het prognostisch potentieel van UH-CIS6.

Een tweede doel van deze studie was de identificatie van het *in vivo* target voor UH-CIS6. Hiervoor maakten we gebruik van een polyklonaal konijn anti-UH-CIS6 antilichaam. Dit antilichaam was in staat om hetzelfde epitoop te binden als humane anti-UH-CIS6 antilichamen. Hiermee bewezen we dat dit konijn anti-UH-CIS6 antilichaam geschikt was voor verdere experimenten. Om het *in vivo* target voor UH-CIS6 te identificeren, werd het konijn anti-UH-CIS6 antilichaam gebruikt voor immuunprecipitatie op een humaan hersenlysaat. Analyse van de immuunprecipitatie output identificeerde *coronin-1a* als een kandidaat target voor anti-UH-CIS6 antilichamen. Binding van zowel konijn als humane anti-UH-CIS6 antilichamen aan *coronin-1a* kon worden bevestigd via Western blot analyse en ELISA. Daarnaast werden T-cellen en macrofagen geïdentificeerd die *coronin-1a* tot expressie brachten in een MS hersen laesie, waarmee de aanwezigheid van *coronin-1a* in MS hersenweefsel werd aangetoond. In conclusie, kunnen we stellen dat *coronin-1a* een nieuw antilichaam target is voor CIS en MS. Toekomstige studies moeten meer inzicht verschaffen in de rol van *coronin-1a* en de antilichaamrespons tegen dit eiwit in CIS en MS. Onder meer kunnen antilichamen gericht tegen *coronin-1a* worden gebruikt voor

experimenten in een diermodel voor MS, genaamd *experimental autoimmune encephalomyelits*. Op deze manier kan het effect van anti-*coronin-1a* antilichamen worden bestudeerd op het ziekteverloop.

Doelstelling 4: Analyse van de antilichaamrespons gericht tegen MS antilichaam targets in CIS

In een eerdere studie (**hoofdstuk 2**) identificeerden we UH-CIS6 als een nieuw kandidaat antilichaam target voor CIS en RR-MS en slaagden we er in om een gevoelige UH-CIS6 peptide ELISA te ontwikkelen voor de detectie van antilichaam reactiviteit tegen UH-CIS6 (**hoofdstuk 4**). Naast de antilichaamrespons gericht tegen UH-CIS6 in CSV, waren we ook geïnteresseerd in de analyse van de anti-UH-CIS6 antilichaamrespons in serum van CIS-patiënten. Aangezien CIS een mogelijke eerste manifestatie van MS is, is het aannemelijk dat de antilichaamrespons in CIS en MS gericht is tegen een vergelijkbaar antigen repertoire. Inderdaad werd er voor enkele antilichaam targets, zoals bijvoorbeeld *myelin oligodendrocyte glycoprotein* (MOG), *myelin basic protein* (MBP) en het kalium kanaal *KIR 4.1*, antilichaam reactiviteit waargenomen in zowel CIS- als MS-patiënten (samenvatting is weergegeven in Tabel 1.1). Echter voor enkele MS antilichaam targets is de antilichaamrespons in CIS onbekend. Om deze reden onderzochten we in **hoofdstuk 5** de antilichaamrespons tegen MS antilichaam targets *sperm associated antigen 16* (SPAG16), *neurofilament-light* (NF-L) en *neurofascin* (NF) in serum van CIS-patiënten en vergeleken dit met UH-CIS6.

We analyseerden de antilichaamrespons tegen het panel van 4 antilichaam targets in serum van CIS-patiënten en gezonde controles. Voor UH-CIS6 werd een sensitiviteit van 15% en een specificiteit van 90% bekomen. Tevens was de gemiddelde antilichaam reactiviteit tegen UH-CIS6 verhoogd, hoewel niet significant, in de CIS-groep vergeleken met de gezonde controles (p=0.08). Anti-UH-CIS6 antilichaam reactiviteit correleerde met een stijging van de immunoglobuline G (IgG) concentratie in gepaarde CSV samples (p=0.012), wat mogelijk kan wijzen op de productie van anti-UH-CIS6 antilichamen in CSV. Verder testte 13% van de CIS patiënten positief voor antilichaam reactiviteit

tegen SPAG16 (specficiteit 95%), 7% voor NF-L (specificiteit 94%) en 8% voor NF (specificiteit 95%).

De afwezigheid van een verschil in de antilichaam respons tegen NF in CIS-patiënten en gezonde controles kan mogelijk te wijten zijn aan het NF eiwit dat werd gebruikt in deze studie. Recent werd namelijk aangetoond dat dit NF eiwit niet geschikt is om antilichaam reactiviteit tegen NF te meten [118]. Om deze reden zijn in de toekomst nieuwe studies noodzakelijk om de anti-NF antilichaam respons in CIS-patiënten te bestuderen. Verder werd antilichaam reactiviteit gericht tegen meerdere targets zelden waargenomen in CIS-patiënten. De bevindingen beschreven in deze studie tonen aan dat UH-CIS6 en SPAG16 mogelijk serum antilichaam targets zijn voor CIS.

Eindconclusies

In deze thesis hebben we nieuwe antilichaam targets geïdentificeerd voor CIS en MS door gebruik te maken van de krachtige SAS technologie. Voor de meerderheid van de geïdentificeerde antilichaam targets kon antilichaam reactiviteit worden aangetoond in CSV en/of serum in een subpopulatie van de geteste CIS- en MS-patiënten. Dit kan er mogelijk op wijzen dat de geïdentificeerde targets algemene antilichaam targets zijn binnen CIS en MS. Bovendien bleek antilichaam reactiviteit tegen één van onze targets, UH-CIS6, te corresponderen met antilichaam reactiviteit tegen *coronin-1a*. Hierdoor werd *coronin-1a* als een nieuwe antilichaam target voor CIS en MS geïdentificeerd. Met de verkregen resultaten kunnen toekomstige studies worden uitgevoerd, enerzijds gericht op de verdere analyse van de antilichaamrespons tegen *coronin-1a* en andere targets in CIS en MS. Anderzijds kunnen studies worden aangevangen om de rol van de antilichaamrespons tegen deze targets in het ziekteproces van CIS en MS te onderzoeken.

References

1. Pugliatti M, Rosati G, Carton H, Riise T, Drulovic J, Vecsei L*, et al.* The epidemiology of multiple sclerosis in Europe. *Eur J Neurol* 2006, **13**(7)**:** 700-722.

2. Compston A, Coles A. Multiple sclerosis. *Lancet* 2002, **359**(9313)**:** 1221-1231.

3. Compston A, Coles A. Multiple sclerosis. *Lancet* 2008, **372**(9648)**:** 1502-1517.

4. Miller DH, Chard DT, Ciccarelli O. Clinically isolated syndromes. *Lancet neurology* 2012, **11**(2)**:** 157-169.

5. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M*, et al.* Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol* 2011, **69**(2)**:** 292-302.

6. Miller DH, Weinshenker BG, Filippi M, Banwell BL, Cohen JA, Freedman MS*, et al.* Differential diagnosis of suspected multiple sclerosis: a consensus approach. *Mult Scler* 2008, **14**(9)**:** 1157-1174.

7. Miller D, Barkhof F, Montalban X, Thompson A, Filippi M. Clinically isolated syndromes suggestive of multiple sclerosis, part I: natural history, pathogenesis, diagnosis, and prognosis. *Lancet neurology* 2005, **4**(5)**:** 281-288.

8. Dobson R, Ramagopalan S, Giovannoni G. The effect of gender in clinically isolated syndrome (CIS): a meta-analysis. *Mult Scler* 2012, **18**(5)**:** 600-604.

9. Mowry EM, Pesic M, Grimes B, Deen SR, Bacchetti P, Waubant E. Clinical predictors of early second event in patients with clinically isolated syndrome. *J Neurol* 2009, **256**(7)**:** 1061-1066.

10. Fisniku LK, Brex PA, Altmann DR, Miszkiel KA, Benton CE, Lanyon R*, et al.* Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain* 2008, **131**(Pt 3)**:** 808-817.

11. Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC*, et al.* New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983, **13**(3)**:** 227-231.

12. Link H, Huang YM. Oligoclonal bands in multiple sclerosis cerebrospinal fluid: an update on methodology and clinical usefulness. *J Neuroimmunol* 2006, **180**(1-2)**:** 17-28.

13. Reiber H, Peter JB. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. *J Neurol Sci* 2001, **184**(2)**:** 101-122.

14. McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD*, et al.* Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol* 2001, **50**(1)**:** 121-127.

15. Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L*, et al.* Diagnostic criteria for multiple sclerosis: 2005 revisions to the "McDonald Criteria". *Ann Neurol* 2005, **58**(6)**:** 840-846.

16. Wucherpfennig KW, Strominger JL. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 1995, **80**(5)**:** 695-705.

17. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. *N Engl J Med* 2000, **343**(13)**:** 938-952.

18. Nylander A, Hafler DA. Multiple sclerosis. *J Clin Invest* 2012, **122**(4)**:** 1180-1188.

19. Hemmer B, Nessler S, Zhou D, Kieseier B, Hartung HP. Immunopathogenesis and immunotherapy of multiple sclerosis. *Nature clinical practice Neurology* 2006, **2**(4)**:** 201-211.

20. Miller E. Multiple sclerosis. *Adv Exp Med Biol* 2012, **724:** 222-238.

21. Husain J, Juurlink BH. Oligodendroglial precursor cell susceptibility to hypoxia is related to poor ability to cope with reactive oxygen species. *Brain Res* 1995, **698**(1-2)**:** 86-94.

22. van Horssen J, Witte ME, Ciccarelli O. The role of mitochondria in axonal degeneration and tissue repair in MS. *Mult Scler* 2012, **18**(8)**:** 1058-1067.

23. Stys PK, Zamponi GW, van Minnen J, Geurts JJ. Will the real multiple sclerosis please stand up? *Nature reviews Neuroscience* 2012, **13**(7)**:** 507-514.

24. Filippi M, Rocca MA, Barkhof F, Bruck W, Chen JT, Comi G*, et al.* Association between pathological and MRI findings in multiple sclerosis. *Lancet neurology* 2012, **11**(4)**:** 349-360.

25. Jadasz JJ, Aigner L, Rivera FJ, Kury P. The remyelination Philosopher's Stone: stem and progenitor cell therapies for multiple sclerosis. *Cell Tissue Res* 2012, **349**(1)**:** 331-347.

26. 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**(6)**:** 707-717.

27. Breij EC, Brink BP, Veerhuis R, van den Berg C, Vloet R, Yan R*, et al.* Homogeneity of active demyelinating lesions in established multiple sclerosis. *Ann Neurol* 2008, **63**(1)**:** 16-25.

28. Geurts JJ, Barkhof F. Grey matter pathology in multiple sclerosis. *Lancet neurology* 2008, **7**(9)**:** 841-851.

29. Pirko I, Lucchinetti CF, Sriram S, Bakshi R. Gray matter involvement in multiple sclerosis. *Neurology* 2007, **68**(9)**:** 634-642.

30. Robertson NP, Fraser M, Deans J, Clayton D, Walker N, Compston DA. Age-adjusted recurrence risks for relatives of patients with multiple sclerosis. *Brain* 1996, **119 (Pt 2):** 449-455.

31. Sadovnick AD, Armstrong H, Rice GP, Bulman D, Hashimoto L, Paty DW*, et al.* A population-based study of multiple sclerosis in twins: update. *Ann Neurol* 1993, **33**(3)**:** 281-285.

32. Oksenberg JR, Baranzini SE, Sawcer S, Hauser SL. The genetics of multiple sclerosis: SNPs to pathways to pathogenesis. *Nature reviews Genetics* 2008, **9**(7)**:** 516-526.

33. Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL*, et al.* Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 2007, **357**(9)**:** 851-862.

34. Hoppenbrouwers IA, Aulchenko YS, Ebers GC, Ramagopalan SV, Oostra BA, van Duijn CM*, et al.* EVI5 is a risk gene for multiple sclerosis. *Genes and immunity* 2008, **9**(4)**:** 334-337.

35. De Jager PL, Baecher-Allan C, Maier LM, Arthur AT, Ottoboni L, Barcellos L*, et al.* The role of the CD58 locus in multiple sclerosis. *Proc Natl Acad Sci U S A* 2009, **106**(13)**:** 5264-5269.

36. Rubio JP, Stankovich J, Field J, Tubridy N, Marriott M, Chapman C*, et al.* Replication of KIAA0350, IL2RA, RPL5 and CD58 as multiple sclerosis susceptibility genes in Australians. *Genes and immunity* 2008, **9**(7)**:** 624-630.

37. Aulchenko YS, Hoppenbrouwers IA, Ramagopalan SV, Broer L, Jafari N, Hillert J*, et al.* Genetic variation in the KIF1B locus influences susceptibility to multiple sclerosis. *Nat Genet* 2008, **40**(12)**:** 1402-1403.

38. Vandenbroeck K, Alvarez J, Swaminathan B, Alloza I, Matesanz F, Urcelay E*, et al.* A cytokine gene screen uncovers SOCS1 as genetic risk factor for multiple sclerosis. *Genes and immunity* 2012, **13**(1)**:** 21-28.

39. Ban M, Goris A, Lorentzen AR, Baker A, Mihalova T, Ingram G*, et al.* Replication analysis identifies TYK2 as a multiple sclerosis susceptibility factor. *Eur J Hum Genet* 2009, **17**(10)**:** 1309-1313.

40. Sawcer S, Hellenthal G, Pirinen M, Spencer CC, Patsopoulos NA, Moutsianas L*, et al.* Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* 2011, **476**(7359)**:** 214-219.

41. Baranzini SE, Mudge J, van Velkinburgh JC, Khankhanian P, Khrebtukova I, Miller NA*, et al.* Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. *Nature* 2010, **464**(7293)**:** 1351-1356.

42. Jelcic I, Hsu KC, Kakalacheva K, Breiden P, Dupont B, Uhrberg M*, et al.* Killer immunoglobulin-like receptor locus polymorphisms in multiple sclerosis. *Mult Scler* 2012, **18**(7)**:** 951-958.

43. Middleton D, Meenagh A, Moscoso J, Arnaiz-Villena A. Killer immunoglobulin receptor gene and allele frequencies in Caucasoid, Oriental and Black populations from different continents. *Tissue Antigens* 2008, **71**(2)**:** 105-113.

44. Kelly MA, Cavan DA, Penny MA, Mijovic CH, Jenkins D, Morrissey S*, et al.* The influence of HLA-DR and -DQ alleles on progression to multiple sclerosis following a clinically isolated syndrome. *Hum Immunol* 1993, **37**(3)**:** 185-191.

45. Pender MP. The essential role of Epstein-Barr virus in the pathogenesis of multiple sclerosis. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 2011, **17**(4)**:** 351-367.

46. Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P*, et al.* Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *J Exp Med* 2007, **204**(12)**:** 2899-2912.

47. Serafini B, Severa M, Columba-Cabezas S, Rosicarelli B, Veroni C, Chiappetta G*, et al.* Epstein-Barr virus latent infection and BAFF expression in B cells in the multiple sclerosis brain: implications for viral persistence and intrathecal B-cell activation. *J Neuropathol Exp Neurol* 2010, **69**(7)**:** 677-693.

48. Lassmann H, Niedobitek G, Aloisi F, Middeldorp JM. Epstein-Barr virus in the multiple sclerosis brain: a controversial issue--report on a focused workshop held in the Centre for Brain Research of the Medical University of Vienna, Austria. *Brain* 2011, **134**(Pt 9)**:** 2772-2786.

49. Peferoen LA, Lamers F, Lodder LN, Gerritsen WH, Huitinga I, Melief J*, et al.* Epstein Barr virus is not a characteristic feature in the central nervous system in established multiple sclerosis. *Brain* 2010, **133**(Pt 5)**:** e137.

50. Willis SN, Stadelmann C, Rodig SJ, Caron T, Gattenloehner S, Mallozzi SS*, et al.* Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain. *Brain* 2009, **132**(Pt 12)**:** 3318-3328.

51. Ordonez G, Pineda B, Garcia-Navarrete R, Sotelo J. Brief presence of varicella-zoster vral DNA in mononuclear cells during relapses of multiple sclerosis. *Arch Neurol* 2004, **61**(4)**:** 529-532.

52. Mancuso R, Delbue S, Borghi E, Pagani E, Calvo MG, Caputo D*, et al.* Increased prevalence of varicella zoster virus DNA in cerebrospinal fluid from patients with multiple sclerosis. *J Med Virol* 2007, **79**(2)**:** 192-199.

53. Sotelo J, Ordonez G, Pineda B. Varicella-zoster virus at relapses of multiple sclerosis. *J Neurol* 2007, **254**(4)**:** 493-500.

54. Burgoon MP, Cohrs RJ, Bennett JL, Anderson SW, Ritchie AM, Cepok S*, et al.* Varicella zoster virus is not a disease-relevant antigen in multiple sclerosis. *Ann Neurol* 2009, **65**(4)**:** 474-479.

55. Kim JS, Lee KS, Park JH, Kim MY, Shin WS. Detection of human herpesvirus 6 variant A in peripheral blood mononuclear cells from multiple sclerosis patients. *Eur Neurol* 2000, **43**(3)**:** 170-173.

56. Mancuso R, Hernis A, Cavarretta R, Caputo D, Calabrese E, Nemni R*, et al.* Detection of viral DNA sequences in the cerebrospinal fluid of patients with multiple sclerosis. *J Med Virol* 2010, **82**(6)**:** 1051-1057.

57. Tuke PW, Hawke S, Griffiths PD, Clark DA. Distribution and quantification of human herpesvirus 6 in multiple sclerosis and control brains. *Mult Scler* 2004, **10**(4)**:** 355-359.

58. Taus C, Pucci E, Cartechini E, Fie A, Giuliani G, Clementi M*, et al.* Absence of HHV-6 and HHV-7 in cerebrospinal fluid in relapsing-remitting multiple sclerosis. *Acta Neurol Scand* 2000, **101**(4)**:** 224-228.

59. Mirandola P, Stefan A, Brambilla E, Campadelli-Fiume G, Grimaldi LM. Absence of human herpesvirus 6 and 7 from spinal fluid and serum of multiple sclerosis patients. *Neurology* 1999, **53**(6)**:** 1367-1368.

60. Harberts E, Yao K, Wohler JE, Maric D, Ohayon J, Henkin R*, et al.* Human herpesvirus-6 entry into the central nervous system through the olfactory pathway. *Proc Natl Acad Sci U S A* 2011, **108**(33)**:** 13734-13739.

61. Opsahl ML, Kennedy PG. Early and late HHV-6 gene transcripts in multiple sclerosis lesions and normal appearing white matter. *Brain* 2005, **128**(Pt 3)**:** 516-527.

62. Kakalacheva K, Munz C, Lunemann JD. Viral triggers of multiple sclerosis. *Biochim Biophys Acta* 2011, **1812**(2)**:** 132-140.

63. Simpson S, Jr., Taylor B, Dwyer DE, Taylor J, Blizzard L, Ponsonby AL*, et al.* Anti-HHV-6 IgG titer significantly predicts subsequent relapse risk in multiple sclerosis. *Mult Scler* 2012, **18**(6)**:** 799-806.

64. Pakpoor J, Pakpoor J, Disanto G, Giovannoni G, Ramagopalan SV. Cytomegalovirus and multiple sclerosis risk. *J Neurol* 2013, **260**(6)**:** 1685-1660.

65. Buljevac D, Verkooyen RP, Jacobs BC, Hop W, van der Zwaan LA, van Doorn PA*, et al.* Chlamydia pneumoniae and the risk for exacerbation in multiple sclerosis patients. *Ann Neurol* 2003, **54**(6)**:** 828-831.

66. Pohl D, Rostasy K, Jacobi C, Lange P, Nau R, Krone B*, et al.* Intrathecal antibody production against Epstein-Barr and other neurotropic viruses in pediatric and adult onset multiple sclerosis. *J Neurol* 2010, **257**(2)**:** 212-216.

67. Garcia-Montojo M, Dominguez-Mozo M, Arias-Leal A, Garcia-Martinez A, De las Heras V, Casanova I*, et al.* The DNA copy number of human endogenous retrovirus-W (MSRV-type) is increased in multiple sclerosis patients and is influenced by gender and disease severity. *PloS one* 2013, **8**(1)**:** e53623.

68. Brettschneider J, Tumani H, Kiechle U, Muche R, Richards G, Lehmensiek V*, et al.* IgG antibodies against measles, rubella, and varicella zoster virus predict conversion to multiple sclerosis in clinically isolated syndrome. *PloS one* 2009, **4**(11)**:** e7638.

69. Miller DH, Hammond SR, McLeod JG, Purdie G, Skegg DC. Multiple sclerosis in Australia and New Zealand: are the determinants genetic or environmental? *J Neurol Neurosurg Psychiatry* 1990, **53**(10)**:** 903-905.

70. Correale J, Ysrraelit MC, Gaitan MI. Immunomodulatory effects of Vitamin D in multiple sclerosis. *Brain* 2009, **132**(Pt 5)**:** 1146-1160.

71. Hedstrom AK, Baarnhielm M, Olsson T, Alfredsson L. Tobacco smoking, but not Swedish snuff use, increases the risk of multiple sclerosis. *Neurology* 2009, **73**(9)**:** 696-701.

72. Rook GA. Hygiene hypothesis and autoimmune diseases. *Clin Rev Allergy Immunol* 2012, **42**(1)**:** 5-15.

73. Jacobs LD, Cookfair DL, Rudick RA, Herndon RM, Richert JR, Salazar AM*, et al.* Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Ann Neurol* 1996, **39**(3)**:** 285-294.

74. Interferon beta-1b in the treatment of multiple sclerosis: final outcome of the randomized controlled trial. The IFNB Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology* 1995, **45**(7)**:** 1277-1285.

75. Randomised double-blind placebo-controlled study of interferon beta-1a in relapsing/remitting multiple sclerosis. PRISMS (Prevention of Relapses and Disability by Interferon beta-1a Subcutaneously in Multiple Sclerosis) Study Group. *Lancet* 1998, **352**(9139)**:** 1498-1504.

76. Kieseier BC. The mechanism of action of interferon-beta in relapsing multiple sclerosis. *CNS drugs* 2011, **25**(6)**:** 491-502.

77. Johnson KP, Brooks BR, Cohen JA, Ford CC, Goldstein J, Lisak RP*, et al.* Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group. *Neurology* 1995, **45**(7)**:** 1268-1276.

78. Comi G, Filippi M, Wolinsky JS. European/Canadian multicenter, double-blind, randomized, placebo-controlled study of the effects of glatiramer acetate on magnetic resonance imaging--measured disease activity and burden in patients with relapsing multiple sclerosis. European/Canadian Glatiramer Acetate Study Group. *Ann Neurol* 2001, **49**(3)**:** 290-297.

79. Blanchette F, Neuhaus O. Glatiramer acetate: evidence for a dual mechanism of action. *J Neurol* 2008, **255 Suppl 1:** 26-36.

80. Martinelli V, Radaelli M, Straffi L, Rodegher M, Comi G. Mitoxantrone: benefits and risks in multiple sclerosis patients. *Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology* 2009, **30 Suppl 2:** S167-170.

81. Cohen BA, Mikol DD. Mitoxantrone treatment of multiple sclerosis: safety considerations. *Neurology* 2004, **63**(12 Suppl 6)**:** S28-32.

82. Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P*, et al.* A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *N Engl J Med* 2010, **362**(5)**:** 387-401.

83. Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH*, et al.* Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* 2006, **355**(11)**:** 1124-1140.

84. Graler MH, Goetzl EJ. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J* 2004, **18**(3)**:** 551-553.

85. Gold R, Kappos L, Arnold DL, Bar-Or A, Giovannoni G, Selmaj K*, et al.* Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. *N Engl J Med* 2012, **367**(12)**:** 1098-1107.

86. O'Connor P, Wolinsky JS, Confavreux C, Comi G, Kappos L, Olsson TP*, et al.* Randomized trial of oral teriflunomide for relapsing multiple sclerosis. *N Engl J Med* 2011, **365**(14)**:** 1293-1303.

87. Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH*, et al.* A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 2006, **354**(9)**:** 899-910.

88. Langer-Gould A, Atlas SW, Green AJ, Bollen AW, Pelletier D. Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. *N Engl J Med* 2005, **353**(4)**:** 375-381.

89. Kleinschmidt-DeMasters BK, Tyler KL. Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 2005, **353**(4)**:** 369-374.

90. Chen Y, Bord E, Tompkins T, Miller J, Tan CS, Kinkel RP*, et al.* Asymptomatic reactivation of JC virus in patients treated with natalizumab. *N Engl J Med* 2009, **361**(11)**:** 1067-1074.

91. Coles AJ, Twyman CL, Arnold DL, Cohen JA, Confavreux C, Fox EJ*, et al.* Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet* 2012, **380**(9856)**:** 1829-1839.

92. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ*, et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* 2008, **358**(7)**:** 676-688.

93. Kappos L, Li D, Calabresi PA, O'Connor P, Bar-Or A, Barkhof F*, et al.* Ocrelizumab in relapsing-remitting multiple sclerosis: a phase 2, randomised, placebo-controlled, multicentre trial. *Lancet* 2011, **378**(9805)**:** 1779-1787.

94. Lulu S, Waubant E. Humoral-targeted immunotherapies in multiple sclerosis. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* 2013, **10**(1)**:** 34-43.

95. Bielekova B, Richert N, Howard T, Blevins G, Markovic-Plese S, McCartin J*, et al.* Humanized anti-CD25 (daclizumab) inhibits disease activity in multiple sclerosis patients failing to respond to interferon beta. *Proc Natl Acad Sci U S A* 2004, **101**(23)**:** 8705-8708.

96. Warnke C, Leussink VI, Goebels N, Aktas O, Boyko A, Kieseier BC*, et al.* Cladribine as a therapeutic option in multiple sclerosis. *Clin Immunol* 2012, **142**(1)**:** 68-75.

97. Giovannoni G, Comi G, Cook S, Rammohan K, Rieckmann P, Soelberg Sorensen P*, et al.* A placebo-controlled trial of oral cladribine for relapsing multiple sclerosis. *N Engl J Med* 2010, **362**(5)**:** 416-426.

98. Giacomini PS, Bar-Or A. Laquinimod in multiple sclerosis. *Clin Immunol* 2012, **142**(1)**:** 38-43.

99. Jacobs LD, Beck RW, Simon JH, Kinkel RP, Brownscheidle CM, Murray TJ*, et al.* Intramuscular interferon beta-1a therapy initiated during a first demyelinating event in multiple sclerosis. CHAMPS Study Group. *N Engl J Med* 2000, **343**(13)**:** 898-904.

100. Kinkel RP, Kollman C, O'Connor P, Murray TJ, Simon J, Arnold D*, et al.* IM interferon beta-1a delays definite multiple sclerosis 5 years after a first demyelinating event. *Neurology* 2006, **66**(5)**:** 678-684.

101. Kinkel RP, Dontchev M, Kollman C, Skaramagas TT, O'Connor PW, Simon JH*, et al.* Association between immediate initiation of intramuscular interferon beta-1a at the time of a clinically isolated syndrome and long-term outcomes: a 10-year follow-up of the Controlled High-Risk Avonex Multiple Sclerosis Prevention Study in Ongoing Neurological Surveillance. *Arch Neurol* 2012, **69**(2)**:** 183-190.

102. Comi G, Filippi M, Barkhof F, Durelli L, Edan G, Fernandez O*, et al.* Effect of early interferon treatment on conversion to definite multiple sclerosis: a randomised study. *Lancet* 2001, **357**(9268)**:** 1576-1582.

103. Filippi M, Rovaris M, Inglese M, Barkhof F, De Stefano N, Smith S*, et al.* Interferon beta-1a for brain tissue loss in patients at presentation with syndromes suggestive of multiple sclerosis: a randomised, double-blind, placebo-controlled trial. *Lancet* 2004, **364**(9444)**:** 1489-1496.

104. Kappos L, Polman CH, Freedman MS, Edan G, Hartung HP, Miller DH*, et al.* Treatment with interferon beta-1b delays conversion to clinically definite and

McDonald MS in patients with clinically isolated syndromes. *Neurology* 2006, **67**(7)**:** 1242-1249.

105. Kappos L, Freedman MS, Polman CH, Edan G, Hartung HP, Miller DH*, et al.* Effect of early versus delayed interferon beta-1b treatment on disability after a first clinical event suggestive of multiple sclerosis: a 3-year follow-up analysis of the BENEFIT study. *Lancet* 2007, **370**(9585)**:** 389-397.

106. Kappos L, Freedman MS, Polman CH, Edan G, Hartung HP, Miller DH*, et al.* Long-term effect of early treatment with interferon beta-1b after a first clinical event suggestive of multiple sclerosis: 5-year active treatment extension of the phase 3 BENEFIT trial. *Lancet neurology* 2009, **8**(11)**:** 987-997.

107. Comi G, Martinelli V, Rodegher M, Moiola L, Bajenaru O, Carra A*, et al.* Effect of glatiramer acetate on conversion to clinically definite multiple sclerosis in patients with clinically isolated syndrome (PreCISe study): a randomised, double-blind, placebo-controlled trial. *Lancet* 2009, **374**(9700)**:** 1503-1511.

108. Janeway CA, Travers P, Walport M, Shlomchik MJ. *Immunobiology*, 2005.

109. Ireland SJ, Blazek M, Harp CT, Greenberg B, Frohman EM, Davis LS*, et al.* Antibody-independent B cell effector functions in relapsing remitting multiple sclerosis: clues to increased inflammatory and reduced regulatory B cell capacity. *Autoimmunity* 2012, **45**(5)**:** 400-414.

110. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H*, et al.* Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J Immunol* 2007, **178**(10)**:** 6092-6099.

111. Bar-Or A, Fawaz L, Fan B, Darlington PJ, Rieger A, Ghorayeb C*, et al.* Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Ann Neurol* 2010, **67**(4)**:** 452-461.

112. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol* 2004, **14**(2)**:** 164-174.

113. von Budingen HC, Bar-Or A, Zamvil SS. B cells in multiple sclerosis: connecting the dots. *Curr Opin Immunol* 2011, **23**(6)**:** 713-720.

114. Obermeier B, Mentele R, Malotka J, Kellermann J, Kumpfel T, Wekerle H*, et al.* Matching of oligoclonal immunoglobulin transcriptomes and proteomes of cerebrospinal fluid in multiple sclerosis. *Nat Med* 2008, **14**(6)**:** 688-693.

115. Somers V, Govarts C, Somers K, Hupperts R, Medaer R, Stinissen P. Autoantibody profiling in multiple sclerosis reveals novel antigenic candidates. *J Immunol* 2008, **180**(6)**:** 3957-3963.

116. Srivastava R, Aslam M, Kalluri SR, Schirmer L, Buck D, Tackenberg B*, et al.* Potassium channel KIR4.1 as an immune target in multiple sclerosis. *N Engl J Med* 2012, **367**(2)**:** 115-123.

117. Mayer MC, Meinl E. Glycoproteins as targets of autoantibodies in CNS inflammation: MOG and more. *Therapeutic advances in neurological disorders* 2012, **5**(3)**:** 147-159.

118. Ng JK, Malotka J, Kawakami N, Derfuss T, Khademi M, Olsson T*, et al.* Neurofascin as a target for autoantibodies in peripheral neuropathies. *Neurology* 2012, **79**(23)**:** 2241-2248.

119. Couratier P, Yi FH, Preud'homme JL, Clavelou P, White A, Sindou P*, et al.* Serum autoantibodies to neurofilament proteins in sporadic amyotrophic lateral sclerosis. *J Neurol Sci* 1998, **154**(2)**:** 137-145.

120. Bartos A, Fialova L, Svarcova J, Ripova D. Patients with Alzheimer disease have elevated intrathecal synthesis of antibodies against tau protein and heavy neurofilament. *J Neuroimmunol* 2012, **252**(1-2)**:** 100-105.

121. Berger T, Rubner P, Schautzer F, Egg R, Ulmer H, Mayringer I*, et al.* Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. *N Engl J Med* 2003, **349**(2)**:** 139-145.

122. Mader S, Gredler V, Schanda K, Rostasy K, Dujmovic I, Pfaller K*, et al.* Complement activating antibodies to myelin oligodendrocyte glycoprotein in neuromyelitis optica and related disorders. *Journal of neuroinflammation* 2011, **8:** 184.

123. Menge T, Lalive PH, von Budingen HC, Genain CP. Conformational epitopes of myelin oligodendrocyte glycoprotein are targets of potentially pathogenic antibody responses in multiple sclerosis. *Journal of neuroinflammation* 2011, **8:** 161.

124. 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**(2)**:** 91-96.

125. Reindl M, Linington C, Brehm U, Egg R, Dilitz E, Deisenhammer F*, et al.* Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain* 1999, **122 (Pt 11):** 2047-2056.

126. Di Pauli F, Mader S, Rostasy K, Schanda K, Bajer-Kornek B, Ehling R*, et al.* Temporal dynamics of anti-MOG antibodies in CNS demyelinating diseases. *Clin Immunol* 2011, **138**(3)**:** 247-254.

127. Klawiter EC, Piccio L, Lyons JA, Mikesell R, O'Connor KC, Cross AH. Elevated intrathecal myelin oligodendrocyte glycoprotein antibodies in multiple sclerosis. *Arch Neurol* 2010, **67**(9)**:** 1102-1108.

128. Tomassini V, De Giglio L, Reindl M, Russo P, Pestalozza I, Pantano P*, et al.* Anti-myelin antibodies predict the clinical outcome after a first episode suggestive of MS. *Mult Scler* 2007, **13**(9)**:** 1086-1094.

129. Kuhle J, Pohl C, Mehling M, Edan G, Freedman MS, Hartung HP*, et al.* Lack of association between antimyelin antibodies and progression to multiple sclerosis. *N Engl J Med* 2007, **356**(4)**:** 371-378.

130. Greeve I, Sellner J, Lauterburg T, Walker U, Rosler KM, Mattle HP. Anti-myelin antibodies in clinically isolated syndrome indicate the risk of multiple sclerosis in a Swiss cohort. *Acta Neurol Scand* 2007, **116**(4)**:** 207-210.

131. Rauer S, Euler B, Reindl M, Berger T. Antimyelin antibodies and the risk of relapse in patients with a primary demyelinating event. *J Neurol Neurosurg Psychiatry* 2006, **77**(6)**:** 739-742.

132. Cruz M, Olsson T, Ernerudh J, Hojeberg B, Link H. Immunoblot detection of oligoclonal anti-myelin basic protein IgG antibodies in cerebrospinal fluid in multiple sclerosis. *Neurology* 1987, **37**(9)**:** 1515-1519.

133. Belogurov AA, Jr., Kurkova IN, Friboulet A, Thomas D, Misikov VK, Zakharova MY*, et al.* Recognition and degradation of myelin basic protein peptides by serum autoantibodies: novel biomarker for multiple sclerosis. *J Immunol* 2008, **180**(2)**:** 1258-1267.

134. Ponomarenko NA, Durova OM, Vorobiev, II, Belogurov AA, Jr., Kurkova IN, Petrenko AG*, et al.* Autoantibodies to myelin basic protein catalyze site-specific degradation of their antigen. *Proc Natl Acad Sci U S A* 2006, **103**(2)**:** 281-286.

135. Moller JR, Johnson D, Brady RO, Tourtellotte WW, Quarles RH. Antibodies to myelin-associated glycoprotein (MAG) in the cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol* 1989, **22**(1)**:** 55-61.

136. Baig S, Olsson T, Yu-Ping J, Hojeberg B, Cruz M, Link H. Multiple sclerosis: cells secreting antibodies against myelin-associated glycoprotein are present in cerebrospinal fluid. *Scand J Immunol* 1991, **33**(1)**:** 73-79.

137. Sun JB, Olsson T, Wang WZ, Xiao BG, Kostulas V, Fredrikson S*, et al.* Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls. *Eur J Immunol* 1991, **21**(6)**:** 1461-1468.

138. Mathey EK, Derfuss T, Storch MK, Williams KR, Hales K, Woolley DR*, et al.* Neurofascin as a novel target for autoantibody-mediated axonal injury. *J Exp Med* 2007, **204**(10)**:** 2363-2372.

139. Derfuss T, Parikh K, Velhin S, Braun M, Mathey E, Krumbholz M*, et al.* Contactin-2/TAG-1-directed autoimmunity is identified in multiple sclerosis patients and mediates gray matter pathology in animals. *Proc Natl Acad Sci U S A* 2009, **106**(20)**:** 8302-8307.

140. Boronat A, Sepulveda M, Llufriu S, Sabater L, Blanco Y, Gabilondo I*, et al.* Analysis of antibodies to surface epitopes of contactin-2 in multiple sclerosis. *J Neuroimmunol* 2012, **244**(1-2)**:** 103-106.

141. Aslam M, Kalluri SR, Cepok S, Kraus V, Buck D, Srivastava R*, et al.* The antibody response to oligodendrocyte specific protein in multiple sclerosis. *J Neuroimmunol* 2010, **221**(1-2)**:** 81-86.

142. Fialova L, Bartos A, Svarcova J, Malbohan I. Increased intrathecal high-avidity anti-tau antibodies in patients with multiple sclerosis. *PloS one* 2011, **6**(11)**:** e27476.

143. Bartos A, Fialova L, Svarcova J, Dolezil D, Malbohan I. Light neurofilament and antibodies against them in patients with multiple sclerosis. *Mult Scler* 2011, **17**(Supplement 10).

144. Silber E, Semra YK, Gregson NA, Sharief MK. Patients with progressive multiple sclerosis have elevated antibodies to neurofilament subunit. *Neurology* 2002, **58**(9)**:** 1372-1381.

145. Ehling R, Lutterotti A, Wanschitz J, Khalil M, Gneiss C, Deisenhammer F*, et al.* Increased frequencies of serum antibodies to neurofilament light in patients with primary chronic progressive multiple sclerosis. *Mult Scler* 2004, **10**(6)**:** 601-606.

146. Bartos A, Fialova L, Soukupova J, Kukal J, Malbohan I, Pitha J. Antibodies against light neurofilaments in multiple sclerosis patients. *Acta Neurol Scand* 2007, **116**(2)**:** 100-107.

147. Bartos A, Fialova L, Soukupova J, Kukal J, Malbohan I, Pit'ha J. Elevated intrathecal antibodies against the medium neurofilament subunit in multiple sclerosis. *J Neurol* 2007, **254**(1)**:** 20-25.

148. Terryberry JW, Thor G, Peter JB. Autoantibodies in neurodegenerative diseases: antigen-specific frequencies and intrathecal analysis. *Neurobiol Aging* 1998, **19**(3)**:** 205-216.

149. Fialova L, Bartos A, Svarcova J, Zimova D, Kotoucova J. Serum and cerebrospinal fluid heavy neurofilaments and antibodies against them in early multiple sclerosis. *J Neuroimmunol* 2013.

150. Svarcova J, Fialova L, Bartos A, Steinbachova M, Malbohan I. Cerebrospinal fluid antibodies to tubulin are elevated in the patients with multiple sclerosis. *Eur J Neurol* 2008, **15**(11)**:** 1173-1179.

151. Lovato L, Cianti R, Gini B, Marconi S, Bianchi L, Armini A*, et al.* Transketolase and 2',3'-cyclic-nucleotide 3'-phosphodiesterase type I isoforms are specifically recognized by IgG autoantibodies in multiple sclerosis patients. *Molecular & cellular proteomics : MCP* 2008, **7**(12)**:** 2337-2349.

152. Sadatipour BT, Greer JM, Pender MP. Increased circulating antiganglioside antibodies in primary and secondary progressive multiple sclerosis. *Ann Neurol* 1998, **44**(6)**:** 980-983.

153. Kanter JL, Narayana S, Ho PP, Catz I, Warren KG, Sobel RA*, et al.* Lipid microarrays identify key mediators of autoimmune brain inflammation. *Nat Med* 2006, **12**(1)**:** 138-143.

154. Brennan KM, Galban-Horcajo F, Rinaldi S, O'Leary CP, Goodyear CS, Kalna G*, et al.* Lipid arrays identify myelin-derived lipids and lipid complexes as prominent targets for oligoclonal band antibodies in multiple sclerosis. *J Neuroimmunol* 2011, **238**(1-2)**:** 87-95.

155. Menge T, Lalive PH, von Budingen HC, Cree B, Hauser SL, Genain CP. Antibody responses against galactocerebroside are potential stage-specific biomarkers in multiple sclerosis. *J Allergy Clin Immunol* 2005, **116**(2)**:** 453-459.

156. Lolli F, Mazzanti B, Pazzagli M, Peroni E, Alcaro MC, Sabatino G*, et al.* The glycopeptide CSF114(Glc) detects serum antibodies in multiple sclerosis. *J Neuroimmunol* 2005, **167**(1-2)**:** 131-137.

157. Otto C, Oltmann A, Stein A, Frenzel K, Schroeter J, Habbel P*, et al.* Intrathecal EBV antibodies are part of the polyspecific immune response in multiple sclerosis. *Neurology* 2011, **76**(15)**:** 1316-1321.

158. Lunemann JD, Tintore M, Messmer B, Strowig T, Rovira A, Perkal H*, et al.* Elevated Epstein-Barr virus-encoded nuclear antigen-1 immune responses predict conversion to multiple sclerosis. *Ann Neurol* 2010, **67**(2)**:** 159-169.

159. Farrell RA, Antony D, Wall GR, Clark DA, Fisniku L, Swanton J*, et al.* Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI. *Neurology* 2009, **73**(1)**:** 32-38.

160. Cepok S, Zhou D, Srivastava R, Nessler S, Stei S, Bussow K*, et al.* Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. *J Clin Invest* 2005, **115**(5)**:** 1352-1360.

161. Ahlgren C, Oden A, Bergstrom T, Lycke J. Serum and CSF measles antibody levels increase over time in patients with multiple sclerosis or clinically isolated syndrome. *J Neuroimmunol* 2012, **247**(1-2)**:** 70-74.

162. Reiber H, Ungefehr S, Jacobi C. The intrathecal, polyspecific and oligoclonal immune response in multiple sclerosis. *Mult Scler* 1998, **4**(3)**:** 111-117.

163. Villoslada P, Juste C, Tintore M, Llorenc V, Codina G, Pozo-Rosich P*, et al.* The immune response against herpesvirus is more prominent in the early stages of MS. *Neurology* 2003, **60**(12)**:** 1944-1948.

164. Zivadinov R, Nasuelli D, Tommasi MA, Serafin M, Bratina A, Ukmar M*, et al.* Positivity of cytomegalovirus antibodies predicts a better clinical and radiological outcome in multiple sclerosis patients. *Neurol Res* 2006, **28**(3)**:** 262-269.

165. Sanadgol N, Ramroodi N, Ahmadi GA, Komijani M, Moghtaderi A, Bouzari M*, et al.* Prevalence of cytomegalovirus infection and its role in total immunoglobulin pattern in Iranian patients with different subtypes of multiple sclerosis. *New Microbiol* 2011, **34**(3)**:** 263-274.

166. Krametter D, Niederwieser G, Berghold A, Birnbaum G, Strasser-Fuchs S, Hartung HP*, et al.* Chlamydia pneumoniae in multiple sclerosis: humoral immune responses in serum and cerebrospinal fluid and correlation with disease activity marker. *Mult Scler* 2001, **7**(1)**:** 13-18.

167. van Noort JM, Bsibsi M, Gerritsen WH, van der Valk P, Bajramovic JJ, Steinman L*, et al.* Alphab-crystallin is a target for adaptive immune responses and a trigger of innate responses in preactive multiple sclerosis lesions. *J Neuropathol Exp Neurol* 2010, **69**(7)**:** 694-703.

168. Cid C, Garcia-Villanueva M, Salinas M, Alcazar A. Detection of anti-heat shock protein 90 beta (Hsp90beta) antibodies in cerebrospinal fluid. *J Immunol Methods* 2007, **318**(1-2)**:** 153-157.

169. Prabhakar S, Kurien E, Gupta RS, Zielinski S, Freedman MS. Heat shock protein immunoreactivity in CSF: correlation with oligoclonal banding and demyelinating disease. *Neurology* 1994, **44**(9)**:** 1644-1648.

170. Sakurai T, Kimura A, Yamada M, Koumura A, Hayashi Y, Tanaka Y*, et al.* Identification of antibodies as biological markers in serum from multiple sclerosis patients by immunoproteomic approach. *J Neuroimmunol* 2011, **233**(1-2)**:** 175-180.

171. Yokota S, Chiba S, Furuyama H, Fujii N. Cerebrospinal fluids containing anti-HSP70 autoantibodies from multiple sclerosis patients augment HSP70-induced proinflammatory cytokine production in monocytic cells. *J Neuroimmunol* 2010, **218**(1-2)**:** 129-133.

172. Williamson RA, Burgoon MP, Owens GP, Ghausi O, Leclerc E, Firme L*, et al.* Anti-DNA antibodies are a major component of the intrathecal B cell response in multiple sclerosis. *Proc Natl Acad Sci U S A* 2001, **98**(4)**:** 1793-1798.

173. Kimura A, Sakurai T, Koumura A, Yamada M, Hayashi Y, Tanaka Y*, et al.* High prevalence of autoantibodies against phosphoglycerate mutase 1 in patients with autoimmune central nervous system diseases. *J Neuroimmunol* 2010, **219**(1-2)**:** 105-108.

174. Banki K, Colombo E, Sia F, Halladay D, Mattson DH, Tatum AH*, et al.* Oligodendrocyte-specific expression and autoantigenicity of transaldolase in multiple sclerosis. *J Exp Med* 1994, **180**(5)**:** 1649-1663.

175. Esposito M, Venkatesh V, Otvos L, Weng Z, Vajda S, Banki K*, et al.* Human transaldolase and cross-reactive viral epitopes identified by autoantibodies of multiple sclerosis patients. *J Immunol* 1999, **163**(7)**:** 4027-4032.

176. Kolln J, Ren HM, Da RR, Zhang Y, Spillner E, Olek M*, et al.* Triosephosphate isomerase- and glyceraldehyde-3-phosphate dehydrogenase-reactive autoantibodies in the cerebrospinal fluid of patients with multiple sclerosis. *J Immunol* 2006, **177**(8)**:** 5652-5658.

177. Freedman MS, Laks J, Dotan N, Altstock RT, Dukler A, Sindic CJ. Anti-alpha-glucose-based glycan IgM antibodies predict relapse activity in multiple sclerosis after the first neurological event. *Mult Scler* 2009, **15**(4)**:** 422-430.

178. Schwarz M, Spector L, Gortler M, Weisshaus O, Glass-Marmor L, Karni A*, et al.* Serum anti-Glc(alpha1,4)Glc(alpha) antibodies as a biomarker for relapsing-remitting multiple sclerosis. *J Neurol Sci* 2006, **244**(1-2)**:** 59-68.

179. Brettschneider J, Jaskowski TD, Tumani H, Abdul S, Husebye D, Seraj H*, et al.* Serum anti-GAGA4 IgM antibodies differentiate relapsing remitting and secondary progressive multiple sclerosis from primary progressive multiple sclerosis and other neurological diseases. *J Neuroimmunol* 2009, **217**(1-2)**:** 95-101.

180. Yukitake M, Sueoka E, Sueoka-Aragane N, Sato A, Ohashi H, Yakushiji Y*, et al.* Significantly increased antibody response to heterogeneous nuclear

ribonucleoproteins in cerebrospinal fluid of multiple sclerosis patients but not in patients with human T-lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis. *J Neurovirol* 2008, **14**(2)**:** 130-135.

181. Sueoka E, Yukitake M, Iwanaga K, Sueoka N, Aihara T, Kuroda Y. Autoantibodies against heterogeneous nuclear ribonucleoprotein B1 in CSF of MS patients. *Ann Neurol* 2004, **56**(6)**:** 778-786.

182. Beyer NH, Milthers J, Bonde Lauridsen AM, Houen G, Lautrup Frederiksen J. Autoantibodies to the proteasome in monosymptomatic optic neuritis may predict progression to multiple sclerosis. *Scand J Clin Lab Invest* 2007, **67**(7)**:** 696-706.

183. Reindl M, Khantane S, Ehling R, Schanda K, Lutterotti A, Brinkhoff C*, et al.* Serum and cerebrospinal fluid antibodies to Nogo-A in patients with multiple sclerosis and acute neurological disorders. *J Neuroimmunol* 2003, **145**(1-2)**:** 139-147.

184. Erdag E, Tuzun E, Ugurel E, Cavus F, Sehitoglu E, Giris M*, et al.* Switch-associated protein 70 antibodies in multiple sclerosis: relationship between increased serum levels and clinical relapse. *Inflamm Res* 2012, **61**(9)**:** 927-930.

185. Archelos JJ, Trotter J, Previtali S, Weissbrich B, Toyka KV, Hartung HP. Isolation and characterization of an oligodendrocyte precursor-derived B-cell epitope in multiple sclerosis. *Ann Neurol* 1998, **43**(1)**:** 15-24.

186. Pandey S, Dioni I, Lambardi D, Real-Fernandez F, Peroni E, Pacini G*, et al.* Alpha Actinin is Specifically Recognized by Multiple Sclerosis Autoantibodies Isolated Using an N-Glucosylated Peptide Epitope. *Molecular & cellular proteomics : MCP* 2013, **12**(2)**:** 277-282.

187. Hvaring C, Vujicic S, Aasly JO, Feinstein DL, White LR, Boullerne AI. IgM to S-nitrosylated protein is found intrathecally in relapsing-remitting multiple sclerosis. *J Neuroimmunol* 2013, **256**(1-2)**:** 77-83.

188. Niehaus A, Shi J, Grzenkowski M, Diers-Fenger M, Archelos J, Hartung HP*, et al.* Patients with active relapsing-remitting multiple sclerosis synthesize antibodies recognizing oligodendrocyte progenitor cell surface protein: implications for remyelination. *Ann Neurol* 2000, **48**(3)**:** 362-371.

189. Zhou D, Srivastava R, Nessler S, Grummel V, Sommer N, Bruck W*, et al.* Identification of a pathogenic antibody response to native myelin oligodendrocyte glycoprotein in multiple sclerosis. *Proc Natl Acad Sci U S A* 2006, **103**(50)**:** 19057-19062.

190. Huizinga R, Heijmans N, Schubert P, Gschmeissner S, t Hart BA, Herrmann H*, et al.* Immunization with neurofilament light protein induces spastic paresis and axonal degeneration in Biozzi ABH mice. *J Neuropathol Exp Neurol* 2007, **66**(4)**:** 295-304.

191. Somers K, Hendriks JJ, Hupperts R, Zwanikken C, Verbeek MM, Stinissen P*, et al.* SPAG16 isoform 2 as a novel autoantigen in multiple sclerosis. *J Neuroimmunol* 2010, **228**(1-2)**:** 162.

192. Elliott C, Lindner M, Arthur A, Brennan K, Jarius S, Hussey J*, et al.* Functional identification of pathogenic autoantibody responses in patients with multiple sclerosis. *Brain* 2012, **135**(Pt 6)**:** 1819-1833.

193. Xu X, Denic A, Warrington AE, Bieber AJ, Rodriguez M. Therapeutics to promote CNS repair: a natural human neuron-binding IgM regulates membrane-raft dynamics and improves motility in a mouse model of multiple sclerosis. *J Clin Immunol* 2013, **33 Suppl 1:** S50-56.

194. Bielekova B, Martin R. Development of biomarkers in multiple sclerosis. *Brain* 2004, **127**(Pt 7)**:** 1463-1478.

195. Stoop MP, Dekker LJ, Titulaer MK, Burgers PC, Sillevis Smitt PA, Luider TM*, et al.* Multiple sclerosis-related proteins identified in cerebrospinal fluid by advanced mass spectrometry. *Proteomics* 2008, **8**(8)**:** 1576-1585.

196. Stoop MP, Dekker LJ, Titulaer MK, Lamers RJ, Burgers PC, Sillevis Smitt PA*, et al.* Quantitative matrix-assisted laser desorption ionization-fourier transform ion cyclotron resonance (MALDI-FT-ICR) peptide profiling and identification of multiple-sclerosis-related proteins. *Journal of proteome research* 2009, **8**(3)**:** 1404-1414.

197. Broadwater L, Pandit A, Clements R, Azzam S, Vadnal J, Sulak M*, et al.* Analysis of the mitochondrial proteome in multiple sclerosis cortex. *Biochim Biophys Acta* 2011, **1812**(5)**:** 630-641.

198. Robotti A, Natale M, Albo AG, Lis K, Perga S, Marnetto F*, et al.* Acute-phase proteins investigation based on lectins affinity capture prior to 2-DE separation: application to serum from multiple sclerosis patients. *Electrophoresis* 2010, **31**(17)**:** 2882-2893.

199. Noben JP, Dumont D, Kwasnikowska N, Verhaert P, Somers V, Hupperts R*, et al.* Lumbar cerebrospinal fluid proteome in multiple sclerosis: characterization by ultrafiltration, liquid chromatography, and mass spectrometry. *Journal of proteome research* 2006, **5**(7)**:** 1647-1657.

200. Stoop MP, Singh V, Dekker LJ, Titulaer MK, Stingl C, Burgers PC*, et al.* Proteomics comparison of cerebrospinal fluid of relapsing remitting and primary progressive multiple sclerosis. *PloS one* 2010, **5**(8)**:** e12442.

201. De Masi R, Pasca S, Scarpello R, Idolo A, De Donno A. The clinical potential of blood-proteomics in multiple sclerosis. *BMC neurology* 2013, **13**(1)**:** 45.

202. Stoop MP, Singh V, Stingl C, Martin R, Khademi M, Olsson T*, et al.* Effects of natalizumab treatment on the cerebrospinal fluid proteome of multiple sclerosis patients. *Journal of proteome research* 2013, **12**(3)**:** 1101-1107.

203. Ottervald J, Franzen B, Nilsson K, Andersson LI, Khademi M, Eriksson B*, et al.* Multiple sclerosis: Identification and clinical evaluation of novel CSF biomarkers. *Journal of proteomics* 2010, **73**(6)**:** 1117-1132.

204. Ramanathan M, Weinstock-Guttman B, Nguyen LT, Badgett D, Miller C, Patrick K*, et al.* In vivo gene expression revealed by cDNA arrays: the pattern in relapsing-remitting multiple sclerosis patients compared with normal subjects. *J Neuroimmunol* 2001, **116**(2)**:** 213-219.

205. Bomprezzi R, Ringner M, Kim S, Bittner ML, Khan J, Chen Y*, et al.* Gene expression profile in multiple sclerosis patients and healthy controls: identifying pathways relevant to disease. *Hum Mol Genet* 2003, **12**(17)**:** 2191-2199.

206. Infante-Duarte C, Weber A, Kratzschmar J, Prozorovski T, Pikol S, Hamann I*, et al.* Frequency of blood CX3CR1-positive natural killer cells correlates with disease activity in multiple sclerosis patients. *FASEB J* 2005, **19**(13)**:** 1902-1904.

207. Satoh J, Nakanishi M, Koike F, Miyake S, Yamamoto T, Kawai M*, et al.* Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis. *Neurobiol Dis* 2005, **18**(3)**:** 537-550.

208. Booth DR, Arthur AT, Teutsch SM, Bye C, Rubio J, Armati PJ*, et al.* Gene expression and genotyping studies implicate the interleukin 7 receptor in the pathogenesis of primary progressive multiple sclerosis. *J Mol Med (Berl)* 2005, **83**(10)**:** 822-830.

209. Lock C, Hermans G, Pedotti R, Brendolan A, Schadt E, Garren H*, et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med* 2002, **8**(5)**:** 500-508.

210. Wandinger KP, Sturzebecher CS, Bielekova B, Detore G, Rosenwald A, Staudt LM*, et al.* Complex immunomodulatory effects of interferon-beta in multiple sclerosis include the upregulation of T helper 1-associated marker genes. *Ann Neurol* 2001, **50**(3)**:** 349-357.

211. Comabella M, Lunemann JD, Rio J, Sanchez A, Lopez C, Julia E*, et al.* A type I interferon signature in monocytes is associated with poor response to interferon-beta in multiple sclerosis. *Brain* 2009, **132**(Pt 12)**:** 3353-3365.

212. Lim ET, Berger T, Reindl M, Dalton CM, Fernando K, Keir G*, et al.* Anti-myelin antibodies do not allow earlier diagnosis of multiple sclerosis. *Mult Scler* 2005, **11**(4)**:** 492-494.

213. Pelayo R, Tintore M, Montalban X, Rovira A, Espejo C, Reindl M*, et al.* Antimyelin antibodies with no progression to multiple sclerosis. *N Engl J Med* 2007, **356**(4)**:** 426-428.

214. Freedman M, Metzig C, Kappos L, Polman C, Edan G, Hartung HP*, et al.* Predictive nature of IgM anti-alpha-glucose serum biomarker for relapse activity and EDSS progression in CIS patients: a BENEFIT study analysis. *Mult Scler* 2011, **18**(7)**:** 966-973.

215. Chan A, Decard BF, Franke C, Grummel V, Zhou D, Schottstedt V*, et al.* Serum antibodies to conformational and linear epitopes of myelin oligodendrocyte glycoprotein are not elevated in the preclinical phase of multiple sclerosis. *Mult Scler* 2010, **16**(10)**:** 1189-1192.

216. Bosca I, Magraner MJ, Coret F, Alvarez-Cermeno JC, Simo-Castello M, Villar LM*, et al.* The risk of relapse after a clinically isolated syndrome is related to the pattern of oligoclonal bands. *J Neuroimmunol* 2010, **226**(1-2)**:** 143-146.

217. Villar LM, Sadaba MC, Roldan E, Masjuan J, Gonzalez-Porque P, Villarrubia N*, et al.* Intrathecal synthesis of oligoclonal IgM against myelin lipids predicts an aggressive disease course in MS. *J Clin Invest* 2005, **115**(1)**:** 187-194.

218. Zephir H, Lefranc D, Dubucquoi S, de Seze J, Boron L, Prin L*, et al.* Serum IgG repertoire in clinically isolated syndrome predicts multiple sclerosis. *Mult Scler* 2009, **15**(5)**:** 593-600.

219. Villar LM, Espino M, Costa-Frossard L, Muriel A, Jimenez J, Alvarez-Cermeno JC. High Levels Of Cerebrospinal Fluid Free Kappa Chains Predict Conversion To Multiple Sclerosis. *Clin Chim Acta* 2012, **413**(23-24)**:** 1813-1816.

220. Ignacio RJ, Liliana P, Edgardo C. Oligoclonal bands and MRI in clinically isolated syndromes: predicting conversion time to multiple sclerosis. *J Neurol* 2010, **257**(7)**:** 1188-1191.

221. Tintore M, Rovira A, Martinez MJ, Rio J, Diaz-Villoslada P, Brieva L*, et al.* Isolated demyelinating syndromes: comparison of different MR imaging criteria to predict conversion to clinically definite multiple sclerosis. *AJNR Am J Neuroradiol* 2000, **21**(4)**:** 702-706.

222. Masjuan J, Alvarez-Cermeno JC, Garcia-Barragan N, Diaz-Sanchez M, Espino M, Sadaba MC*, et al.* Clinically isolated syndromes: a new oligoclonal band test accurately predicts conversion to MS. *Neurology* 2006, **66**(4)**:** 576-578.

223. Miller DH, Ormerod IE, Rudge P, Kendall BE, Moseley IF, McDonald WI. The early risk of multiple sclerosis following isolated acute syndromes of the brainstem and spinal cord. *Ann Neurol* 1989, **26**(5)**:** 635-639.

224. Villar LM, Espino M, Roldan E, Marin N, Costa-Frossard L, Muriel A*, et al.* Increased peripheral blood CD5+ B cells predict earlier conversion to MS in high-risk clinically isolated syndromes. *Mult Scler* 2011, **17**(6)**:** 690-694.

225. Ristori G, Brescianini S, Pino A, Visconti A, Vittori D, Coarelli G*, et al.* Serum elements and oxidative status in clinically isolated syndromes: imbalance and predictivity. *Neurology* 2011, **76**(6)**:** 549-555.

226. Khademi M, Kockum I, Andersson ML, Iacobaeus E, Brundin L, Sellebjerg F*, et al.* Cerebrospinal fluid CXCL13 in multiple sclerosis: a suggestive prognostic marker for the disease course. *Mult Scler* 2011, **17**(3)**:** 335-343.

227. Brettschneider J, Czerwoniak A, Senel M, Fang L, Kassubek J, Pinkhardt E*, et al.* The chemokine CXCL13 is a prognostic marker in clinically isolated syndrome (CIS). *PloS one* 2010, **5**(8)**:** e11986.

228. Comabella M, Fernandez M, Martin R, Rivera-Vallve S, Borras E, Chiva C*, et al.* Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis. *Brain* 2010, **133**(Pt 4)**:** 1082-1093.

229. Tumani H, Lehmensiek V, Rau D, Guttmann I, Tauscher G, Mogel H*, et al.* CSF proteome analysis in clinically isolated syndrome (CIS): candidate markers for conversion to definite multiple sclerosis. *Neurosci Lett* 2009, **452**(2)**:** 214-217.

230. Martinez-Yelamos A, Rovira A, Sanchez-Valle R, Martinez-Yelamos S, Tintore M, Blanco Y*, et al.* CSF 14-3-3 protein assay and MRI as prognostic markers in patients with a clinically isolated syndrome suggestive of MS. *J Neurol* 2004, **251**(10)**:** 1278-1279.

231. Martinez-Yelamos A, Saiz A, Sanchez-Valle R, Casado V, Ramon JM, Graus F*, et al.* 14-3-3 protein in the CSF as prognostic marker in early multiple sclerosis. *Neurology* 2001, **57**(4)**:** 722-724.

232. Avsar T, Korkmaz D, Tutuncu M, Demirci NO, Saip S, Kamasak M*, et al.* Protein biomarkers for multiple sclerosis: semi-quantitative analysis of cerebrospinal fluid candidate protein biomarkers in different forms of multiple sclerosis. *Mult Scler* 2012, **18**(8)**:** 1081-1091.

233. Corvol JC, Pelletier D, Henry RG, Caillier SJ, Wang J, Pappas D*, et al.* Abrogation of T cell quiescence characterizes patients at high risk for multiple sclerosis after the initial neurological event. *Proc Natl Acad Sci U S A* 2008, **105**(33)**:** 11839-11844.

234. Frisullo G, Nociti V, Iorio R, Patanella AK, Marti A, Mirabella M*, et al.* The persistency of high levels of pSTAT3 expression in circulating CD4+ T cells from

CIS patients favors the early conversion to clinically defined multiple sclerosis. *J Neuroimmunol* 2008, **205**(1-2)**:** 126-134.

235. Wattjes MP, Harzheim M, Lutterbey GG, Bogdanow M, Schmidt S, Schild HH*, et al.* Prognostic value of high-field proton magnetic resonance spectroscopy in patients presenting with clinically isolated syndromes suggestive of multiple sclerosis. *Neuroradiology* 2008, **50**(2)**:** 123-129.

236. Dalton CM, Chard DT, Davies GR, Miszkiel KA, Altmann DR, Fernando K*, et al.* Early development of multiple sclerosis is associated with progressive grey matter atrophy in patients presenting with clinically isolated syndromes. *Brain* 2004, **127**(Pt 5)**:** 1101-1107.

237. Barkhof F, Filippi M, Miller DH, Scheltens P, Campi A, Polman CH*, et al.* Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. *Brain* 1997, **120 (Pt 11):** 2059-2069.

238. Bennett JL, Haubold K, Ritchie AM, Edwards SJ, Burgoon M, Shearer AJ*, et al.* CSF IgG heavy-chain bias in patients at the time of a clinically isolated syndrome. *J Neuroimmunol* 2008, **199**(1-2)**:** 126-132.

239. Di Pauli F, Reindl M, Ehling R, Schautzer F, Gneiss C, Lutterotti A*, et al.* Smoking is a risk factor for early conversion to clinically definite multiple sclerosis. *Mult Scler* 2008, **14**(8)**:** 1026-1030.

240. Lalive PH, Menge T, Delarasse C, Della Gaspera B, Pham-Dinh D, Villoslada P*, et al.* Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis. *Proc Natl Acad Sci U S A* 2006, **103**(7)**:** 2280-2285.

241. Quintana FJ, Farez MF, Viglietta V, Iglesias AH, Merbl Y, Izquierdo G*, et al.* Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis. *Proc Natl Acad Sci U S A* 2008, **105**(48)**:** 18889-18894.

242. Quintana FJ, Farez MF, Izquierdo G, Lucas M, Cohen IR, Weiner HL. Antigen microarrays identify CNS-produced autoantibodies in RRMS. *Neurology* 2012, **78**(8)**:** 532-539.

243. Beyer NH, Lueking A, Kowald A, Frederiksen JL, Heegaard NH. Investigation of autoantibody profiles for cerebrospinal fluid biomarker discovery in patients with relapsing-remitting multiple sclerosis. *J Neuroimmunol* 2012, **242**(1-2)**:** 26-32.

244. Lolli F, Mulinacci B, Carotenuto A, Bonetti B, Sabatino G, Mazzanti B*, et al.* An N-glucosylated peptide detecting disease-specific autoantibodies, biomarkers of multiple sclerosis. *Proc Natl Acad Sci U S A* 2005, **102**(29)**:** 10273-10278.

245. Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 1985, **228**(4705)**:** 1315-1317.

246. Smith GP, Petrenko VA. Phage Display. *Chem Rev* 1997, **97**(2)**:** 391-410.

247. Hufton SE, Moerkerk PT, Meulemans EV, de Bruine A, Arends JW, Hoogenboom HR. Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. *J Immunol Methods* 1999, **231**(1-2)**:** 39-51.

248. Russel M, Lowman HB, Clackson T. *Introduction to phage biology and phage display*. Oxford University Press, 2004.

249. Cortese I, Capone S, Luchetti S, Cortese R, Nicosia A. Cross-reactive phage-displayed mimotopes lead to the discovery of mimicry between HSV-1 and a brain-specific protein. *J Neuroimmunol* 2001, **113**(1)**:** 119-128.

250. Hoogenboom HR, de Bruine AP, Hufton SE, Hoet RM, Arends JW, Roovers RC. Antibody phage display technology and its applications. *Immunotechnology* 1998, **4**(1)**:** 1-20.

251. Rodi DJ, Makowski L. Phage-display technology--finding a needle in a vast molecular haystack. *Curr Opin Biotechnol* 1999, **10**(1)**:** 87-93.

252. Cortese I, Tafi R, Grimaldi LM, Martino G, Nicosia A, Cortese R. Identification of peptides specific for cerebrospinal fluid antibodies in multiple sclerosis by using phage libraries. *Proc Natl Acad Sci U S A* 1996, **93**(20)**:** 11063-11067.

253. Cortese I, Capone S, Luchetti S, Grimaldi LM, Nicosia A, Cortese R. CSF-enriched antibodies do not share specificities among MS patients. *Mult Scler* 1998, **4**(3)**:** 118-123.

254. Jolivet-Reynaud C, Perron H, Ferrante P, Becquart L, Dalbon P, Mandrand B. Specificities of multiple sclerosis cerebrospinal fluid and serum antibodies against mimotopes. *Clin Immunol* 1999, **93**(3)**:** 283-293.

255. Rand KH, Houck H, Denslow ND, Heilman KM. Molecular approach to find target(s) for oligoclonal bands in multiple sclerosis. *J Neurol Neurosurg Psychiatry* 1998, **65**(1)**:** 48-55.

256. Fujimori J, Nakashima I, Fujihara K, Misu T, Sato S, Itoyama Y. Epitope analysis of cerebrospinal fluid IgG in Japanese multiple sclerosis patients using phage display method. *Mult Scler Int* 2011, **2011:** 353417.

257. Yu X, Gilden DH, Ritchie AM, Burgoon MP, Keays KM, Owens GP. Specificity of recombinant antibodies generated from multiple sclerosis cerebrospinal fluid probed with a random peptide library. *J Neuroimmunol* 2006, **172**(1-2)**:** 121-131.

258. Owens GP, Burgoon MP, Devlin ME, Gilden DH. Strategies to identify sequences or antigens unique to multiple sclerosis. *Mult Scler* 1996, **2**(4)**:** 184-194.

259. Hufton SE, Moerkerk P, de Bruine A, Arends JW, Hoogenboom HR. Serological antigen selection of phage displayed colorectal tumour cDNA libraries. *Biochem Soc Trans* 1998, **26**(1)**:** S5.

260. Somers VA, Brandwijk RJ, Joosten B, Moerkerk PT, Arends JW, Menheere P*, et al.* A panel of candidate tumor antigens in colorectal cancer revealed by the serological selection of a phage displayed cDNA expression library. *J Immunol* 2002, **169**(5)**:** 2772-2780.

261. Cleutjens KB, Faber BC, Rousch M, van Doorn R, Hackeng TM, Vink C*, et al.* Noninvasive diagnosis of ruptured peripheral atherosclerotic lesions and myocardial infarction by antibody profiling. *J Clin Invest* 2008, **118**(8)**:** 2979-2985.

262. Somers K, Geusens P, Elewaut D, De Keyser F, Rummens JL, Coenen M*, et al.* Novel autoantibody markers for early and seronegative rheumatoid arthritis. *J Autoimmun* 2011, **36**(1)**:** 33-46.

263. Soares MB, Bonaldo MF, Jelene P, Su L, Lawton L, Efstratiadis A. Construction and characterization of a normalized cDNA library. *Proc Natl Acad Sci U S A* 1994, **91**(20)**:** 9228-9232.

264. Becker KG, Mattson DH, Powers JM, Gado AM, Biddison WE. Analysis of a sequenced cDNA library from multiple sclerosis lesions. *J Neuroimmunol* 1997, **77**(1)**:** 27-38.

265. Govarts C, Somers K, Hupperts R, Stinissen P, Somers V. Analysis of antibody reactivity in paired cerebrospinal fluid and serum of a relapsing remitting multiple sclerosis patient. *Autoimmunity* 2009, **42**(8)**:** 699-704.

266. Awad A, Hemmer B, Hartung HP, Kieseier B, Bennett JL, Stuve O. Analyses of cerebrospinal fluid in the diagnosis and monitoring of multiple sclerosis. *J Neuroimmunol* 2010, **219**(1-2)**:** 1-7.

267. Szmyrka-Kaczmarek M, Pokryszko-Dragan A, Pawlik B, Gruszka E, Korman L, Podemski R*, et al.* Antinuclear and antiphospholipid antibodies in patients with multiple sclerosis. *Lupus* 2012, **21**(4)**:** 412-420.

268. Lunemann JD, Ascherio A. Immune responses to EBNA1: biomarkers in MS? *Neurology* 2009, **73**(1)**:** 13-14.

269. Govarts C, Somers K, Hupperts R, Stinissen P, Somers V. Exploring cDNA phage display for autoantibody profiling in the serum of multiple sclerosis patients: optimization of the selection procedure. *Ann N Y Acad Sci* 2007, **1109:** 372-384.

270. Somers K, Stinissen P, Somers V. Optimization of high-throughput autoantibody profiling for the discovery of novel antigenic targets in rheumatoid arthritis. *Ann N Y Acad Sci* 2009, **1173:** 92-102.

271. Somers V, Govarts C, Hellings N, Hupperts R, Stinissen P. Profiling the autoantibody repertoire by serological antigen selection. *J Autoimmun* 2005, **25**(3)**:** 223-228.

272. Rovaris M, Confavreux C, Furlan R, Kappos L, Comi G, Filippi M. Secondary progressive multiple sclerosis: current knowledge and future challenges. *Lancet neurology* 2006, **5**(4)**:** 343-354.

273. Fredrikson S, Baig S, Link H. Immunoglobulin producing cells in bone marrow and blood of patients with multiple sclerosis and controls. *J Neurol Neurosurg Psychiatry* 1991, **54**(5)**:** 412-414.

274. Mahajan SS, Little MM, Vazquez R, Wilson AC. Interaction of HCF-1 with a cellular nuclear export factor. *J Biol Chem* 2002, **277**(46)**:** 44292-44299.

275. Black JA, Newcombe J, Waxman SG. Astrocytes within multiple sclerosis lesions upregulate sodium channel Nav1.5. *Brain* 2010, **133**(Pt 3)**:** 835-846.

276. Cavone L, Chiarugi A. Targeting poly(ADP-ribose) polymerase-1 as a promising approach for immunomodulation in multiple sclerosis? *Trends Mol Med* 2012, **18**(2)**:** 92-100.

277. Xiao Q, Hartzell HC, Yu K. Bestrophins and retinopathies. *Pflugers Arch* 2010, **460**(2)**:** 559-569.

278. Steinberg SJ, Dodt G, Raymond GV, Braverman NE, Moser AB, Moser HW. Peroxisome biogenesis disorders. *Biochim Biophys Acta* 2006, **1763**(12)**:** 1733-1748.

279. Ng B, Yang F, Huston DP, Yan Y, Yang Y, Xiong Z*, et al.* Increased noncanonical splicing of autoantigen transcripts provides the structural basis for expression of untolerized epitopes. *J Allergy Clin Immunol* 2004, **114**(6)**:** 1463-1470.

280. Evsyukova I, Somarelli JA, Gregory SG, Garcia-Blanco MA. Alternative splicing in multiple sclerosis and other autoimmune diseases. *RNA Biol* 2010, **7**(4)**:** 462-473.

281. Antel J, Antel S, Caramanos Z, Arnold DL, Kuhlmann T. Primary progressive multiple sclerosis: part of the MS disease spectrum or separate disease entity? *Acta Neuropathol* 2012, **123**(5)**:** 627-638.

282. Miller DH, Leary SM. Primary-progressive multiple sclerosis. *Lancet neurology* 2007, **6**(10)**:** 903-912.

283. Rouwette M, Somers K, Govarts C, De Deyn PP, Hupperts R, Van Wijmeersch B*, et al.* Novel cerebrospinal fluid and serum autoantibody targets for clinically isolated syndrome. *J Neurochem* 2012, **123**(4)**:** 568-577.

284. Briggs FB, Goldstein BA, McCauley JL, Zuvich RL, De Jager PL, Rioux JD*, et al.* Variation within DNA repair pathway genes and risk of multiple sclerosis. *Am J Epidemiol* 2010, **172**(2)**:** 217-224.

285. Vladimirova O, O'Connor J, Cahill A, Alder H, Butunoi C, Kalman B. Oxidative damage to DNA in plaques of MS brains. *Mult Scler* 1998, **4**(5)**:** 413-418.

286. Vanheel A, Daniels R, Plaisance S, Baeten K, Hendriks JJ, Leprince P*, et al.* Identification of protein networks involved in the disease course of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. *PloS one* 2012, **7**(4)**:** e35544.

287. Gatfield J, Albrecht I, Zanolari B, Steinmetz MO, Pieters J. Association of the leukocyte plasma membrane with the actin cytoskeleton through coiled coil-mediated trimeric coronin 1 molecules. *Mol Biol Cell* 2005, **16**(6)**:** 2786-2798.

288. Mueller P, Massner J, Jayachandran R, Combaluzier B, Albrecht I, Gatfield J*, et al.* Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering. *Nature immunology* 2008, **9**(4)**:** 424-431.

289. Mugnier B, Nal B, Verthuy C, Boyer C, Lam D, Chasson L*, et al.* Coronin-1A links cytoskeleton dynamics to TCR alpha beta-induced cell signaling. *PloS one* 2008, **3**(10)**:** e3467.

290. Ferrari G, Langen H, Naito M, Pieters J. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell* 1999, **97**(4)**:** 435-447.

291. Jayachandran R, Sundaramurthy V, Combaluzier B, Mueller P, Korf H, Huygen K*, et al.* Survival of mycobacteria in macrophages is mediated by coronin 1-dependent activation of calcineurin. *Cell* 2007, **130**(1)**:** 37-50.

292. Holtta-Vuori M, Vainio S, Kauppi M, Van Eck M, Jokitalo E, Ikonen E. Endosomal actin remodeling by coronin-1A controls lipoprotein uptake and degradation in macrophages. *Circ Res* 2012, **110**(3)**:** 450-455.

293. Grogan A, Reeves E, Keep N, Wientjes F, Totty NF, Burlingame AL*, et al.* Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *J Cell Sci* 1997, **110 (Pt 24):** 3071-3081.

294. Shiow LR, Roadcap DW, Paris K, Watson SR, Grigorova IL, Lebet T*, et al.* The actin regulator coronin 1A is mutant in a thymic egress-deficient mouse strain and in a patient with severe combined immunodeficiency. *Nature immunology* 2008, **9**(11)**:** 1307-1315.

295. Shiow LR, Paris K, Akana MC, Cyster JG, Sorensen RU, Puck JM. Severe combined immunodeficiency (SCID) and attention deficit hyperactivity disorder (ADHD) associated with a Coronin-1A mutation and a chromosome 16p11.2 deletion. *Clin Immunol* 2009, **131**(1)**:** 24-30.

296. Moshous D, Martin E, Carpentier W, Lim A, Callebaut I, Canioni D*, et al.* Whole-exome sequencing identifies Coronin-1A deficiency in 3 siblings with immunodeficiency and EBV-associated B-cell lymphoproliferation. *J Allergy Clin Immunol* 2013.

297. Siegmund K, Zeis T, Kunz G, Rolink T, Schaeren-Wiemers N, Pieters J. Coronin 1-mediated naive T cell survival is essential for the development of autoimmune encephalomyelitis. *J Immunol* 2011, **186**(6)**:** 3452-3461.

298. Kaminski S, Hermann-Kleiter N, Meisel M, Thuille N, Cronin S, Hara H*, et al.* Coronin 1A is an essential regulator of the TGFbeta receptor/SMAD3 signaling pathway in Th17 CD4(+) T cells. *J Autoimmun* 2011, **37**(3)**:** 198-208.

299. Xu W, Yuan Z, Gao X. Immunoproteomic analysis of the antibody response obtained in mouse following vaccination with a T-cell vaccine. *Proteomics* 2011, **11**(22)**:** 4368-4375.

300. Kallenberg CG. Pathogenesis of ANCA-associated vasculitides. *Ann Rheum Dis* 2011, **70 Suppl 1:** i59-63.

301. Kurita N, Mise N, Fujii A, Mori M, Sai K, Nishi T*, et al.* Myeloperoxidase-antineutrophil cytoplasmic antibody-associated crescentic glomerulonephritis with rheumatoid arthritis: a comparison of patients without rheumatoid arthritis. *Clinical and experimental nephrology* 2010, **14**(4)**:** 325-332.

302. Roozendaal C, Kallenberg CG. Are anti-neutrophil cytoplasmic antibodies (ANCA) clinically useful in inflammatory bowel disease (IBD)? *Clin Exp Immunol* 1999, **116**(2)**:** 206-213.

303. Kettritz R. How anti-neutrophil cytoplasmic autoantibodies activate neutrophils. *Clin Exp Immunol* 2012, **169**(3)**:** 220-228.

304. Racanelli V, Prete M, Musaraj G, Dammacco F, Perosa F. Autoantibodies to intracellular antigens: generation and pathogenetic role. *Autoimmunity reviews* 2011, **10**(8)**:** 503-508.

305. Tintore M, Rovira A, Rio J, Tur C, Pelayo R, Nos C*, et al.* Do oligoclonal bands add information to MRI in first attacks of multiple sclerosis? *Neurology* 2008, **70**(13 Pt 2)**:** 1079-1083.

306. Somers K. *Novel molecular markers for rheumatoid arthritis and multiple sclerosis by application of phage display*, 2009.

307. Virtanen JO, Pietilainen-Nicklen J, Uotila L, Farkkila M, Vaheri A, Koskiniemi M. Intrathecal human herpesvirus 6 antibodies in multiple sclerosis and other demyelinating diseases presenting as oligoclonal bands in cerebrospinal fluid. *J Neuroimmunol* 2011, **237**(1-2)**:** 93-97.

308. Zhang Z, Kostetskii I, Tang W, Haig-Ladewig L, Sapiro R, Wei Z*, et al.* Deficiency of SPAG16L causes male infertility associated with impaired sperm motility. *Biol Reprod* 2006, **74**(4)**:** 751-759.

309. Zhang Z, Sapiro R, Kapfhamer D, Bucan M, Bray J, Chennathukuzhi V*, et al.* A sperm-associated WD repeat protein orthologous to Chlamydomonas PF20 associates with Spag6, the mammalian orthologue of Chlamydomonas PF16. *Mol Cell Biol* 2002, **22**(22)**:** 7993-8004.

310. Bornstein NM, Aronovich B, Korczyn AD, Shavit S, Michaelson DM, Chapman J. Antibodies to brain antigens following stroke. *Neurology* 2001, **56**(4)**:** 529-530.

311. Pruss H, Schwab JM, Derst C, Gortzen A, Veh RW. Neurofascin as target of autoantibodies in Guillain-Barre syndrome. *Brain* 2011, **134**(Pt 5)**:** e173; author reply e174.

312. Knevel R, Klein K, Somers K, Ospelt C, Houwing-Duistermaat JJ, van Nies J*, et al.* Genome-Wide Association Study On the Severity of Joint Destruction in Autoantibody Positive Rheumatoid Arthritis Identifies a Role for Sperm Associated Antigen 16. *Arthritis Rheum* 2012, **64**(10)**:** S1132-S1132.

313. Diez J, Park Y, Zeller M, Brown D, Garza D, Ricordi C*, et al.* Differential splicing of the IA-2 mRNA in pancreas and lymphoid organs as a permissive genetic mechanism for autoimmunity against the IA-2 type 1 diabetes autoantigen. *Diabetes* 2001, **50**(4)**:** 895-900.

314. Larman HB, Laserson U, Querol L, Verhaeghen K, Solimini NL, Xu GJ, *et al.* PhIP-Seq characterization of autoantibodies from patients with multiple sclerosis, type 1 diabetes and rheumatoid arthritis. *J Autoimmun* 2013.

315. von Budingen HC, Kuo TC, Sirota M, van Belle CJ, Apeltsin L, Glanville J, *et al.* B cell exchange across the blood-brain barrier in multiple sclerosis. *J Clin Invest* 2012, **122**(12)**:** 4533-4543.

316. Obermeier B, Lovato L, Mentele R, Bruck W, Forne I, Imhof A, *et al.* Related B cell clones that populate the CSF and CNS of patients with multiple sclerosis produce CSF immunoglobulin. *J Neuroimmunol* 2011, **233**(1-2)**:** 245-248.

317. Alarcon-Segovia D, Ruiz-Arguelles A, Fishbein E. Antibody to nuclear ribonucleoprotein penetrates live human mononuclear cells through Fc receptors. *Nature* 1978, **271**(5640)**:** 67-69.

318. Song YC, Sun GH, Lee TP, Huang JC, Yu CL, Chen CH, *et al.* Arginines in the CDR of anti-dsDNA autoantibodies facilitate cell internalization via electrostatic interactions. *Eur J Immunol* 2008, **38**(11)**:** 3178-3190.

319. Jang JY, Jeong JG, Jun HR, Lee SC, Kim JS, Kim YS, *et al.* A nucleic acid-hydrolyzing antibody penetrates into cells via caveolae-mediated endocytosis, localizes in the cytosol and exhibits cytotoxicity. *Cell Mol Life Sci* 2009, **66**(11-12)**:** 1985-1997.

320. Selter RC, Biberacher V, Grummel V, Buck D, Eienbroker C, Oertel WH, *et al.* Natalizumab treatment decreases serum IgM and IgG levels in multiple sclerosis patients. *Mult Scler* 2013, **Epub ahead of print**.

321. Garren H, Robinson WH, Krasulova E, Havrdova E, Nadj C, Selmaj K, *et al.* Phase 2 trial of a DNA vaccine encoding myelin basic protein for multiple sclerosis. *Ann Neurol* 2008, **63**(5)**:** 611-620.

322. Hudson AM, Cooley L. Phylogenetic, structural and functional relationships between WD- and Kelch-repeat proteins. *Subcell Biochem* 2008, **48:** 6-19.

323. Gandhi M, Goode BL. Coronin: the double-edged sword of actin dynamics. *Subcell Biochem* 2008, **48:** 72-87.

324. Nal B, Carroll P, Mohr E, Verthuy C, Da Silva MI, Gayet O, *et al.* Coronin-1 expression in T lymphocytes: insights into protein function during T cell development and activation. *Int Immunol* 2004, **16**(2)**:** 231-240.

325. Foger N, Rangell L, Danilenko DM, Chan AC. Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. *Science* 2006, **313**(5788)**:** 839-842.

326. Yokoyama K, Kaji H, He J, Tanaka C, Hazama R, Kamigaki T*, et al.* Rab27a negatively regulates phagocytosis by prolongation of the actin-coating stage around phagosomes. *J Biol Chem* 2011, **286**(7)**:** 5375-5382.

Curriculum Vitae

Myrthe Rouwette werd geboren op 12 maart 1985 in Weert. In 2003 behaalde zij haar Gymnasium diploma aan de scholengemeenschap Sint Ursula te Horn. Vervolgens startte zij haar studie Medische Biologie aan de Radboud Universiteit Nijmegen, waar zij in 2006 haar bachelor Medische Biologie behaalde. Aansluitend behaalde zij haar master Medische Biologie (onderzoeksvariant) in 2008. Tijdens haar master Medische Biologie heeft zij stage gelopen op de afdelingen Antropogenetica (o.l.v. Prof Dr. Hannie Kremer) en Kindergeneeskunde (o.l.v. Dr. Richard Rodenburg) van het Universitair Medisch Centrum Sint Radboud. In september 2008 startte zij haar doctoraat aan het Biomedisch Onderzoeksinstituut van de Universiteit Hasselt, waarin zij de antilichaamrespons in clinically isolated syndrome en multiple sclerose onderzocht. Tijdens dit doctoraat behaalde ze het certificaat van de doctoral school for Medicine and Life Sciences en volgde ze de cursussen qPCR experimental design and data analysis, parametrische en niet-parametrische statische methoden voor de levenswetenschappen (deel I en II), academisch engels, scientific writing and oral presentation, lab book taking, good scientific conduct en lab safety. Bovendien ontving ze reisbeurzen om de volgende congressen bij te wonen: ESNI 2009 (Istanbul) en ECTRIMS 2011 (Amsterdam) en won ze presentatie en posterprijzen, respectievelijk op de Immunology of autoimmunity meeting 2009 (Leuven) en Biomedica 2012 (Luik).

Bibliography

Publications

Rouwette M, Somers K, Govarts C, De Deyn PP, Hupperts R, Van Wijmeersch B, De Jong BA, Verbeek MM, van Pesch V, Sindic C, Villar LM, Álvarez-Cermeño JC, Stinissen P and Somers V
Novel cerebrospinal fluid and serum autoantibody targets for clinically isolated syndrome
J Neurochem. 2012;123(4):568-77

Rouwette M, Noben JP, Van Horssen J, Van Wijmeersch B, Hupperts R, Jongen PJ, Verbeek MM, De Deyn PP, Stinissen P and Somers V
Identification of coronin-1a as a novel antibody target for clinically isolated syndrome and multiple sclerosis
J Neurochem, 2013;126(4):483-492

Rouwette M, et *al*
Comparison of a novel candidate CIS marker to a panel of MS antibody targets in serum from CIS patients.
In preparation

de Bock L[*], Somers K[*], Fraussen J, Hendriks J, van Horssen J, **Rouwette M**, Hellings N, Hupperts R, Jongen P, Damoiseaux J, Verbeek M, De Deyn P, Villar LM, Álvarez-Cermeño JC, D'Hooghe M, Van Wijmeersch B, Stinissen P and Somers V
Sperm-associated antigen 16 (SPAG16): a novel autoantigen in multiple sclerosis
In preparation

Somers K and Fraussen J, **Rouwette M**, Govarts C, Stinissen P and Somers V
B cells and autoantibodies in multiple sclerosis.
In: Multiple Sclerosis: Causes, Diagnosis and management: 2011

de Heer AM, Collin RW, Huygen PL, Schraders M, Oostrik J, **Rouwette M**, Kunst HP, Kremer H, Cremers CW
Progressive sensorineural hearing loss and normal vestibular function in a Dutch DFNB7/11 family
with a novel mutation in TMC1
Audiol Neurootol. 2011;16(2):93-105

Published abstracts

Rouwette M, Somers K, Govarts C, De Deyn PP, Hupperts R, De Jong BA, Verbeek MM, Van Pesch V, Sindic C, Villar LM, Alvarez-Cermeno JC, Stinissen, P, and Somers V
Novel cerebrospinal fluid and serum autoantibody targets for clinically isolated syndrome
J Neuroimmunol, 2012, 253(1-2). P22-22

Rouwette M, Somers K, Govarts C, Hupperts R, Van Wijmeersch B, De Jong BA, Verbeek MM, Van Pesch V, Sindic C, Villar LM, Stinissen P and Somers V
Novel potential autoantibody biomarkers for clinically isolated syndrome
Mult Scler, 2011, 17(10). P S273-274

Rouwette M, Somers K, Govarts C, Hupperts R, Van Wijmeersch B, Zwanikken C, Verbeek MM, Stinissen P and Somers V
Identification of novel potential autoantibody biomarkers in multiple sclerosis
J Neuroimmunol, 2010, 228(1-2). P158-158

Oral presentations

Rouwette M, Somers K, Govarts C, Hupperts R, van Wijmeersch B, Zwanikken C, Verbeek MM, Stinissen P and Somers V
Profiling the autoantibody response in early multiple sclerosis and clinically isolated syndrome by use of serological antigen selection.
MS research days 2009, 12/11/09-13/11/09, Groningen, the Netherlands

Rouwette M, Somers K, Govarts C, Hupperts R, van Wijmeersch B, Zwanikken C, Verbeek MM, Stinissen P and Somers V
Profiling the autoantibody response in early multiple sclerosis and clinically isolated syndrome by use of serological antigen selection
Immunology of Autoimmunity meeting 2009, 16/11/2009, Leuven, Belgium

Rouwette M, Somers K, Govarts C, Hupperts R, van Wijmeersch B, de Jong BA, Verbeek MM, van Pesch V, Sindic C, Stinissen P and Somers V
Novel potential autoantibody biomarkers for clinically isolated syndrome
Minisymposium on Neuroimmunology, 20/05/2011, Brussels, Belgium

Rouwette M, Somers K, Govarts C, Hupperts R, van Wijmeersch B, de Jong BA, Verbeek MM, van Pesch V, Sindic C, Villar LM, Stinissen P and Somers V
Discovery of novel antigenic targets for clinically isolated syndrome
10th Dresden symposium on autoantibodies,22/09/11-25/09/11, Dresden, Germany

Rouwette M, Somers K, Hupperts R, Van Wijmeersch B, De Jong BA, Verbeek MM, Van Pesch V, Sindic C, Villar LM, Álvarez-Cermeño JC, De Deyn PP, Stinissen P and Somers V
Identification of novel antigens for clinically isolated syndrome
Biomedica, 2012, 18/04/12-19/04/12, Liège, Belgium

Rouwette M, Somers K, Hupperts R, Van Wijmeersch B, de Jong BA, Verbeek MM, Van Pesch V, Sindic C, Villar LM, Álvarez-Cermeño JC, De Deyn PP, Stinissen P and Somers V
Identification of novel antigens for clinically isolated syndrome
NUBIN, 14/06/12-15/06/12, Amsterdam, the Netherlands

Poster presentations

Rouwette M, Somers K, Govarts C, Hupperts R, Van Wijmeersch B, Zwanikken C, Verbeek MM, Stinissen P and Somers V

Identification of biomarkers for clinically isolated syndrome

- Biomedica 2010, 17/03/2010-18/03/2010, Aachen, Germany

Rouwette M, Somers K, Govarts C, Hupperts R, Van Wijmeersch B, Zwanikken C, Verbeek MM, Stinissen P and Somers V

Identification of novel potential antibody biomarkers in early multiple sclerosis

- 14th euron PhD days, 7/10/2010-8/10/2010, Diepenbeek, Belgium
- BSCDB meeting, 22/10/2010, Diepenbeek, Belgium
- ISNI, 26/10/10-30/10/10, Sitges, Spain
- FWO-WOG meeting, 9/12/2010, Diepenbeek, Belgium
- MS research days 2010, 14/12/2010-15/12/2010, Alphen aan den Rijn, the Netherlands

Rouwette M, Somers K, Govarts C, Hupperts R, Van Wijmeersch B, de Jong BA, Verbeek MM, Van Pesch V, Sindic C, Stinissen P and Somers V

Novel antigenic targets for early and progressive multiple sclerosis

- Knowledge for growth, 5/5/11, Gent, Belgium

Rouwette M, Somers K, Hupperts R, Van Wijmeersch B, de Jong BA, Verbeek MM, Van Pesch V, Sindic C, Villar LM, Álvarez-Cermeño JC, De Deyn PP, Stinissen P and Somers V

Discovery of novel antigenic targets for clinically isolated syndrome

- 10th Dresden symposium on autoantibodies,22/09/11-25/09/11, Dresden, Germany
- ECTRIMS/ACTRIMS, 19/10/2011-22/10/2011, Amsterdam, the Netherlands
- BIS meeting, 18/11/2011, Diepenbeek, Belgium
- FWO-WOG meeting, 1/12/2011, Brussels, Belgium
- PhD symposium Doctoral School for Medicine and Life Sciences, 09/02/2012, Diepenbeek Belgium

Rouwette M, Somers K, Govarts C, Hupperts R, Van Wijmeersch B, de Jong BA, Verbeek MM, Van Pesch V, Sindic C, Villar LM, Álvarez-Cermeño JC, De Deyn PP, Stinissen P and Somers V
Identification of novel antigens for clinically isolated syndrome

- Biomedica, 2012, 18/04/12-19/04/12, Liège, Belgium
- ISNI, 4/11/2012-8/11/2012, Boston, USA

Awards

Poster award
Biomedica, 18/04/2012-19/04/2012, Liège, Belgium

Travel grant
ECTRIMS/ACTRIMS, 19/10/11-22/10/11, Amsterdam, the Netherlands

Science communication award
Immunology of autoimmunity meeting, 16/11/2009, Leuven, Belgium

Travel grant
ESNI, 01/09/2009-04/09/2009, Istanbul, Turkey

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"Science, you don't know, looks like magic."

Christopher Moore