cDNA phage display as a tool to analyze antibody reactivity in multiple sclerosis

Proefschrift voorgelegd tot het behalen van de graad van Doctor in de Biomedische Wetenschappen, te verdedigen door:

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not “Eureka!” (I found it!) but rather “hmm... that's funny ...”

*Isaac Asimov*
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List of Abbreviations

2D-GE  two-dimensional gel electrophoresis
AA    amino acids
aBC   αB-crystallin
AGA   anti-ganglioside antibody
AN2   progenitor cell-surface glycoprotein
AP    alkaline phosphatase
APC   antigen presenting cell
ApoE  apolipoprotein E
APRIL a proliferating inducing ligand
BAFF  B cell-activating factor
BBB   blood-brain barrier
BDNF  brain-derived neurotrophic factor
BLAST Basic Local Alignment and Search Tool
BMK1  big MAP kinase 1
BRRF  early Epstein-Barr virus protein
BSA   bovine serum albumin
CD    cluster of differentiation
CDMS  clinically definite multiple sclerosis
cDNA  complementary deoxyribonucleic acid
CDR   complementarity-determining region
CFU   colony forming units
CIS   clinically isolated syndrome
CNPase 2',3'-cyclic nucleotide 3' phosphodiesterase
CNS   central nervous system
CSF   cerebrospinal fluid
DAB   3,3'-Diaminobenzidine
dNTP  deoxyribonucleotide triphosphate
E. coli Escherichia coli
EAE   experimental autoimmune encephalomyelitis
EBNA  Epstein-Barr nuclear antigen
EBV   Epstein-Barr virus
EDSS  expanded disability status scale
ELISA enzyme-linked immunosorbent assay
ERK   extracellular signal-regulated kinase
EST   expressed sequence tag
FDA   Food and drug administration
FW    forward primer
GA    glatiramer acetate
gVI   gene VI
HBV   hepatitis B virus
HC    healthy control
HCV   hepatitis C virus
HHV   human herpes virus
HLA   human leukocyte antigen
HRP   horseradish peroxidase
Hsp   heat shock protein
HTLV  human T-lymphotropic virus
IEF   iso-electric focusing
IFN   interferon
Ig    immunoglobulin
IL    interleukin
LS    lipid-specific
mAb   monoclonal antibody
MAG   myelin-associated-glycoprotein
MAPK  mitogen activated protein kinase
MBP   myelin basic protein
MCS   multiple cloning site
MHC   major histo-compatibility complex
MMP   matrix metalloproteinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRZ</td>
<td>measles, rubella, varicella zoster</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>NC</td>
<td>negative control</td>
</tr>
<tr>
<td>NCB1</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NCL</td>
<td>neuronal cell line</td>
</tr>
<tr>
<td>ND</td>
<td>neurodegenerative disease</td>
</tr>
<tr>
<td>NFH</td>
<td>neurofilament heavy chain</td>
</tr>
<tr>
<td>NFL</td>
<td>neurofilament light chain</td>
</tr>
<tr>
<td>NFM</td>
<td>neurofilament medium chain</td>
</tr>
<tr>
<td>NGR</td>
<td>Nogo-receptor</td>
</tr>
<tr>
<td>NIND</td>
<td>non-inflammatory neurological disease</td>
</tr>
<tr>
<td>NMO</td>
<td>neuromyelitis optica</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OAD</td>
<td>other autoimmune diseases</td>
</tr>
<tr>
<td>OCB</td>
<td>oligoclonal bands</td>
</tr>
<tr>
<td>OD</td>
<td>optic density</td>
</tr>
<tr>
<td>OIND</td>
<td>other inflammatory neurological disease</td>
</tr>
<tr>
<td>OND</td>
<td>other neurological diseases</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendrocyte progenitor cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>positive control</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>P3</td>
<td>minor coat protein 3</td>
</tr>
<tr>
<td>p6</td>
<td>minor coat protein 6</td>
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<td>p7</td>
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<tr>
<td>p8</td>
<td>major coat protein 8</td>
</tr>
<tr>
<td>P9</td>
<td>minor coat protein 9</td>
</tr>
<tr>
<td>PML</td>
<td>progressive multifocal leucoencephalopathy</td>
</tr>
<tr>
<td>PP</td>
<td>primary progressive</td>
</tr>
<tr>
<td>PR</td>
<td>progressive relapsing</td>
</tr>
<tr>
<td>RaH</td>
<td>rabbit anti-human</td>
</tr>
<tr>
<td>RBA</td>
<td>radiobinding assay</td>
</tr>
<tr>
<td>REV</td>
<td>reverse primer</td>
</tr>
<tr>
<td>RF</td>
<td>reading frame</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>RPL</td>
<td>random peptide library</td>
</tr>
<tr>
<td>RR</td>
<td>relapsing remitting</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAS</td>
<td>serological antigen selection</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>secondary progressive</td>
</tr>
<tr>
<td>SSPE</td>
<td>subacute sclerosing panencephalitis</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5' tetramethyl-benzidine dihydrochloride</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VH</td>
<td>heavy chain variable region</td>
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General introduction and aims
1.1 Immunopathogenesis of MS

1.1.1 Clinical features

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the central nervous system (CNS). It is the most common disabling neurological disease affecting mostly young adults with a predominance for females and a prevalence in the USA and Northern Europe of ~100 per 100,000 people\textsuperscript{1,2}. Clinical manifestations of MS are variable between patients and include sensory and visual impairment, paralysis and other neurological deficits, sometimes accompanied with considerable cognitive dysfunction. Diagnosis of MS is made based on the McDonald criteria and is comprised of a combination of magnetic resonance imaging (MRI), clinical examination and cerebrospinal fluid (CSF) analysis\textsuperscript{3,4}.

At the onset of disease, about 85 - 90 % of patients are suffering from the relapsing remitting (RR) form of MS which is characterized by recurrent relapses followed by complete or partial recovery\textsuperscript{5}. The reversible disability in RR MS is caused by inflammatory demyelination in the brain that appears as sclerotic lesions or ‘plaques’ as demonstrated by MRI. As a consequence of inflammation, myelin, oligodendrocytes and axons are destroyed resulting in neurological disability\textsuperscript{6}. Decline of inflammation in combination with remyelination helps to restore axonal conduction and contributes to clinical recovery. Furthermore, due to plasticity of the human CNS, neuronal dysfunction and loss will not result in permanent disability in an early phase\textsuperscript{6}. When the CNS can no longer compensate for additional neuronal loss a transition from RR MS to a more progressive phase occurs\textsuperscript{7}. This progressive stage, called secondary progressive (SP) MS is characterized by a continuous deterioration in the absence of new inflammatory demyelinating lesions\textsuperscript{8}. Neurodegenerative processes prevail, leading to irreversible disability\textsuperscript{9,10}. Some patients (10 - 15 %) immediately start with a progressive disease course which is called primary progressive (PP) MS in which relapses are rare or nonexistent\textsuperscript{11}. Interestingly, PP MS patients
tend to be older than RR MS patients and prevalence is equal between females and males\textsuperscript{12}. A minority of patients (approximately 5\%) has progressive relapsing (PR) MS in which a gradual progression of disability from disease onset occurs, later accompanied by one or more relapses\textsuperscript{12,13}.

1.1.2 Genetic and environmental factors
MS predisposition is thought to be influenced by a complex, yet unclear interaction of genetic and environmental factors. Family members of MS patients have an increased risk of developing MS\textsuperscript{14,15} while twin studies indicate a 31\% concordance in monozygotic twins and 5\% concordance in dizygotic twins\textsuperscript{16,17}. These observations support the role of a genetic background in MS. Probably multiple interacting polymorphic genes are involved, each having a small effect on the overall disease risk. The strongest association has been found for the human leukocyte antigen (HLA)-DRB1*1501 gene indicating that antigen presentation to T cells is an important feature in the susceptibility for MS\textsuperscript{18,19}. Two additional MS-associated genes are the interleukin (IL)-7 receptor alpha chain and the IL-2 receptor alpha chain\textsuperscript{20-22}. Many other genes have been proposed to be associated with MS of which several await confirmation in additional studies. Some examples of proposed genetic markers are the HLA-DRB5 gene\textsuperscript{23} and a single nucleotide polymorphism (SNP) in the 3' untranslated region (UTR) of the IL-23 receptor\textsuperscript{24}.

The influence of environmental factors is demonstrated by the geographical incidence of MS which increases with distance from the equator\textsuperscript{2,25,26}. In addition, migration from a high to low prevalence area before puberty results in a reduction in the risk of developing MS\textsuperscript{27-29}. Candidate environmental factors influencing disease onset and exacerbations are metabolic and lifestyle factors such as vitamin D status and smoking, together with the influential effect of viruses such as Epstein-Barr virus (EBV)\textsuperscript{30-32}.

1.1.3 Role of viruses in MS
The involvement of viruses in the pathogenesis of MS is still a matter of debate. Evidence for a role of viruses as environmental or causal triggers of MS stem from the animal model for MS, experimental autoimmune encephalomyelitis
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(EAE). Transgenic mice expressing a T cell receptor (TCR) specific for an encephalitogenic peptide of myelin basic protein (MBP), develop EAE when they are housed under nonpathogen-free conditions, whereas the same animals housed in a pathogen-free facility remained disease free\textsuperscript{33}. Furthermore, one of the animal models for MS is generated by infection of mice with Theiler’s murine encephalomyelitis virus\textsuperscript{34}. In MS, the occurrence of oligoclonal bands (OCB) in the CSF of more than 90 % of MS patients\textsuperscript{35,36} may point to a viral involvement since diseases in which most patients have OCB in their CSF are in many cases of an infectious nature\textsuperscript{37}.

A viral agent involved in MS could be persistently present in the brain or it could cause a transient infection (hit-and-run hypothesis) in which the virus can act as a triggering agent and may be undetectable when clinical symptoms arise\textsuperscript{38}. There are several mechanisms in which a virus can cause autoimmunity and subsequent demyelination. An aberrant immune response can be caused as a result of direct viral damage and as an attempt of the immune response to clear the infectious agent. Alternatively, an autoimmune response could be triggered through the release or alteration of previously sequestered self-antigens (epitope spreading) or as a result of molecular mimicry in which the immune system causes self-damage by cross reactivity with viral epitopes\textsuperscript{38}. Tissue damage could also be the result of priming of non-encephalitogenic immune cells resulting in a nonspecific or bystander immune response that changes the local cytokine environment\textsuperscript{37}.

Some lines of evidence point towards a role of EBV in the pathogenesis of MS. EBV is a ubiquitous B-lymphotrophic herpes virus infecting more than 84-95% of the world’s adult population\textsuperscript{39}. Nevertheless, studies have shown that essentially all MS patients are EBV seropositive\textsuperscript{40-42}. Elevated EBV antibody titres can be detected years prior to MS onset and they confer an increased risk of developing MS\textsuperscript{43}. EBV association is found in epidemiological surveys where late EBV infection confers a higher risk of MS\textsuperscript{44} and EBV reactivation also appears to be linked to disease activity in early MS\textsuperscript{45,46}. Furthermore, Epstein-Barr virus nuclear antigen-1 (EBNA-1) associated OCB were detected in the CSF of a subset of MS patients\textsuperscript{47,48}. Recently, EBV-infected B cells and plasma cells were
found to accumulate in perivascular areas of white matter and meninges in MS brains\textsuperscript{45}. Although these results point towards a perturbed EBV-response in MS, the precise link between EBV-related immune alterations and the pathogenesis of MS remains unclear.

### 1.1.4 Therapy in MS

Current therapy in MS is mainly focused on treating symptoms and slowing the process of permanent neurological disability. Recent studies indicated that treating patients as early as possible increases the efficacy of the treatment and has important consequences on further disease development\textsuperscript{49-51}.

**Approved therapies for MS**

At present, six available disease modifying agents are approved for use in RR MS. These include: three interferon (IFN)-β preparations, glatiramer acetate (GA), the monoclonal antibody (mAb) natalizumab and the chemotherapeutic agent mitoxantrone\textsuperscript{52}.

IFN-β is a member of the type I interferon family and is the most frequently described therapeutic to control exacerbations in RR MS. It reduces major histocompatibility complex (MHC)-upregulation on antigen-presenting cells (APC), alters the cytokine expression profile, modulates apoptotic pathways and blocks migration of immune cells across endothelia. Overall, IFN-β has been shown to reduce clinical relapses, decrease MRI activity and possibly slow progression of permanent neurological disability\textsuperscript{12}. However, effects are only partial and a substantial proportion of patients are non-responders. Furthermore, IFN-β therapy has been associated with a number of adverse reactions including flu-like symptoms and the induction of neutralizing antibodies\textsuperscript{53}. Also, the significance of long-term effects has not been determined.

GA has been evaluated in three randomized, double-blind placebo-controlled clinical trials which all demonstrated the clinical efficacy of GA in reducing the frequency of relapses and progression of neurological disability\textsuperscript{54-56}. Long-term clinical outcome of GA was evaluated by one study that indicated that disability progression is less rapid in patients who were still receiving treatment after 10
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years compared to untreated patients\textsuperscript{57}. Glatiramer acetate's mechanism of action involves the induction of a phenotypic shift in the T cells from a pro-inflammatory T helper 1 (Th1) phenotype to a more anti-inflammatory Th2 phenotype\textsuperscript{58-60}. Besides the production of anti-inflammatory cytokines, GA-activated Th2 cells are also stimulated to secrete neurotrophines like brain-derived neurotrophic factor (BDNF) that may promote neuronal repair\textsuperscript{52,61,62}. The most common adverse reaction to GA is a local reaction of erythema and induration due to the injection\textsuperscript{63}.

Natalizumab, a humanized mAb specific for α4-integrin, was developed to suppress the binding of leukocytes to vascular endothelia thereby inhibiting immune cell entry into the CNS\textsuperscript{64}. Studies have shown that administration of natalizumab also resulted in a marked decrease in the number of white blood cells in the CSF, such as T cells, B cells and plasma cells\textsuperscript{65}. Despite its beneficial effects, natalizumab was withdrawn from the market in 2005 due to the development of progressive multifocal leukoencephalopathy (PML) in 2 MS patients after administering natalizumab. It was relaunched in 2006 and the Food and Drug administration (FDA) approved the drug as a monotherapy for RR MS patients with severe disease characterized by 2 or more severe relapses per year who have failed other therapies\textsuperscript{65,66}.

Mitoxantrone, an immunosuppressant that is also applied in cancer chemotherapy, is used in the treatment of SP MS, PR MS and worsening RR MS. Mitoxantrone reduces neurological disability and relapse frequency of patients by inhibition of T cell, B cell and macrophage proliferation. However, use of mitoxantrone can lead to serious adverse effects, particularly cardiotoxicity, myelosuppression, and, rarely, leukemia\textsuperscript{52,67}.

**Monoclonal antibodies in clinical development**

Although currently used therapies have demonstrated beneficial effects on the disease, more effective therapies such as humanized mAb are needed. mAb have revolutionised the treatment of autoimmune diseases since they enable a more specific approach by targeting molecules or cells directly involved in the
disease process. Natalizumab was the first mAb approved as a therapy for MS but many more are now being evaluated in clinical trials.

Daclizumab is an anti-CD25 antibody that blocks the proliferation of activated autoreactive T cells\textsuperscript{68}. It has already been used for the treatment of human T-cell lymphotrophic virus 1-induced adult T-cell leukemia and for the prevention of allograft rejection. It has been tested in several phase II clinical trials that all MS patients showed similar results manifested by a reduction in relapse rate and inflammatory activity. Treatment effects were seen after a delay of 4 to 6 weeks\textsuperscript{69-71}. No serious adverse effects were reported.

Alemtuzumab is a humanized mAb that targets CD52 which is a cell-surface glycoprotein with unknown function that is abundantly expressed on mature T and B cells, monocytes and eosinophils but is absent on lymphoid progenitor cells\textsuperscript{68}. Alemtuzumab has a cytotoxic effect on lymphocytes through the mechanism of antibody-dependent cell-mediated cytotoxicity and complement-dependent cytolysis\textsuperscript{72}. Clinical trials in MS have shown no clinical improvement on SP MS patients\textsuperscript{73,74}. However, phase II clinical trials in RR MS patients have shown a marked reduction in relapse rate and improvement in disability\textsuperscript{75-77}. Despite these positive results, severe side-effects including secondary autoimmunity have been reported implying the need for strategies to monitor or avoid these side-effects.

Rituximab\textsuperscript{\textregistered} is directed against CD20 which is exclusively expressed on B cells. Since antibody secreting plasma cells and stem cells do not express CD20 on their surface antibody production and B-cell recovery is not impaired\textsuperscript{68}. Target cells are lysed by complement-dependent cytolysis, antibody-dependent cell-mediated cytotoxicity and possible through direct induction of apoptosis\textsuperscript{78}. Clinical trials in MS have shown beneficial effects on relapse rate and number of MRI lesions\textsuperscript{79-82}. Treatment of MS patients with rituximab\textsuperscript{\textregistered} induces a pronounced depletion of circulating B cells without affecting antibody production\textsuperscript{81,83}. No adverse side-effects were reported. The positive effects of rituximab\textsuperscript{\textregistered} indicate an essential role of B cells in the pathogenesis of MS that is not restricted to antibody production.
1.1.5 Pathogenesis of MS

The aetiology of MS is still unclear but substantial evidence supports an autoimmune nature of the disease which is directed against components of the CNS. Besides inflammation, neurodegenerative processes occur early in the disease process resulting in significant axonal damage which is believed to be the cause of irreversible neurological deficits\textsuperscript{52,84}.

At present, it is not clear whether the invasion of the CNS by T and B cells is the initiating event of MS, or whether it is secondary to the activation of resident microglia and the local release of self and foreign antigens. Heterogeneity in lesion pathology has been described before\textsuperscript{85} indicating that inflammation might play a less dominant role in a subgroup of patients or at certain disease stages.

Autoreactive cells circulate in the blood of both healthy subjects and patients with autoimmune diseases, indicating that they are part of the normal immune cell repertoire\textsuperscript{86}. Normally autoreactive T cells are silenced by complex mechanisms of negative selection and peripheral tolerance mechanisms but in MS deficits in the peripheral regulation have been detected\textsuperscript{87-89}. In combination with a genetic predisposition, this can lead to an altered immune response when encountering infectious agents. Because of the degeneracy of the TCR, a T cell can be activated by bacterial and viral peptides that cross-react with self-antigens through the process of molecular mimicry (reviewed in \textsuperscript{90}). Activated antigen-specific T cells cross the blood-brain barrier (BBB) and are reactivated by an yet unknown antigen in the CNS presented by resident APC like microglial cells or perivascular macrophages\textsuperscript{91,92}. This initiates an inflammatory reaction that is directed to the myelin sheets and axons. Through the production of pro-inflammatory cytokines and chemokines other T cells, B cells and macrophages are attracted to the CNS and resident cells like microglia and astrocytes become activated\textsuperscript{93}. An amplification of the inflammatory reaction occurs and demyelination in combination with neurodegeneration is initiated through the joined efforts of cytotoxic cells, antibodies, complement fixation, apoptosis and phagocytosis\textsuperscript{1,5}. In addition, indirect mechanisms, such as loss of myelin, mitochondrial dysfunction and production of nitric oxide (NO) and glutamate can further contribute to substantial axonal damage which is considered to be the
reason for progressive and irreversible neurological disability in MS\textsuperscript{94}. Following the inflammatory damage, oligodendrocytes proliferate and partially remyelinate the demyelinated axon\textsuperscript{95}.

**Figure 1.1: Mechanisms of B cell function in MS**

B cells contribute on several levels to the pathogenesis of MS. As efficient antigen-presenting cells they can activate autoreactive T cells, thereby perpetuating the immune response. Autoantibody production can result in complement activation, ADCC of CNS cells and opsonisation of myelin. Studies also suggest that antibodies can promote remyelination by stimulating clearance of myelin debris by macrophages. B cells produce cytokines that can activate macrophages and T cells and enhance tissue damage. Production of IL-10 on the other hand would have a regulatory effect by influencing the $T_{H1}$ and $T_{H2}$ balance.

ADCC: antibody-dependent cell mediated cytotoxicity, CNS: central nervous system, IFN: interferon, IL: interleukin, TGF: transforming growth factor,

Although T cells are thought to make a major contribution to MS pathology, B cells, plasma cells and antibodies were proven to be involved in many levels of the pathogenic process. They are essential in processes including antigen capture and presentation, cytokine production, antibody secretion, opsonisation, tissue damage and remyelination (Figure 1.1)\textsuperscript{96}. It has also been suggested that B cells have regulatory capacity through the production of IL-10\textsuperscript{97,98}. Furthermore, the specificity of B cell responses reflects the overall specificity of
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the autoimmune response. B cells can only provide help to, and receive help from T cells that recognize MHC-bound antigens presented by B cells. This reciprocal activation of T and B cells results in an efficient immune response which is directed against invading pathogens.
1.2 B cells and antibodies in MS

The most compelling and early evidence for a role of the humoral immune response in the pathogenesis of MS is the intrathecal antibody production by plasma cells in the CSF, resulting in OCBs in more than 90% of MS patients. The intrathecally produced immunoglobulins (Ig) are mainly IgG1 and IgG3 although the presence of IgM OCB has also been described. OCB differ between patients but remain constant over time in each individual patient, indicating a long-lived chronic intrathecal immune response. OCB antibodies are a general feature of chronic brain inflammation in infectious CNS diseases were they represent antibodies directed towards the disease-causing agent like the measles virus in subacute sclerosing panencephalitis (SSPE). However, despite extensive research, there is still no general consensus on the nature of the antigens that react with the OCB present in the CSF. This raises the question whether OCB are the result of an MS-related antigen-driven response or whether OCB are a result of unspecific B cell activation.

Several lines of evidence support the hypothesis that B-cell activation is the result of a specific antigen-driven humoral immune response. Recent studies have demonstrated that the CSF but not the peripheral blood of MS patients is enriched with plasma blasts and B cells with a memory phenotype. The number of plasma blasts correlated not only with local IgG synthesis but also with the extent of CNS inflammation indicating an ongoing local exposure of disease-relevant antigens to the immune system. Studies on B cell receptor rearrangements further support these findings by demonstrating that the plasma blast population in MS CSF was characterized by a prominent clonal expansion. When studying the Ig heavy chain variable regions (VH) repertoire, a preferential usage of VH4 chains was noticed. The existence of a clonal B cell population in both CSF and MS brain plaques was demonstrated before (reviewed in ). In addition, Obermeier and colleagues were able to match the oligoclonal Ig transcriptome with the CSF B cell proteome thereby...
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providing direct evidence that CSF B cells are the primary source of the detected OCB\textsuperscript{112}. The clonal expansion of B cells together with the preferential use of VH4 chains and numerous somatic hypermutations in the complementarity-determining region (CDR)3 regions\textsuperscript{113-119} are consistent with germinal center selection\textsuperscript{120} as a result of a T-cell dependent antigen-driven humoral immune response.

The presence of long-lived memory B cells and the continued production of antibodies implies a B cell fostering environment in the inflamed CNS of MS patients. Indeed, several studies have suggested the presence of ectopic secondary lymphoid structures in the meninges of EAE animals and MS patients that may be involved in maintaining the intrathecal B cell response\textsuperscript{121-124}. In the MS lesion, B cells are attracted by the expression of several chemokines that regulate migration across the BBB\textsuperscript{108,125}. In addition, a B cell favoring environment is created by the presence of soluble factors including IL-6 and IL-10\textsuperscript{108,126}, two B cell survival and differentiation cytokines. B cell-activating factor (BAFF) and a proliferating inducing ligand (APRIL) are upregulated in MS lesions promoting the survival and clonal expansion of B cells within the CNS\textsuperscript{121,127,128}. An effector function of antibodies is provided by the presence of upregulated Fc receptors on microglia and perivascular macrophages\textsuperscript{129}. Furthermore, macrophages and activated microglia engaged in demyelination show capping of surface IgG suggesting a specific antibody-mediated process\textsuperscript{130}. Deposition of antibodies and activated complement bound to disintegrating myelin was described, implicating IgG-antigen complexes in myelin breakdown\textsuperscript{131,132}. Histopathological studies have shown that of the 4 distinct lesion patterns described in MS the most common pattern (pattern II) is characterized by the presence of antibodies and complement in addition to T cells and macrophages\textsuperscript{85}.

Together, these results point towards an important role for B cells in the pathogenesis of MS. Although the results indicate a major role for antibodies in MS, the recent success of the B-cell depleting mAb rituximab\textsuperscript{8} indicates that antibody-independent B cell functions such as antigen-presentation and cytokine production are as well critical in the disease process of MS\textsuperscript{79}.
1.3 Antibodies as biomarkers for MS

Bodily fluids like tears, urine, CSF and blood can be used in the search for biomarkers for MS with CSF and serum being the most frequently used media. Blood or serum is an easily accessible medium that can be obtained in large quantities. CSF on the other hand may be a better reflection of disease-associated alterations due to its close proximity to the CNS\textsuperscript{133}. Besides protecting the brain from trauma, the CSF functions as a transport medium for neurosecreted, biosynthesised and metabolic cellular products\textsuperscript{134}. However, CSF collection can be a painful and invasive technique and can therefore be used only in the experimental setting for a selected patient population and sampled only for a limited number of time points\textsuperscript{135}.

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\textsuperscript{135}. Biomarkers can be used as indicators of disease activity and/or prognosis of MS. Subtyping patients according to their immunopathogenic profile and subsequent adjustment of therapy is another potential application. However, because of the complexity of the MS pathogenesis, it is highly unlikely that a single biomarker will predict the outcome and prognosis of MS. Rather, a panel of biomarkers must be used to give a good indication of the different disease processes. Since a diagnostic test for MS is still unavailable and prediction of the disease course is difficult, many studies have been performed in the search for biomarkers for MS.

Autoantibodies may serve as biomarkers that may be useful to study disease activity and to define patient heterogeneity. Many proteins have been investigated as targets for autoantibodies in MS. Since myelin is the main target in the pathogenesis of MS, a major focus was led on several myelin antigens including MBP, myelin oligodendrocyte glycoprotein (MOG), proteolipid-protein (PLP) and myelin-associated-glycoprotein (MAG). Other potential candidates are
axonal proteins and proteins exposed at the surface of CNS-associated cells including oligodendrocytes and oligodendrocyte progenitor cells (OPC). Table 1.1_S in the addendum gives an overview of performed studies concerning antibodies as biomarkers in MS since 1990. In the following part, an overview will be given concerning the use of antibodies as markers for diagnosis, prognosis, repair and defining different disease subtypes of MS.

1.3.1 Autoantibodies as diagnostic markers in MS
The best known biomarker in MS with established diagnostic utility is the presence of intrathecally produced Ig (mainly IgG₁ and IgG₃) in the CSF of more than 90% of MS patients. Although it is an important diagnostic marker, the antigen specificity of the OCB still remains to be defined (reviewed in^136,137). In addition, OCB are not an unique feature of the MS pathogenesis as they are a common aspect of chronic brain inflammation in infectious CNS diseases^137. The presence of OCB is frequently associated with a polyclonal intrathecal immune response with the simultaneous production of specific antibodies against measles, rubella and varicella zoster (MRZ reaction)^105. The MRZ reaction is considered to represent non-specific activation of a part of the B cell population in the CNS whereas in other CNS infections mostly specific antibodies towards a single antigen are produced^138. Although the MRZ reaction is less sensitive than iso-electric focusing (IEF) it can be used as an additional confirmatory assay after the presence of OCB has been detected^136.

Antibodies towards EBV have also been proposed as biomarkers for MS. Studies have shown that essentially all MS patients are EBV seropositive^42,139,140 whereas 84-95% of individuals worldwide are infected^39. The detection of an increased anti-EBV antibody titres long before disease onset^43 and EBV-associated OCB^47 further supports the use of EBV antibodies as biomarkers. However, increased seropositivity is also associated with other autoimmune diseases and therefore not specific for MS^141.

MOG is a highly immunogenic CNS-specific transmembrane protein that has gained much interest. Although MOG is only a minor component of myelin, its position on the outermost surface of the myelin sheath ensures an easy
accessibility for autoantibodies\textsuperscript{142}. The important role of anti-MOG antibodies in EAE\textsuperscript{143} is another aspect that has led to the extensive study of the presence of anti-MOG antibodies in the CSF and serum of MS patients versus control patients (table 1.1). The results of these studies are ambiguous as antibody frequencies ranging from 0-80\% in MS patients and 0-60\% in healthy controls (HC) were found. These differences in antibody positivity are probably the result of the usage of different antigen preparations and methods to detect these antibodies. Furthermore, studies indicated that only antibodies against conformational epitopes of MOG can mediate demyelination in vivo and in vitro\textsuperscript{144,145}. High-affinity antibodies have been found in MS-lesions while in CSF and serum only low-affinity antibodies were found\textsuperscript{146}. Other studies have indicated that MS antibodies recognize different epitopes than antibodies present in HC\textsuperscript{147,148}. In the future it will be necessary to reach a methodological consensus to detect anti-MOG antibodies to rule out inconsistency about their presence in the serum or CSF of MS and control patients.

1.3.2 Autoantibodies as markers for prognosis and progression

The presence of an intrathecal IgM synthesis has been correlated with a higher expanded disability status scale (EDSS) score in patients with clinically definite MS (CDMS)\textsuperscript{149,150}. In addition, detection of IgM OCB shortly after disease onset predicted a worse evolution during the first years of the disease\textsuperscript{99} although others did not find a correlation\textsuperscript{151}. This can be explained by a recent study which showed that only lipid-specific IgM antibodies were correlated with poor prognosis whereas non-lipid-specific IgM antibodies represented a transient immune response\textsuperscript{152,153}. By combining the IgM OCB with clinical parameters including the presence of pyramidal and cerebellar symptoms at onset, Mandriolo and colleagues have developed a multifactorial prognostic index of the probability, at diagnosis, of developing a severe or benign course of MS\textsuperscript{154}. The presence of IgM OCB in combination with pyramidal signs at onset and 2 acute episodes over 6 months was found to be predictive of a severe disease course with a probability of 99.8 \%. Although the patient number included in the study was small, these results are in concordance with other studies correlating pyramidal and cerebellar signs at onset with a high EDDS score and a rapid shift to a progressive disease stage\textsuperscript{155}. 
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Since the initial finding of a correlation between the presence of anti-MOG and anti-MBP antibodies and the development of CDMS after the first clinical symptoms\(^{156}\), several groups have addressed the predictive role of anti-MOG and anti-MBP antibodies (table 1.1). Studies indicating that IgG antibodies directed against native MOG are serological markers for early inflammation in MS provide further evidence for a possible prognostic role of anti-MOG antibody\(^{157}\). A study performed by Zhou and colleagues demonstrated elevated IgG antibody levels to native MOG in serum of MS patients, with the highest prevalence in PP MS patients, compared to controls. These high-affinity antibodies were shown to induce death of MOG-expressing target cells while serum from MS patients with high anti-MOG antibody titres stained white matter myelin in rat brain and enhanced demyelination and axonal damage when transferred to EAE animals\(^{158}\). Together, these results indicate an important role for anti-MOG antibodies in the disease process of MS. However, conflicting results were again obtained concerning the presence of anti-MOG and anti-MBP antibodies in the CSF and serum of clinically isolated syndrome (CIS) patients. Since in most studies the same type of assay was used, the controversial results could be the result of differences in study cohorts. Therefore, the prognostic value of these antibodies is not clear.

Another promising marker for monitoring axonal damage and therefore for the conversion to chronic progressive MS are the cytoskeletal neurofilament proteins which are localized within axons\(^{159}\). Neurofilament proteins have been detected in CSF and serum of MS patients and increasing levels of antibodies to the neurofilament light subunit (NFL) have been found in the PP or SP phase of the disease course\(^{160,161}\) or were correlated with brain atrophy\(^{162}\). It is highly unlikely that these antibodies are the cause of MS, but their increasing presence in the serum or CSF of MS patients could be a result of increasing damage to axons during the disease process of MS.

**1.3.3 Autoantibodies as markers for repair and remyelination**

Nogo-A is a protein associated with CNS myelin and plays a key role in inhibition of axonal regeneration and in suppressing sprouting and plastic changes of synaptic terminals. Although serum anti-Nogo-A IgG antibody levels were not
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shown to be specific for MS\textsuperscript{163}, significantly elevated serum levels of anti-Nogo-A IgM antibodies were detected in MS and acute inflammatory and noninflammatory neurological diseases but not in neurodegenerative diseases, systemic inflammatory disease or HC\textsuperscript{164}. Furthermore, these antibodies were significantly more frequent in younger patients and in MS patients with RR disease course. Hence, anti-Nogo-A antibodies might be used to assess the endogenous potential of axonal regrowth and therefore repair in MS.

In MS, remyelination of demyelinated lesions by OPC diminishes with disease progression for unknown reasons\textsuperscript{12}. Antibodies specific for oligodendrocyte progenitor antigens like progenitor cell-surface glycoprotein AN2\textsuperscript{165} and heat shock protein (Hsp) 90\textsuperscript{166,167} have been detected in the CSF of MS patients. These antibodies could be involved in the selective depletion of cells involved in remyelination and repair and may thus contribute to the development and progression of chronically demyelinating lesions.

1.3.4 Autoantibodies as markers for classification of MS subtypes

Although some interindivudual differences in autoimmune reactivity are found in serum antibodies, several studies have demonstrated a consensual immune pattern of self-recognition defined as the immunological homunculus\textsuperscript{168-170}. However, pathological conditions can result in a modified pattern of antibody responses to self-antigens\textsuperscript{171}. This was shown by several studies in which the antibody profile towards a panel of CNS proteins, heat shock proteins, lipid autoantigens and brain self-antigens was analyzed using western blotting\textsuperscript{172} or antigen microarrays\textsuperscript{173,174}. Different antibody profiles were identified that were able to discriminate between MS patients, healthy controls and patients with other neurological or autoimmune diseases. Moreover, their approach separated the three main clinical forms of MS namely RR, SP and PP MS. These results show the importance of antibody profiles and suggest that patterns of IgG reactivity could model the pathological processes underlying the various forms of MS.
1.4 Biomarker discovery tools

In recent years numerous efforts have been undertaken to identify both antigenic targets and biomarkers for MS that could play a role in diagnosis, prognosis and monitoring of patients. Several techniques including transcriptomics and proteomics can be used allowing the simultaneous analysis of thousands of genes and proteins.

1.4.1 Proteomics
Proteomics is the large-scale study of expression, function and interactions of proteins\textsuperscript{175}. There are several approaches to identify proteins that are differentially expressed or modified in MS patients but not in healthy controls including two-dimensional gel electrophoresis (2D-GE), 2D-DIGE (difference in gel electrophoresis) or liquid chromatography in tandem with mass spectrometry. Studies that have applied these techniques to explore the proteome in CSF of MS patients and controls identified several proteins that showed a distinct expression profile between MS patients and controls\textsuperscript{176-180}. However, further studies are needed to examine the exact role of these proteins in the pathogenesis of MS. Because of the laborious protocol and poor sensitivity, new high-throughput methods including microarrays and phage display have been developed. Furthermore, these techniques can also be used for the profiling of antibody signatures in CSF and serum of MS patients.

1.4.2 Transcriptomics
Many gene expression studies have been performed to look at the specific patterns of gene transcript levels in MS or in its animal model, EAE. Transcriptional profiling studies were performed on peripheral blood cells, brain lesions and normal appearing white matter of MS patients and in the EAE model (reviewed in \textsuperscript{181} and \textsuperscript{182}). In general, genes showing variable expression include mainly immunological and inflammatory genes, stress and anti-oxidant genes, as well as metabolic and CNS markers. However, due to the complexity of the
disease and the many genes involved, it is difficult to decipher and define the gene pathways involved in the disorder. Although several unexpected molecules associated with MS pathogenesis have been identified including osteopontin\textsuperscript{183}, aBC\textsuperscript{184} and clotting cascade proteins\textsuperscript{185}, gene expression profiling is not suitable for the search of soluble disease markers present in body fluids. In addition, discrepancy exists between mRNA and protein expression levels because of the susceptibility of mRNA to degradation. Posttranscriptional regulation of gene expression and alternative splicing of mRNA can result in the generation of a different protein profile. Therefore, it is crucial to support gene expression data with identification and characterization of the translated proteins at the site of the disease.

1.4.3 Protein microarrays

Microarrays have become a widely used technology in molecular biology research. Protein arrays are produced by attaching hundreds of proteins or peptides to planar surfaces using a robotic arrayer. Arrays can then be incubated with a test sample, e.g. patient serum or CSF, and fluorescent labels are used to detect autoantibody binding to specific autoantigens on the array\textsuperscript{186}. Protein arrays have been shown to be well suited for the study of autoantibody responses\textsuperscript{187}. They reduce costs by decreasing reagents consumption and improve efficiency by increasing the number of assays that can be performed by a single serum sample. Some of the bottlenecks of the technology are complex data analysis, protein instability, problems with nonspecific interactions, and the lack of amplification techniques to generate sufficient amounts of the lower abundance proteins\textsuperscript{188}.

Protein arrays have been applied to study antibody profiles in both MS patients and EAE animals. Steinman and colleagues have developed a myelin proteome microarray containing 232 peptide and protein antigens derived from the myelin sheath to study the anti-myelin antibody response in EAE mice\textsuperscript{189}. In acute EAE, the diversity of the anti-myelin antibody responses predicted subsequent disease activity while in chronic EAE an extensive intra- and intermolecular epitope spreading to overlapping but distinct sets of epitopes on myelin proteins was observed. These data suggest that diverse autoreactive B cell responses precede
and contribute to autoimmune disease progression, at least in EAE. By developing a lipid microarray, antibody reactivity to lipids was studied in CSF of MS patients and EAE mice \(^{190}\). A panel of lipids targeted by antibodies in MS was identified, including sulfatide, sphingomyelin and several oxidized lipids. Sulfatide-specific antibodies were also detected in EAE mice suggesting that autoimmune responses directed against lipid components contribute to the demyelination in MS. Others have applied a protein expression array derived from a human brain cDNA expression library to decipher the specificity of the CSF IgG in MS patients \(^{48}\). Twenty-one protein sequences were identified that showed a higher reactivity in MS patients than in controls. The 2 peptides that showed the highest IgG reactivity in MS patients in comparison with those of control patients were shown to contain a motif found in EBV proteins EBNA-1 and an early EBV protein, BRRF-2. Intrathecal synthesis and binding of OCB were confirmed for both proteins. Quintana and colleagues have used protein arrays to compare serum antibody profiles from 35 RR MS, 29 SP MS and 32 PP MS patients and 30 healthy controls. Unique antibody patterns were identified that were able to distinguish MS patients from controls. Antibody reactivity was principally found towards CNS antigens and heat shock proteins. Antibody reactivity towards heat shock proteins seemed to be more specific for RR MS. In addition, they were able to associate specific antibody patterns with high lesion load and with brain atrophy as evaluated by MRI \(^{173,174}\). Together, these results indicate that antibody profiles in MS can be used to identify different types and stages of MS.

### 1.4.4 Phage display

Phage display was introduced by Smith et al. in 1985 \(^{191}\) and has since then successfully been used to identify different types of target molecules. Most phage display work has been performed with filamentous phage strains such as M13 or its close relatives fd and f1. The M13 virus particle is made up of a single stranded circular DNA (about 6400 nucleotides), surrounded by a protein coat consisting of \(\pm 2700\) copies of major coat protein 8 (p8) and 5 copies each of minor coat proteins 3 (p3), 6 (p6), p7 (p7) and 9 (p9) \(^{192,193}\). Display of proteins on the surface of filamentous phage is possible by fusion of the gene of interest to one of the phage coat genes including gene III, VIII or VI. Expression of the
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fusion product and its subsequent incorporation into the mature phage coat
results in the target being displayed on the phage surface while its genetic
material resides within the phage particle, thereby providing a physical link
between genotype and phenotype. This direct link between genotype and
phenotype allows the enrichment of specific phage by performing several
consecutive rounds of affinity selection towards a given ligand. During affinity
selections, phage that display a relevant target will be retained and amplified,
while non-relevant phage will be washed away. After several consecutive rounds
of affinity selection, target-specific phage populations can be obtained and the
selected gene products can be analyzed and identified.

Phage display has the advantage of rapid isolation of interacting partners
combined with an efficient handling of large molecular libraries in small volumes.
Furthermore, the conditions for selection can be controlled to recover either high
or low affinity binding partners. However, due to the use of a bacterial
expression system, no post-translational modifications will occur although the
formation of disulphide bonds will occur in the periplasm of the bacterial host194.

The value of phage display has already been shown by the selection of binders
from linear or constraint peptide libraries195-197 and by the display of functional
protein domains such as antibody fragments198-200, enzymes201-203,
hormones204,205, and DNA-binding proteins206-208. cDNA phage display libraries
are less commonly used due to the technical difficulties in their construction and
expression. Most of the phage display libraries make use of the direct fusion of
the encoding DNA to the N-terminus of phage coat proteins p3 or p8. N-terminal
fusion of cDNA is not possible due to inherent stop codons present in the cDNA.
Although C-terminal fusion to p3 or p8 interferes with the incorporation into the
phage coat209, this fusion can be performed with p6, which has a surface-
exposed C-terminus allowing the attachment of foreign proteins at this site210.
Another possibility of cDNA phage display is by indirect linkage to p3 via the
interaction of Jun and Fos leucine zippers211.

Several groups have demonstrated the efficacy of the display system by the
functional expression of enzymes194 and by the successful identification of serine
protease inhibitors\textsuperscript{210}, candidate biomarkers of colorectal cancer\textsuperscript{212} and antigenic targets associated with the presence of ruptured peripheral atherosclerotic lesions and myocardial infarction\textsuperscript{213}. Together, these results clearly demonstrate the usefulness of the p6-phage display system in identifying specific binding interactions.

Phage display has already been used in the search for biomarkers for MS. Antibodies present in CSF or serum were used to screen random peptide libraries (RPL) to enrich for MS-related antigens\textsuperscript{214-219}. Although Jolivet-Reynaud and colleagues found a higher immunoreactivity in CSF of MS patients compared to controls by combining 4 CSF-specific motifs\textsuperscript{218}, other groups did not retrieve MS-specific antigens\textsuperscript{214} or did not perform a comparative immunoreactive study\textsuperscript{217,219}. Motifs with significant homology to human collagen, a 68 kDa neurofilament protein, and several herpes viruses\textsuperscript{217} were identified. A motif which showed similarity to EBNA1 and aB-crystallin was shown to correspond to a subset of OCBs in the CSF of the same patient used in the original selection procedure\textsuperscript{219,220}.

With regard to RPL, cDNA phage display libraries have the advantage of displaying naturally occurring peptides instead of synthetic peptide fragments that can be selected by antibodies present in CSF or serum. Gilden and colleagues have constructed a cDNA expression library prepared from MS brain plaques and used MS CSF or serum to screen the cDNA library. Although several antigens were identified, none displayed MS-specific immunoreactivity\textsuperscript{221,222}. When a cDNA library obtained from an oligodendrocyte precursor lines was used, selections on MS CSF retrieved an epitope of 7 amino acids (AA) which was highly homologous to a translated consensus Alu repeat epitope. When tested in CSF or serum from a larger group of MS patients, approximately 44% reacted with these so-called Alu peptides\textsuperscript{223}. However, both studies used an elaborate filter screening approach which can disrupt the conformation of the antigens and result in nonspecific binding of antibodies.

To fully explore the autoantibody repertoire present in CSF and serum of MS patients, our group has applied a new and powerful technique called serological
antigen selection (SAS). SAS involves the use of a cDNA phage display library for affinity selections on pooled MS CSF and serum leading to the selective enrichment of antigens recognized by antibodies present in MS CSF and serum. This approach has already resulted in the identification of 8 antigenic targets that were able to discriminate MS patients from controls with 86 % specificity and 45 % sensitivity\textsuperscript{224}.
1.5 Conclusion

It is becoming more and more evident that MS is a very complex disease of the CNS. Because of its heterogeneity, making a diagnose is a difficult and long-term process which is merely based on clinical symptoms rather than on a specific diagnostic test. Prediction of the disease process is even more complicated which makes it difficult to adjust therapy according to the needs of the patient. As a result, this can have serious effects on the further course of the disease process. Since biomarkers could have a great impact on diagnosis, prognosis and subtyping of patients, a major effort has been undertaken in the search for these markers. Although this research has led to a better understanding of the disease process, few of the identified candidates have the potential to be used as a real biomarker for MS. Therefore, a new approach is needed to identify potential biomarkers for MS. In this study, a new and powerful technique called serological antigen selection was applied to characterize the antibody profile in CSF and serum of MS patients.
1.6 Goal of the study

The goal of this study was to apply SAS as a tool to analyze antibody reactivity in MS CSF and serum. Antibodies present in CSF and serum of MS patients can be used to identify antigenic targets that evoke an antibody response. By using a cDNA phage display library derived from MS brain plaques with varying degrees of inflammation and demyelination combined with a solution-based selection procedure with MS CSF and serum, high affinity interactions will be promoted.

In a first part of the study an optimization of the SAS-procedure was necessary for use with pooled MS sera and CSF. After optimization, antibodies present in MS CSF and sera will be used to identify MS-related antigens expressed as fusion products on the surface of an MS cDNA phage display library. On the one hand pooled CSF and serum will be used to search for candidate biomarkers for MS. On the other hand, paired CSF and serum of an individual patient will be used to explore the possibility if cDNA phage display can be used to compare antibody reactivity between these two compartments. Finally, the occurrence of frameshifting in the p6-display system will be analyzed in more detail.
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Aim 1: Optimization of the SAS procedure for pooled body fluids and identification of biomarkers for multiple sclerosis

To apply SAS in pooled serum and CSF, an optimization of the procedure is needed to deplete aspecific cDNA products and to diminish the broad antibody profile existing in serum. In chapter 3, a differential screening procedure will be optimized in which positive selection rounds on MS serum/CSF and negative selection rounds on healthy control serum will be alternated. By reducing the occurrence of nondisease-related cDNA products, the opportunity of identifying candidate MS-related antigenic targets will be substantially increased.

Next, the SAS-procedure will be applied to pooled CSF and serum of 10 RR MS patients to identify possible MS-related antigenic targets. By using pooled CSF and serum samples, patient-specific reactivity will be diluted whereas the opportunity to identify MS-related antigens will be increased. Affinity selections will result in the identification of a panel of enriched phage clones. Immunoreactivity towards this panel will then be further evaluated in CSF or serum of a large group of MS patients and controls to validate their potential as biomarkers in MS.

Aim 2: Comparison of antibody profiles in CSF and serum of a RR MS patient

B cells and antibodies are thought to play an important role in the pathogenesis of MS. However, the precise antigenic targets of these antibodies are still unknown. In chapter 4, we will explore the possibility to apply SAS as a tool to analyze antigen reactivity of antibodies present in the CSF and serum of a RR MS patient. In addition, the identified antibody profile in CSF will be compared with the one existing in serum.

Antigenic targets selected with antibodies present in CSF and serum will be identified and mutually compared to distinguish antibodies with unique or common reactivity. After confirmation of immunoreactivity towards the selected antigenic targets in the CSF and serum of the patient used in the selection
procedure, antibody reactivity will be further analyzed in a larger group of both MS and control patients. Finally, the expression profile of interesting targets will be further validated in MS brain lesions.

**Aim 3: Analysis of frameshifting in the p6-display system**

In this thesis, a gene VI-based phage display system was used for the expression of cDNA fragments on the surface of filamentous phage. During the repeated selection rounds with MS antibodies, enrichment of phage clones targeted by these antibodies occurred. However, we occasionally identified an enriched phage clone that did not express any protein due to out of frame insertion of the cDNA. A possible explanation for this occasional enrichment can be found in the mechanism of frameshifting. Frameshifting has already been described in gene III- and gene VIII-based phage display studies. In chapter 5, the goal is to investigate whether frameshifting can be demonstrated in the gene VI-display system.

Therefore, we focus on a phage clone, not displaying a protein due to an out of frame insertion of the cDNA, which was selected multiple times during affinity selections on MS sera. We examine if we can demonstrate the occurrence of frameshifting in this phage clone by fusing the cDNA insert of this phage clone to an E-tag. After cloning the cDNA – E-tag construct in three pSPVI vectors, each representing a different reading frame, the E-tag signal of each construct can be detected using ELISA. In this way, it would be possible to demonstrate the event of frameshifting in the p6-display system.
General materials and methods
2.1 Materials and methods

This chapter gives an overview and detailed description of the materials and techniques that play a pivotal role throughout this thesis. Study-specific materials and methods are outlined in the corresponding chapters.

2.1.1 Construction of an MS cDNA phage display library
A cDNA library (1.0 x 10^6 primary recombinants), derived from active chronic multiple sclerosis (MS) plaques with varying degrees of demyelination and inflammatory activity was kindly provided by Dr. Soares (University of Iowa). This library was normalized by a procedure based on reassociation kinetics\(^{225}\).

To construct a cDNA phage display library, plasmid DNA was prepared from the normalized MS cDNA library and further digested with EcoRI and NotI. DNA fragments ranging from 500 bp to 2.5 kb were gel purified (GFX gel band purification kit, GE Healthcare, Diegem, Belgium) and ligated at the C-terminus of minor coat protein 6 of the three EcoRI and NotI-digested cDNA phage display vectors pSPVIA, pSPVIB and pSPVIC\(^{194}\), each encoding a different reading frame (figure 2.1). The ligation mixtures were used to transform E.coli TG1 cells by electroporation to obtain libraries MS-pSPVIA, MS-pSPVIB and MS-pSPVIC of 9.0 x 10^5, 1.0 x 10^6 and 1.0 x 10^6 independent clones, respectively.

2.1.2 Growth and rescue of phage particles
To rescue phagemid particles 50 ml of 2 x YT containing ampicillin and glucose (2 x YT-AG: 31 g/l Difco\(^{TM}\) yeast extract tryptone (BD, Erembodegem, Belgium), 100 μg/ml ampicillin (Roche Diagnostics, Vilvoorde, Belgium) and 2 % (w/v) glucose) was inoculated with 10^8 bacteria taken from the library stock and grown for approximately 1.5 h, shaking at 37°C. When an optical density (OD) of 0.5 was reached at 600 nm, a 20:1 ratio of M13K07 helper phage (Pharmacia, Uppsala, Sweden) was added to 2 x 10^10 colony forming units (cfu) (5 ml) of exponential growing bacteria and the mixture was incubated for 30 min
at 37°C without shaking. Cells were centrifuged at 2,800 x g and resuspended in 25 ml of 2 x YT containing 100 μg/ml ampicillin and 25 μg/ml kanamycin (2 x YT-AK, Gibco, Invitrogen Corporation, Paisley, United Kingdom) and grown overnight, shaking at 30°C. Phage particles were purified and concentrated by two PEG-precipitations (20% polyethylene glycol (PEG)-2.5 M NaCl) and resuspended in 1 ml phosphate-buffered saline (PBS, 750 mM NaCl, 40 mM Na₂HPO₄, 7.8 mM KH₂PO₄, pH 7.4) to approximately 10¹³ cfu/ml.

For titration of phagemid particles, 2 x 10⁸ cfu (500 μl) of exponential growing *Escherichia coli* (*E. coli*) 2 x YT-AG agar plates (2xYT-AG containing 15gr/l bacto™ agar (BD)) and incubated overnight at 30°C. Colonies were counted and phage titres determined by following formula:

\[ \text{Phage titre (cfu/ml)} = \frac{\text{number of colonies} \times (1/\text{dilution}) \times (1/\text{fraction plated})}{2} \]

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**Figure 2.1: Construction of the MS cDNA phage display library**

The normalized MS cDNA library was cloned at the C-terminus of gene VI. As a result, the cDNA products will be displayed at the phage surface as a fusion protein with minor coat protein 6 (p6). cDNA phage display vectors pSPVIA, pSPVIB and pSPVIC allow directional cloning of the cDNA in the 3 different reading frames using EcoRI and NotI restriction enzymes (indicated with *). GS linker is a glycine-serine linker to separate p6 from the cloned cDNA. Restriction sites are underlined. Stops represent a stop cassette to ensure efficient termination of cloned cDNA. MCS: multiple cloning site, lacZ: gene VI promoter.
2.1.3 Cerebrospinal fluid and serum samples

All cerebrospinal fluid (CSF) and serum samples used in the selection procedure were first adsorbed to *E. coli* and phage components by repeated passage through columns of Sepharose 6MB (Pharmacia) coupled to lysates of *E. coli* Y1090 and bacteriophage-infected *E. coli* XL1Blue. After adsorption steps, aliquots of CSF and sera were prepared in 0.2 % (w/v) skimmed milk powder in 1 x tris-buffered saline (MTBS) (50 mM Tris-HCl (pH 7.9) and 150 mM NaCl) and stored at -80°C. CSF and serum samples used in the ELISA were aliquoted and stored at -80°C.

2.1.4 Serological antigen selection of cDNA phage display repertoires

CSF and serum samples from relapsing-remitting (RR) MS patients were used for the serological antigen selection (SAS) procedure. In chapter 3 serum and CSF samples from 10 RR MS patients were pooled and used for affinity selections while in chapter 4 paired CSF and serum from a single RR MS patient were used. The SAS procedure is outlined in figure 2.2.

For the selections, immunotubes (Nunc, Roskilde, Denmark) were coated with 10 μg/ml rabbit anti-human (RαH) IgG (Dako, Glostrup, Denmark) in coating buffer (0.1 M NaHCO₃ pH 9.6), overnight at 4°C. After washing the immunotubes with 0.1 % (v/v) Tween 20 in PBS (PBSTween) and PBS, the tubes were blocked for 2h at room temperature (RT) with 2 % (w/v) skimmed milk powder in PBS (MPBS). Phage were purified from each library (MS-pSPVIA, MS-pSPVIB and MS-pSPVIC) as described in 2.1.2. Approximately equal numbers of phage derived from each library (ca. 2 x 10¹² plaque forming units (pfu)) were added to the serum (1:100 diluted in 0.1 % MPBS) or the CSF (1:5 diluted in 0.1 % MPBS) and incubated in a glass tube for 1.5 h at RT on a end-over-end rotating platform. After washing the coated immunotube with 0.1 % PBSTween and PBS, the preincubated serum/CSF and phage mix was transferred to the coated immunotube and incubated for 30 min on a rotating platform and for 2h standing at RT. Tubes were washed 20 times with 0.1 % PBSTween and 20 times with PBS to remove non-bound phage. Bound phage were eluted with 1 ml of 100 mM triethylamine for 10 min on a rotating platform and neutralized with
0.5 ml 1 M Tris-HCl, pH 7.4. Input and output phage were amplified by infecting *E. coli* TG1 cells and were plated on 2 x YT-AG agar plates at each round of selection. Resulting colonies were scraped and phage were rescued for further rounds of affinity selections. Input and output phage from each round of selection were titrated.

**Figure 2.2: Serological antigen selection used for selection of MS-associated antigens**
During a selection round, phage displaying the MS cDNA repertoire were incubated with MS CSF or serum (1). IgGs present in CSF/serum (black) bind MS-specific antigens (black) displayed on phage (2). Phage antigen-IgG complexes were retained using an anti-human IgG capture antibody (3) while non-bound phage were washed away (4). Bound phage were eluted and used to reinfect bacteria (5). Selected phage were amplified and used for a subsequent selection round (6).
2.1.5 Screening for enriched clones with PCR and fingerprinting

After each selection round, individual colonies were picked and the insert size was determined by PCR. Primers (Eurogentec, Seraing, Belgium) flanking the EcoRI and NotI cloning sites of the vector were used (forward: 5' - CTCTCTGTAAAGGCTGC-3' and reverse: 5' - CGCCAGGGTTTCCCCAGTCAGAC-3'). PCR was performed in a 50 μl amplification-mix containing 10 pmol forward and 10 pmol reverse primer, 1 x PCR buffer (Roche Diagnostics), 12.5 pmol dNTP's (Roche Diagnostics) and 2 U of TAQ DNA polymerase (Roche Diagnostics). The PCR reaction consisted of an initial denaturation step for 10 min at 95°C, followed by 35 cycles of amplification (30 s at 94°C, 30 s at 55°C, 1 min at 72°C) and an elongation step for 10 min at 72°C in an ICycler (Biorad, Nazareth Eke, Belgium). Amplification products were analysed by gel electrophoresis on a 1 % agarose gel to confirm the presence and size of the cDNA inserts. PCR products were used for fingerprinting analysis by incubating 10 μl of the amplification product with 5 U of the restriction enzyme BstNI (Roche Diagnostics) for 2h at 37°C. After enzyme digestion, restriction products were analysed on a 2 % agarose gel.

2.1.6 Sequence analysis

PCR products were purified by adding 2 μl of ExoSAP-IT clean-up kit (Exonuclease I and Shrimp Alkaline Phosphatase; GE Healthcare) to 5 μl of amplification products according to manufacturer's instructions. After 15 min incubation at 37°C, the ExoSAP-IT was inactivated at 80°C for 15 min. Concentration of purified PCR product was estimated on 1 % agarose gel by comparison with 5 μl smartladder (Eurogentec). For sequence analysis 50-100 ng purified PCR product was added to the Big Dye TMT terminator cycle sequencing ready reaction kit II (Applied Biosystems, Lenrik, Belgium) in combination with a gene-6-primer (5' - CTCTCTGTAAAGGCTGC-3'; Eurogentec). The sequencing program consisted of an initial denaturation step for 30” at 96°C, followed by 25 cycles of amplification (10 s at 96°C, 5 s at 50°C, 4 min at 60°C) in an ICycler. Sequencing products were purified using self-made Sephadex G50 spin columns (GE Healthcare), dissolved in highly deionized formamide (Applied Biosystems) and analysed using the ABI Prism 310 Genetic Analyser (Applied Biosystems). cDNA sequences were translated according to
Materials and methods

the reading frame of the vector (pSPVIA, pSPVIB and pSPVIC) in which the corresponding peptide/protein is displayed. Obtained sequences were compared with public nucleotide and protein databases using the Basic Local Alignment and Search Tool (BLAST) on the NCBI website (http://www.ncbi.nlm.nih.gov/).

2.1.7 ELISA of ligand-displaying phage

Immunoreactivity towards phage clones was determined with phage ELISA. Immunoreactivity against each phage clone (OD sample) was measured in relation to empty phage (OD background) that was used as an internal control. Ninety-six-well flat-bottomed microtiter plates (Falcon/BD) were coated overnight at 4°C with 200 μl of RαH IgG, 10 μg/ml in coating buffer and blocked with 200 μl of 2 % MPBS for 1h at RT. Following preincubation of 50 μl diluted serum (1:100 in 2 % MPBS) or 50 μl diluted CSF (1:3 in 2 % MPBS) with 100 μl of PEG-purified phage (10^10 phage/well) in a 96-well round-bottomed plate (Nunc) for 1h at 37°C, the plates were shaken for 30 min at RT. After washing three times with 0.1 % PBSTween and PBS, the preincubation mixture was transferred to the RαH IgG-coated plate and incubated for 1h at 37°C and 30 min shaking at RT. After washing the plate 3 times with 0.1 % PBSTween and PBS, 150 μl of a horseradish peroxidase (HRP) conjugated anti-M13 monoclonal antibody (GE Healthcare), diluted 1:5,000 in 2 % MPBS was incubated for 1h shaking at RT. After washing the plate 3 times with 0.1 % PBSTween en PBS, 130 μl of a 3,3′,5,5′ tetramethyl-benzidine dihydrochloride (TMB) chromogen solution (10 mg/ml) was added. Colour development was stopped with 65 μl/well 2 M H₂SO₄. The plates were read at 450 nm in a Bio-Rad Benchmark microplate reader (Bio-Rad).

2.1.8 Immunostaining of MS brain tissue sections

MS brain tissue samples were obtained from the Dutch Brain Bank (Amsterdam). MS tissue samples were taken from lesions located in the subcortical white matter. Immunohistochemistry was performed on frozen brain tissue of a 38 year old female RR MS patient with an expanded disability status scale (EDSS) score of 3 who died of a cardiac arrest after a 9-year disease history. Ten μm sections were cut using a Shandon Cryotome FSE (Thermo Fisher) and placed on Polysine coated TM microscope slides (VWR) which were air dried. Rabbit anti-
phospho-extracellular signal-regulated kinase 5 (p-ERK5) antibody (1:200 diluted in TBS) and anti-p-ERK1/2 antibody (Abcam) was used in combination with an anti-rabbit IgG-horseradish peroxidase secondary antibody (1:300 diluted in PBS; Dako). Colouring was obtained using the avidin-biotin-peroxidase complex (ABC, Dako). Peroxidase was developed with Sigma Fast 3,3’-Diaminobenzidine (DAB) tablets (Sigma, Bornem, Belgium) according to manufacturer’s instructions followed by counterstaining with haematoxylin and dehydration in an ethanol and xylene gradient. For the simultaneous detection of macrophages and p-ERK5 or p-ERK1/2 immuno double staining was performed immediately after DAB-staining by incubating a mouse anti-human CD68 antibody (1:2 diluted in TBS, Abcam) for 1h at RT. Colouring was obtained using Extravidin-alkaline phosphatase (AP, 1:75 diluted in TBS, Sigma) in combination with the Sigma Fast Red TR/Naphtol AS-MX tablets (Sigma) followed by counterstaining with haematoxylin. Finally, sections were mounted and observed using optical microscopy.

2.1.9 Statistical analysis
Statistical analysis was performed using Graphpad Prism version 4.00. Comparison between two patient groups was performed by the student’s T test or Mann-Whitney test when appropriate. The Fisher’s exact test was applied to evaluate a possible relationship between different clinical parameters such as age, gender, age of disease onset, disease duration, EDSS score, clinical subtype, active versus inactive disease, IgG concentration, treatment or no treatment and the presence and number of oligoclonal bands (OCB). A p-value of less than 0.05 was considered to be statistically significant.
Searching for new biomarkers for multiple sclerosis

Parts of these results were based on:
Exploring cDNA phage display for autoantibody profiling in the serum of multiple sclerosis patients: optimization of the selection procedure
Govarts, C.; Somers, K.; Hupperts, R.; Stinissen, P.; Somers, V.
Abstract

In the search for biomarkers for multiple sclerosis (MS), we applied a cDNA phage display method called serological antigen selection (SAS) to identify immunogenic targets that evoke an autoantibody response in serum or cerebrospinal fluid (CSF) of MS patients. This method involves the display of a cDNA expression library, in this study an MS brain library, on filamentous phage and subsequent selection using patient immunoglobulin G (IgG). In a first part of the study, the SAS procedure was optimized in order to deplete aspecific cDNA products. This was achieved by a differential selection procedure in which positive selection rounds on MS serum or CSF and negative selection rounds on healthy control serum were alternated, thereby decreasing the presence of aspecific phage clones. After optimization, affinity selections on pooled MS sera and CSF resulted in the identification of 5 enriched phage clones derived from selections on MS serum whereas selection rounds on MS CSF resulted in the identification of 12 enriched phage clones. Immunoreactivity against a panel of serum- and CSF-derived phage clones was then evaluated in respectively serum and CSF of another group of MS patients and control patients. Whereas immunoreactivity towards the serum-derived phage clones was found in a higher number of MS patients compared to controls, no difference in immunoreactivity could be found towards the CSF-derived phage clones.
3.1 Introduction

Since a correct diagnosis of MS sometimes can take years, MS-specific biomarkers are of great interest to many researchers and clinicians. Biomarkers could not only aid in improving a correct and early diagnosis, they could also be used for the stratification of patients and prediction of disease progression. As a result, a personalized treatment can be implemented very early in the disease course which could have a beneficial effect on further progression of symptoms.

Previously, our group has identified 8 MS-related biomarkers by applying SAS to pooled CSF of 10 relapsing remitting (RR) MS patients. Since these biomarkers were derived from affinity selection on patient CSF, we also wanted to explore the potential of serum antibodies to select candidate biomarkers for MS using cDNA phage display. Therefore, serum and CSF of 10 relapsing remitting (RR) MS patients was pooled and used for affinity selections on our MS cDNA display library. We decided to pool the CSF and serum samples in order to dilute patient-specific reactivity and at the same time increase the opportunity to identify MS-related antigens. In addition, the use of a cDNA library derived from active chronic MS brain plaques with varying degrees of inflammation and demyelination in combination with antibodies present in MS serum and CSF has the advantage of identifying disease-related antigenic targets without knowing the identity of the antigens in advance.

In the first part of the study, the SAS procedure was optimized in order to deplete non-relevant cDNA products. By introducing a differential screening method in which positive selection rounds on MS serum/CSF and negative selection rounds on healthy control (HC) serum were alternated, the occurrence of non-relevant phage clones was diminished. After optimization, affinity selections on pooled MS sera and CSF resulted in the identification of 5 enriched phage clones derived from selections on MS serum whereas selection rounds on MS CSF resulted in the identification of 12 enriched phage clones.
Chapter 3

Immunoreactivity against a panel of serum- and CSF-derived phage clones was then evaluated in another group of MS patients and control patients to determine if antibody reactivity towards the selected antigenic targets was specific for MS.
3.2 Materials and methods

3.2.1 Patient material
For the affinity selection, pooled serum and CSF samples from 10 untreated relapsing remitting (RR) MS patients were used (table 3.1). The negative selection rounds were performed with pooled serum from 10 healthy controls (3 men, 6 women, mean age = 38.0, range 24-52 years). For the ELISA-screening of phage clones derived from selections performed on MS sera, serum samples were obtained from 1 clinically isolated syndrome (CIS), 29 MS patients and 36 patients with other neurological diseases (OND) (hernia, epilepsy, dementia, meningitis, polyneuropathy,...) (table 3.2a). For the ELISA-screening of phage clones derived from selections on MS CSF, CSF samples were obtained from 1 CIS, 54 MS patients and 54 OND patients (table 3.2b).

Table 3.1: Characteristics of RR MS patients used in the selection rounds

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Disease duration (years)</th>
<th>EDSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V</td>
<td>48</td>
<td>11</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>V</td>
<td>43</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>V</td>
<td>51</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>V</td>
<td>35</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>V</td>
<td>28</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>44</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>V</td>
<td>31</td>
<td>9</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>26</td>
<td>6</td>
<td>1.5</td>
</tr>
<tr>
<td>9</td>
<td>V</td>
<td>35</td>
<td>6</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>V</td>
<td>55</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

EDSS: expanded disability status scale
RR MS: relapsing remitting multiple sclerosis
Table 3.2: Characteristics of patients used in the ELISA screening

A) Serological screening on MS and control sera

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>F/M</th>
<th>Mean age (SD) (Range in years)</th>
<th>Mean EDDS (SD) (Range)</th>
<th>Mean disease duration (SD) (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIS</td>
<td>1</td>
<td>0/1</td>
<td>29</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>MS</td>
<td>29</td>
<td>14/15</td>
<td>47.5 (12.0) (15-66)</td>
<td>4.2 (2.0) (0.0-8.0)</td>
<td>11.1 (7.7) (0-25)</td>
</tr>
<tr>
<td>RR MS</td>
<td>11</td>
<td>6/5</td>
<td>39.0 (11.7) (15-55)</td>
<td>2.5 (1.1) (0.0-4.0)</td>
<td>5.4 (4.7) (0-15)</td>
</tr>
<tr>
<td>SP MS</td>
<td>10</td>
<td>6/4</td>
<td>52.8 (11.0) (33-66)</td>
<td>5.3 (1.5) (2.5-7.0)</td>
<td>16.5 (7.0) (6-27)</td>
</tr>
<tr>
<td>PP MS</td>
<td>5</td>
<td>2/3</td>
<td>55.2 (3.8) (51-59)</td>
<td>4.9 (1.3) (3.5-6.5)</td>
<td>13.2 (6.0) (6-20)</td>
</tr>
<tr>
<td>PR MS</td>
<td>3</td>
<td>0/3</td>
<td>48.0 (8.9) (41-58)</td>
<td>5.3 (2.1) (3.0-8.0)</td>
<td>10.7 (5.8) (3-17)</td>
</tr>
<tr>
<td>OND</td>
<td>36</td>
<td>26/10</td>
<td>46.8 (13.2) (19-78)</td>
<td>NA</td>
<td>ND</td>
</tr>
</tbody>
</table>

B) Serological screening on MS and control CSF

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>F/M</th>
<th>Mean age (SD) (Range in years)</th>
<th>Mean EDDS (SD) (Range)</th>
<th>Mean disease duration (SD) (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIS</td>
<td>1</td>
<td>1/0</td>
<td>33</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>MS</td>
<td>54</td>
<td>42/13</td>
<td>42.6 (10.9) (16-71)</td>
<td>3 (2.3) (0.0-8.5)</td>
<td>7.4 (7.8) (0-35)</td>
</tr>
<tr>
<td>RR MS</td>
<td>34</td>
<td>27/8</td>
<td>38.9 (10.6) (16-71)</td>
<td>2.1 (1.5) (0.0-3.5)</td>
<td>7.0 (7.9) (0-30)</td>
</tr>
<tr>
<td>SP MS</td>
<td>9</td>
<td>7/2</td>
<td>51.8 (9.0) (36-64)</td>
<td>5.4 (2.8) (2.0-8.5)</td>
<td>10.6 (11.0) (2-35)</td>
</tr>
<tr>
<td>PP MS</td>
<td>11</td>
<td>8/3</td>
<td>46.9 (7.2) (38-57)</td>
<td>4.1 (1.5) (2.5-6.5)</td>
<td>6.0 (3.2) (1-10)</td>
</tr>
<tr>
<td>OND</td>
<td>54</td>
<td>32/22</td>
<td>49.1 (16.6) (16-85)</td>
<td>NA</td>
<td>ND</td>
</tr>
</tbody>
</table>

N: number of patients, F: female, M: male, SD: standard deviation, EDDS: expanded disability status scale
CIS: clinically isolated syndrome, RR MS: relapsing-remitting multiple sclerosis, SP MS: secondary progressive MS, PP MS: primary progressive MS, PR MS: primary relapsing MS,

3.2.2 Depletion of aspecific phage

During the selection procedure, specific enrichment of phage displaying candidate MS antigens occurred after each selection cycle. However, after a few selection cycles, aspecific cDNA products were identified. For that reason, an alternative selection strategy, called negative selection, was introduced to deplete these antigens. After each positive selection round (with MS sera or CSF) phage were prepared and used for a subsequent negative selection cycle on HC serum (1:100 diluted in 4% MPBS). The positive selection round was performed as described in section 2.1.4 and figure 2.2. The negative selection round was performed with minor modifications (figure 3.1). Briefly, after coating the immunotubes (Nunc) overnight at 4°C with 10 µg/ml rabbit anti-human (RaH) IgG (Dako) in coating buffer the tubes were blocked for 2h at room
temperature (RT) with 2 % (w/v) skimmed milk powder in PBS (MPBS). Approximately $2 \times 10^{12}$ plaque forming units (pfu) of purified phage clones derived from output phage of a positive selection round were added to the HC serum (1:100 diluted in 0.1 % MPBS) and incubated in a glass tube for 1.5 h at RT. After a preincubation for 1.5 h at RT on an end-over-end rotating platform, the preincubated HC serum and phage mix was transferred to the coated immunotube for 30 min on a rotating platform and 2 h standing at RT. Non-bound phage were then recovered and used for infection of Escherichia coli TG1 cells. Phage were rescued and the selection process of alternating selections on MS patient and normal sera was repeated. After each selection round, output phage were used for amplification and titration. PCR and fingerprinting analysis was used as described in 2.1.5 to monitor the depletion of aspecific phage clones and determine the amount of enrichment. Sequence analysis was used for confirmation of enrichment and identification of selected phage clones as described in 2.1.6.

### 3.2.3 Identification of plastic-binding phage clones

For identification of plastic-binders, plastic-binding capacity was assessed with the standardized ELISA-protocol as described in 2.1.5 (in presence of serum) and several experimental conditions (in absence of serum): i) non-coated microtiter plates blocked with 2 % MPBS, ii) non-coated microtiter plates blocked with 2 % bovine serum albumin (BSA; in PBS) and iii) non-coated microtiter plates blocked with PBS.

### 3.2.4 Statistical analysis

Statistical analysis was performed as described in 2.1.9. Evaluated parameters include age, gender, age of disease onset, disease duration, EDSS score, clinical subtype, IgG concentration, treatment or no treatment and the presence of oligoclonal bands (OCB). A $p$-value of less than 0.05 was considered to be statistically significant.
**Figure 3.1: Depletion of aspecific cDNA products by alternating selection rounds**

During a positive selection round, the MS cDNA display library is incubated with MS patient IgG (1). Antibodies bound to their specific antigenic target displayed on the phage (2) are captured with an anti-human IgG antibody (3) and subsequently eluted (4). Phage are amplified through infection of *E. coli* bacteria (5) and used as input phage in a negative selection cycle in which healthy control serum is used (6). After binding of aspecific phage clones (grey) (7), phage antigen-IgG complexes are captured (8). Non-bound phage were recovered (9) and used for infection and amplification (10). Phage were prepared and used for a subsequent positive selection cycle.
3.3 Results

3.3.1 Depletion of aspecific phage clones
An alternative selection procedure using pooled serum from healthy controls was introduced to diminish the occurrence of aspecific phage clones. PCR and fingerprinting analysis were used to monitor the depletion of aspecific phage clones and phage ELISA was used to identify plastic-binding phage clones.

3.3.1.1 Negative selection rounds decrease the presence of aspecific phage clones
The first 4 selection rounds on CSF were positive selection rounds performed with MS CSF. Due to a gradual enrichment of an aspecific phage clone named UH-AS1 (short for UHasselt – aspecific clone 1) encoding an out of frame IgG transcript, negative selection rounds were introduced from the 4th round (table 3.3). Selection rounds on serum demonstrated a higher enrichment of aspecific phage clones, including UH-AS1. Therefore, negative selection rounds were immediately introduced. A total of 10 alternating selection rounds (5 positive and 5 negative rounds) were performed (table 3.3).

Table 3.3: Affinity selection performed with pooled CSF and serum from 10 RR MS patients

<table>
<thead>
<tr>
<th>Serum selection</th>
<th>Round</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS serum / HC serum</td>
<td>MS HC MS HC MS HC MS HC MS HC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CSF selection</th>
<th>Round</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4a</th>
<th>4b</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS CSF / HC serum</td>
<td>MS MS MS HC MS HC MS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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However, most specific phage clones were retrieved from the first 4 selection rounds, both from selections on CSF and serum. In the following section, only the results of the alternative selection procedure on MS sera will be shown since negative selection rounds on CSF were only introduced in later selection rounds. The percentage of aspecific cDNA clones after each selection round was determined with PCR and fingerprinting analysis. As shown in table 3.4 the percentage of UH-AS1 substantially decreased after each negative selection round on serum, thereby increasing the chance to identify MS-related antigenic targets.

Table 3.4: Depletion of aspecific cDNA clones

<table>
<thead>
<tr>
<th>Selection round on serum</th>
<th>Phage clones screened</th>
<th>Non-UH-AS1</th>
<th>% UH-AS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MS</td>
<td>MS</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>2 HC</td>
<td>HC</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>3 MS</td>
<td>MS</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>4 HC</td>
<td>HC</td>
<td>50</td>
<td>39</td>
</tr>
</tbody>
</table>

3.3.1.2 Identification of plastic-binding phage clones by phage ELISA

The efficiency of selection was determined by testing the number of phage clones reactive in ELISA with the MS serum pool used in the selection procedure. Positive clones were identified from the 2nd round of selection. More aspecific phage clones were identified as the number of selection cycles increased, although their enrichment was not as dominant as UH-AS1. Aspecific phage clones were characterized by polyreactivity as a result of plastic-binding capacity. Figure 3.2 illustrates the results of aspecific phage clones UH-AS2 and UH-AS3 which were representative for plastic-binding phage clones. Empty phage, not displaying any protein, was used as a reference value since in normal conditions this clone is used to determine background values. The detected OD-values for UH-AS2 and UH-AS3 were very high in all tested conditions. This was in contrast with the OD-values of empty phage under standard conditions and when the microtiter plate was blocked with 2 % MPBS (in absence of serum). However, when suboptimal blocking with 2 % BSA was applied or in the absence
of blocking buffer the OD-values of empty phage also increased due to aspecific binding.

Figure 3.2: Identification of plastic-binding phage clones by phage ELISA
Reactivity of 2 representative plastic-binders, UH-AS2 and UH-AS3, was determined under several experimental conditions: RαH IgG-coated 96-well plates in presence of serum (standard conditions), non-coated plates without incubation of serum blocked with either 2 % MPBS, 2 % BSA or only PBS. Empty phage was used as a reference value.

3.3.2 Enrichment of candidate MS-related phage clones derived from selection rounds on MS sera
Despite the fact that in later rounds aspecific phage clones were selected, specific immunoreactive phage clones were identified in earlier selection rounds on MS sera. Enriched phage clones were defined as phage clones that were identified at least twice throughout the selection procedure. Enrichment was determined by PCR and fingerprinting analysis and confirmed by sequence analysis. After an initial PCR-screening of 20 output phage clones in the first 2 rounds, approximately 50 output phage clones were screened per round. A total of 5 enriched candidate MS-related phage clones named UH-SAS.SP1 to UH-SAS.SP5 (short for UHasselt – serological antigen selection. serum pool, number of the phage clone) were identified (table 3.5).
### Chapter 3

#### Table 3.5: Sequence analysis of 5 enriched phage clones derived from selections on MS sera

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>cDNA Identity* (accession No.)</th>
<th>Orientation</th>
<th>Translated cDNA product</th>
<th>Size†</th>
<th>BLASTp homology* (accession No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-SAS.SP1</td>
<td>Heat shock protein 90AA1 (NM_005348)</td>
<td>3' UTR</td>
<td>KISVY*</td>
<td>5</td>
<td>100% (5/5) neuraminidase 1 (EAX03537)</td>
</tr>
<tr>
<td>UH-SAS.SP2</td>
<td>RNA binding motif 38 (NM_017495)</td>
<td>3' UTR</td>
<td>ALKLQLFFCNCATKISEVQSIFSADTAATKNPKIFDLERLA FVGSARGDNDGVSV*</td>
<td>58</td>
<td>32% (20/62) hCG2007168 (EAX09667)</td>
</tr>
<tr>
<td>UH-SAS.SP3</td>
<td>UI-CF-FN0 (CB243999)</td>
<td>EST</td>
<td>FFFYIFIVFYFWKDHIHISHQ*</td>
<td>22</td>
<td>45% (10/22) hCG1813824 (EAW64991) Viral: 58% (7/12) Epstein-barr nuclear antigen (EBNA)-1 (YP_001129471.1)</td>
</tr>
<tr>
<td>UH-SAS.SP4</td>
<td>Cyclin dependent kinase 4 (NM_000075)</td>
<td>Out of frame</td>
<td>QSEVGEHSGDKWWNSQAG*</td>
<td>18</td>
<td>72% (8/11) Regulator of G-protein signaling-22 (EAW91797)</td>
</tr>
<tr>
<td>UH-SAS.SP5</td>
<td>NIH_MGC_186 (CD512128)</td>
<td>EST</td>
<td>IND*</td>
<td>3</td>
<td>/</td>
</tr>
</tbody>
</table>

* Sequence similarity on nucleotide level as determined by nucleotide BLAST
† Size of protein product in amino acids fused to pVI coat protein
* Indicates a stop codon

* Sequence similarity at amino acid level as determined by protein BLAST
* EST: expressed sequence tag
Homology search analysis at the nucleotide level revealed a 100% match with the DNA sequence of known genes for 4 phage clones while the nucleotide sequence of UH-SAS.SP5 matched with an expressed sequence tag (EST). cDNA sequences were translated according to the open reading frame of the vector (pSPVIA, pSPVIB or pSPVIC) in which the corresponding peptide/protein is displayed. The length of the translated cDNA products ranged from 3 to 58 amino acids (AA). The translated cDNA products displayed significant homology to several hypothetical proteins (UH-SAS.SP2, UH-SAS.SP3), regulator of G-protein signalling (UH-SAS.SP4) and neuraminidase 1 (Neu1) (UH-SAS.SP1). Regulator of G-protein signalling is involved in intracellular signaling\textsuperscript{226} whereas Neu1 influences cellular activity by removing terminal sialic acid from glycoproteins and glycolipids\textsuperscript{227}. One phage clone also displayed homology with a viral protein derived from Epstein - Barr virus (EBV) (UH-SAS.SP3). The peptide displayed at the surface of UH-MS.SP5 was too small to compare with public databases. Immunoreactivity towards this clone may be directed towards an epitope, also called a phagotope, that is composed of both phage coat proteins and the displayed cDNA insert\textsuperscript{215}.

From our panel of 5 enriched phage clones, serum immunoreactivity towards UH-SAS.SP3, UH-SAS.SP4 and UH-SAS.SP5 was further evaluated. Initially, immunoreactivity towards UH-SAS.SP3, UH-SAS.SP4 and UH-SAS.SP5 was verified in individual serum samples of the 10 RR MS patients used in the selection procedure. Immunoreactivity against our panel of 3 phage clones was detected in 20% (2/10) of the MS patients whose serum was used in the selection procedure. When more MS sera were tested, 18% (7/40) of MS patients displayed immunoreactivity to at least one of the selected phage clones compared to 6% (2/36) of control sera (table 3.6). No correlation could be found between the presence of antibodies in serum of MS patients and clinical parameters.
Chapter 3

Table 3.6: ELISA screening of serum-derived phage clones

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>SAS&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Screening</th>
<th>Total (%)</th>
<th>OND Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-SAS.SP3</td>
<td>1/10</td>
<td>1/30</td>
<td>2/40 (5.0)</td>
<td>0/36 (0)</td>
</tr>
<tr>
<td>UH-SAS.SP4</td>
<td>1/10</td>
<td>3/30</td>
<td>4/40 (10.0)</td>
<td>1/36 (2.8)</td>
</tr>
<tr>
<td>UH-SAS.SP5</td>
<td>1/10</td>
<td>1/30</td>
<td>2/40 (5.0)</td>
<td>1/36 (2.8)</td>
</tr>
</tbody>
</table>

3.3.3 Serological antigen selection performed on pooled MS CSF

A total of 7 selection rounds were performed on pooled MS CSF. After an initial PCR-screening of 20 output phage clones in the first 2 rounds, approximately 50 output phage clones were screened per round. A total of 12 enriched antigenic targets, UH-SAS.CP1 to UH-SAS.CP12 (short for UHasselt, serological antigen selection.CSF pool, number of the phage clone) were identified (table 3.7). The length of the identified cDNA products ranged from 5 to 90 AA. Several of the identified antigenic targets displayed homology to members of intracellular signaling pathways (UH-SAS.CP2, UH-SAS.CP5, UH-SAS.CP6, UH-SAS.CP9). In addition, UH-SAS.CP2 encoded the protein MAPK3, also known as extracellular signal-regulated kinase (ERK) 1. The expression profile of this member of the MAPK family was further analyzed in an MS brain lesion as described in chapter 4 and figure 4.2. Another interesting phage clone is UH-SAS.CP11 which displayed a significant homology to heat shock protein (Hsp)47. Heat shock proteins belong to the superfamily of stress proteins and are important players in the control of immune responses. Other antigenic targets displayed homology to hypothetical proteins (UH-SAS.CP1, UH-SAS.CP4, UH-SAS.CP10 and UH-SAS.CP12) or viral peptides, including human herpes virus 5, human T-lymphotropic virus, hepatitis C and human adenovirus.
Table 3.7: Sequence analysis of 12 enriched phage clones derived from selections on MS CSF

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>cDNA identitya (accession No.)</th>
<th>Orientation</th>
<th>Translated cDNA product</th>
<th>Sizeb</th>
<th>BLASTp homologyc (Accession No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-SAS.CP1</td>
<td>PLACE7 (DA865009)</td>
<td>ESTd</td>
<td>GACVEWEEVALSMGVGLRHPE MINSQRRWQHPQ*</td>
<td>35</td>
<td>40% (13/32) Hypothetical protein (XP_001715006) Viral: 50% (8/16) hemagglutinin (Influenza A virus) (ACI41219)</td>
</tr>
<tr>
<td>UH-SAS.CP2</td>
<td>Mitogen activated kinase 3 (MAPK3) (NM_00110989)</td>
<td>in frame</td>
<td>DLPKERLKE1FQETARFQPGVLE APOQPRQTSLHPGAWTCLLPAPLP PDCXKWLCPARTLAQAGVEH GPXTSLLC*</td>
<td>77</td>
<td>100% (26/26) mitogen activated protein kinase (MAPK) 3; 51% (14/27) potassium channel, subfamily K, member 16 (NM_01103361)</td>
</tr>
<tr>
<td>UH-SAS.CP3</td>
<td>Large tumor suppressor 2 (NM_014572)</td>
<td>out of frame</td>
<td>PSEGSLSMTMATPFDAQSLQEQKL HRLLRAIQKALIWWROIKSACIC TCRWGPQTPTRCLPGQPQAG ALTGQGGRGLFCPNSQVDYFT*</td>
<td>90</td>
<td>43% (13/30) Basic fibroblast growth factor receptor protein 1 (AA750077) Viral: 37% (12/32) polymerase (Hepatitis B virus)</td>
</tr>
<tr>
<td>UH-SAS.CP4</td>
<td>FERM domain containing protein 6 (NM_152330)</td>
<td>3’ UTRb</td>
<td>GYSGT*</td>
<td>5</td>
<td>100% (5/5) hCG1755809 (EAW93068) Viral: 100% (4/4) hexon, human adenovirus -2) (BAG24054)</td>
</tr>
<tr>
<td>UH-SAS.CP5</td>
<td>Ring finger protein 111 (NM_017610)</td>
<td>3’ UTR</td>
<td>VFTQYQQALIMNLSTNL*</td>
<td>20</td>
<td>42% (12/28) transmembrane protein 184B (BAC77406)</td>
</tr>
<tr>
<td>UH-SAS.CP6</td>
<td>Ring finger protein 213 (NM_020914)</td>
<td>3’ UTR</td>
<td>GAKSLIKGTGLPGPGPWSPH A*</td>
<td>25</td>
<td>76% (10/13), 64% (9/14), 100% (6/6) dual specificity phosphatase 8 (EAX02451)</td>
</tr>
<tr>
<td>UH-SAS.CP7</td>
<td>fragile X mental retardation2 (NM_004860)</td>
<td>out of frame</td>
<td>ARGHPWNLNPQKTLPQDRVRT LSASFLRPRMGGPWSWV VW*</td>
<td>46</td>
<td>37% (10/27), 29% (7/24), 42% (8/19) nuclear mitotic apparatus protein (CAA77669) Viral: 30% (11/36) nonstructural protein 5A (hepatitis C virus) (ABF48696)</td>
</tr>
</tbody>
</table>
# Table 3.7 (continued): Sequence analysis of 12 enriched phage clones derived from selections on MS CSF

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>cDNA identity* (accession No.)</th>
<th>Orientation</th>
<th>Translated cDNA product</th>
<th>Size(^b)</th>
<th>BLASTp homology(^c) (Accession No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-SAS.CP8</td>
<td>Protein expressed in non-metastatic cells 5 (NM_003551)</td>
<td>out of frame</td>
<td>WDQQIAQRRHITQTV*</td>
<td>16</td>
<td>75% (9/12) myeloid/lymphoid or mixed-lineage leukemia 5 (EAW83358) Viral: 75% (6/8) reverse transcriptase (human T-lymphotropic virus-1) (AAD39689)</td>
</tr>
<tr>
<td>UH-SAS.CP9</td>
<td>NIH_MGC_229 (CK004732)</td>
<td>EST(^d)</td>
<td>NRGERDSTE*</td>
<td>9</td>
<td>75% (6/8) mitogen-activated protein kinase kinase kinase 4 (BAH12341.1)</td>
</tr>
<tr>
<td>UH-SAS.CP10</td>
<td>Calcyphosine (NM_080590)</td>
<td>out of frame</td>
<td>PGLLQRECLHEIHG*</td>
<td>14</td>
<td>66% (10/15), 80% (4/5) hypothetical protein (XP_001717067) Viral: 64% (9/14) glycoprotein (human herpesvirus-5) (ABU85795)</td>
</tr>
<tr>
<td>UH-SAS.CP11</td>
<td>Platelet-derived growth factor receptor alpha (NM_006206)</td>
<td>3' UTR(^e)</td>
<td>GKMQKQ*</td>
<td>6</td>
<td>100% (5/5) heat shock protein 47 (EAW74978)</td>
</tr>
<tr>
<td>UH-SAS.CP12</td>
<td>BRSSN2003037 (DA343073)</td>
<td>EST</td>
<td>HSTDARHLCYQFLIALYKATMN IFHVFWLIALISGPMTSYSGA MKV5QFSFSGYQIRCQIRPFL SFF*</td>
<td>72</td>
<td>38% (19/50) hCG2038463 (EAW57153)</td>
</tr>
</tbody>
</table>

* Sequence similarity on nucleotide level as determined by nucleotide BLAST
\(^b\) Size of protein product in amino acids fused to pVI coat protein
* Indicates a stop codon
\(^c\) Sequence similarity at amino acid level as determined by protein BLAST
\(^d\) EST: expressed sequence tag
\(^e\) untranslated region
New biomarkers for MS

From our enriched phage clones, a panel of 3 clones (UH-SAS.CP9, UH-SAS.CP11 and UH-SAS.CP12) was chosen for a detailed serological analysis. After an initial screening in individual CSF of the 10 RR MS patients used for the selection procedure, immunoreactivity towards the CSF phage panel was evaluated in the CSF of 55 MS and 54 OND patients (table 3.8). No difference in immunoreactivity could be found between the MS group (6/65 (9%) antibody positive patients) and the control group (3/54 (6%) antibody positive patients). Of note, 5 out of 6 (83%) antibody positive MS patients were in a progressive phase of the disease (SP or PP MS) in contrast to 15 out of 59 (25%) antibody negative MS patients. No correlation could be found between the presence of antibodies in CSF of MS patients and other clinical parameters.

Table 3.8: ELISA screening of CSF-derived phage clones

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>MS patients</th>
<th>OND</th>
<th>Total (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAS&lt;sup&gt;5&lt;/sup&gt;</td>
<td>Screening</td>
<td>Total (%)</td>
<td>Total (%)</td>
</tr>
<tr>
<td>UH-SAS.CP9</td>
<td>1/10</td>
<td>3/55</td>
<td>4/65 (6.2)</td>
<td>1/54 (1.9)</td>
</tr>
<tr>
<td>UH-SAS.CP11</td>
<td>0/10</td>
<td>3/55</td>
<td>3/65 (4.6)</td>
<td>1/54 (1.9)</td>
</tr>
<tr>
<td>UH-SAS.CP12</td>
<td>0/10</td>
<td>4/55</td>
<td>4/65 (6.2)</td>
<td>2/54 (3.7)</td>
</tr>
</tbody>
</table>
3.4 Discussion

In our study, a cDNA phage display-based method called serological antigen selection (SAS) was used as a strategy to identify new antigenic markers in serum and CSF of MS patients. To apply SAS on pooled serum, an optimization of the procedure was needed to deplete aspecific cDNA products. By alternating positive selection rounds, using MS serum or CSF, with negative selection rounds, using HC serum, a substantial decrease of aspecific phage clones was observed after each negative selection round, thereby facilitating the identification of candidate MS-related cDNA phage clones.

The alternative selection procedure allowed the identification of 5 enriched phage clones derived from selections on MS sera whereas selection rounds on MS CSF resulted in the identification of 12 enriched phage clones. Many of the identified phage clones encode polypeptides resulting from expression of 3' untranslated regions of known genes and out of frame sequences. Part of these polypeptides may originate from alternative splicing of mRNA products. Alternative splicing allows one pre-mRNA to be processed into many different mature forms allowing a tissue-specific or temporal expression of a novel gene product. However, the majority of the peptides most likely represent mimotopes which are peptides that mimic the functional binding properties of natural occurring proteins. Although some phage clones may represent mimotopes, significant homology with a heat shock protein and several members of the MAPK family and viral proteins were identified. Of note, the cDNA insert of the serum-derived phage clone UH-SAS.SP3 contained an epitope that displayed homology with Epstein - Barr nuclear antigen (EBNA)-1. This is in line with several other studies that have identified antibody-directed peptide sequences displaying homology with Epstein - Barr virus (EBV)-proteins and adds further evidence to the debate concerning a role for EBV in the pathogenesis of MS. Increased antibody responses in CSF and serum of MS patients and EBNA-specific OCB suggests an increased immune response to
EBV-related proteins\textsuperscript{42,47,48,219}. In addition, epidemiological studies demonstrate that late EBV-infection is associated with an increased risk of developing MS whereas increased EBV-specific antibody titres in serum can already be detected years before clinical symptoms emerge (reviewed in \textsuperscript{231}). However, if these antibodies indeed have a pathological function in MS is not clear.

Immunoreactivity against 3 serum- and 3 CSF-derived phage clones was further evaluated in respectively sera and CSF of both MS patients and control patients. No MS-related immunoreactivity could be found towards the 3 phage clones derived from CSF selections. Of note, most antibody-positive MS patients were in a progressive disease stage which could indicate that these phage clones could be correlated with disease mechanisms associated with a more progressive disease course. However, since this is a very small patient group more patients need to be tested before definite conclusions can be drawn concerning antibody positivity and a correlation with progressive disease stage. Immunoreactivity towards our panel of phage clones derived from serum selections could be found in a subgroup of MS patients. Nevertheless, no correlation existed between antibody positivity towards the panel and available clinical parameters. By testing a larger patient group with different disease subtypes, it may be possible to better correlate antibody positivity with clinical parameters.

Immunoreactivity in serum and CSF was especially found towards phage clones derived from the first 4 selection rounds. Phage clones derived from later selection rounds showed either no reactivity or aspecific polyreactivity against individual serum/CSF samples of the pool. As a result of monovalent display of our cDNA products in combination with stringent selection conditions, only high-affinity antibody-binding interactions are selected\textsuperscript{232}. These high-affinity antibodies could be very relevant regarding pathogenesis and diagnosis of MS, but probably their concentration in serum and CSF is too low for detection in phage ELISA. This can explain the lack of reactivity in the ELISA procedure for some of the selected phage clones in later rounds of selection. Another possibility is that throughout the selection cycles a preferential selection of the best growing phage clones instead of the most relevant ones can occur\textsuperscript{233,234}.
Also, aspecific phage clones, for example plastic-binders, can be enriched, explaining the polyreactivity of some clones\textsuperscript{235}.

In summary, by introducing a differential screening procedure in which positive selection was alternated with negative selection rounds on HC sera, the occurrence of aspecific phage clones was diminished, especially in the first 4 selection rounds. As a result, we could identify a panel of enriched phage clones of which several were further evaluated in a larger patient group. Although immunoreactivity towards the selected phage clones was rather low, more patients with different disease subtypes need to be evaluated to determine the potential of these phage clones as biomarkers for MS.
Analysis of antibody reactivity in cerebrospinal fluid and serum of a relapsing remitting multiple sclerosis patient

Based on:
Analysis of antibody reactivity in cerebrospinal fluid and serum of a relapsing remitting multiple sclerosis patient
Govarts, C.; Somers, K.; Hupperts, R.; Stinissen, P.; Somers, V.
Submitted
Chapter 4

Abstract

Increasing evidence indicates an involvement of B cells in multiple sclerosis (MS). However, little is known about antigenic targets recognized by antibodies present in blood and cerebrospinal fluid (CSF) of MS patients. In this study antigen reactivity of antibodies present in CSF and blood of an MS patient was analyzed using cDNA phage display. Selection rounds on paired CSF and serum of this patient identified 13 antigenic targets of which 5 were enriched by serum antibodies and 2 were identified by CSF antibodies. Interestingly, the 6 remaining antigenic targets were shown to be recognized by both CSF and serum antibodies. These findings point towards antibodies with both common as well as distinct reactivities in CSF and serum of this MS patient.
4.1 Introduction

Identifying the antigenic targets of antibodies present in MS CSF and serum may yield further insights into the pathogenesis of MS. However, despite extensive research, antibody specificity in MS is still unresolved. This is partly due to the lack of powerful technologies to analyze antibody reactivity of MS patients in CSF and serum in parallel. The goal of this chapter was to analyze in parallel the antigen reactivity of antibodies in CSF and serum of a patient with MS to provide more information concerning the correlation between the humoral immune response in CSF and serum. Whereas in the previous chapter pooled patient material was used, in this study paired CSF and serum of a single patient was used. Pooled patient material creates a large diversity of antibodies which is needed in the search for biomarkers for MS. However, this diversity can complicate the comparison of antibody repertoires in CSF and serum.

The patient used in the selection procedure had a rather fast and active disease course. In addition, the patient was suffering from a relapse at time of sampling, thus ensuring an active immune system. Affinity selection of CSF and serum antibodies on an MS cDNA display library resulted in the enrichment of both CSF- and serum-specific antigenic targets. In addition, a panel of antigenic targets was selected by antibodies present in both CSF and serum. These results suggest the presence of both unique and common antibodies in CSF and serum of this MS patient.
4.2 Materials and methods

4.2.1 Patient material
Paired CSF and serum samples were obtained from a 60-year-old male with a 5-
year history of relapsing remitting (RR) MS. The patient was treated in the past
with IFN-β and with methotrexate. At time of sampling the patient had not
received any treatment for 3 months. The patient was chosen because of its fast
disease progression as indicated by an expanded disability status scale (EDSS)
score of 6.5 and an annual relapse rate of 4. In addition, magnetic resonance
imaging (MRI) demonstrated the presence of active lesions pointing towards an
active disease at time of sampling. An involvement of the humoral immune
response is indicated by the presence of 3 to 5 OCB in both CSF and serum with
some unique bands in CSF. IgG concentration was 980 mg/dl and 4 mg/dl for
serum and CSF, respectively. For ELISA-screening, serum samples were
obtained from 1 clinically isolated syndrome (CIS), 21 MS patients, 22 healthy
controls (HC) and 19 patients with other neurological diseases (OND)
(meningitis, polyneuropathy, hernia, epilepsy, dementia,...). Paired CSF from all
CIS, MS and OND patients was also obtained. Patient characteristics are
depicted in table 4.1. After collection, samples were stored at −80°C. Serum and
CSF samples tested showed equivalent total serum and CSF IgG concentrations
and were diluted 1:100 and 1:3 in 2% (w/v) skimmed milk powder phosphate-
buffered saline (MPBS), respectively prior to use in the ELISA.

4.2.2 Serological antigen selection on paired MS CSF and serum
A total of 4 consecutive selection rounds were performed in parallel on paired
MS CSF and serum as described in 2.1.3 with following modification. Pre-
incubation of CSF (diluted 1:5 in 0.1% MPBS) or serum (diluted 1:100 in 0.1%
MPBS) with 2 x 10^{12} plaque forming units (pfu) derived from output phage of the
previous selection round was performed in a glass tube for 1.5 h at RT while
shaking horizontally.
Analysis of antibody reactivity

Table 4.1: Patient characteristics

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>F/M</th>
<th>Mean age (SD) (Range in years)</th>
<th>Mean EDDS (SD) (Range)</th>
<th>Mean disease duration (SD) (Range in years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIS</td>
<td>1</td>
<td>1/0</td>
<td>28 (10.6) (28-66)</td>
<td>2 (0.8) (0-8)</td>
<td>10.4 (6.5) (0-25)</td>
</tr>
<tr>
<td>MS</td>
<td>21</td>
<td>10/11</td>
<td>42.3 (7.3) (32-55)</td>
<td>2.5 (1.2) (0-4)</td>
<td>7.7 (4.3) (0-17)</td>
</tr>
<tr>
<td>RR MS</td>
<td>13</td>
<td>8/5</td>
<td>57.7 (12.7) (43-66)</td>
<td>5.5 (2.1) (4-7)</td>
<td>13.3 (10.2) (6-25)</td>
</tr>
<tr>
<td>SP MS</td>
<td>3</td>
<td>2/1</td>
<td>46.3 (11.1) (36-58)</td>
<td>5.5 (3.5) (3-8)</td>
<td>11 (6.6) (4-17)</td>
</tr>
<tr>
<td>PP MS</td>
<td>2</td>
<td>0/2</td>
<td>44.4 (12.1) (19-68)</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>PR MS</td>
<td>3</td>
<td>0/3</td>
<td>45.6 (13.3) (24-66)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>OND</td>
<td>19</td>
<td>15/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>22</td>
<td>14/8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CIS: clinically isolated syndrome, RR MS: relapsing remitting multiple sclerosis, SP MS: secondary progressive MS, PP MS: primary progressive MS, PR MS: progressive relapsing MS, OND: other neurological disease; HC: healthy control; NA: not applicable; ND: not determined; SD: standard deviation

4.2.3 Immunostaining of tissue sections

Immunostaining was performed on MS brain lesions as described in 2.1.8. Rabbit anti-phospho-extracellular signal-regulated kinase 5 (p-ERK5) and anti-p-ERK1/2 antibody were used as primary antibodies while double staining was performed using a mouse anti-human CD68 antibody.
4.3 Results

4.3.1 Serological antigen selection of an MS cDNA phage display library using paired CSF and serum

Four selection rounds on paired CSF and serum resulted in the identification and enrichment of 13 phage clones, annotated UH-SAS1.1 – UH-SAS1.13 (short for UHasselt-serological antigen selection.number of the phage clone) (table 4.2). Since enrichment of aspecific phage was not as dominant as in the selection rounds with pooled patient material, negative selections were not performed. The lesser enrichment of aspecific phage clones is probably due to a combination of individual patient material which reduces the antibody complexity and the adaptation in the preincubation procedure which is described in section 4.2.2.

Two phage clones, UH-SAS1.1 and UH-SAS1.2 were exclusively found in the output fraction derived from CSF selections while 5 phage clones (UH-SAS1.9 to UH-SAS1.13) were uniquely identified in the output fraction derived from serum selections. Six phage clones (UH-SAS1.3 to UH-SAS1.8) were highly enriched in both CSF and serum outputs, together accounting for 46% of the CSF output screened and 51% of the serum output screened.
### Table 4.2: Enrichment of 13 phage clones after affinity selection on paired MS CSF and serum

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>CSF(^1)</th>
<th>Serum(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-SAS1.1</td>
<td>2/245</td>
<td>0/251</td>
</tr>
<tr>
<td>UH-SAS1.2</td>
<td>4/245</td>
<td>0/251</td>
</tr>
<tr>
<td>UH-SAS1.3</td>
<td>7/245</td>
<td>6/251</td>
</tr>
<tr>
<td>UH-SAS1.4</td>
<td>61/245</td>
<td>22/251</td>
</tr>
<tr>
<td>UH-SAS1.5</td>
<td>9/245</td>
<td>24/251</td>
</tr>
<tr>
<td>UH-SAS1.6</td>
<td>21/245</td>
<td>37/251</td>
</tr>
<tr>
<td>UH-SAS1.7</td>
<td>3/245</td>
<td>24/251</td>
</tr>
<tr>
<td>UH-SAS1.8</td>
<td>18/245</td>
<td>14/251</td>
</tr>
<tr>
<td>UH-SAS1.9</td>
<td>0/245</td>
<td>3/251</td>
</tr>
<tr>
<td>UH-SAS1.10</td>
<td>0/245</td>
<td>2/251</td>
</tr>
<tr>
<td>UH-SAS1.11</td>
<td>0/245</td>
<td>2/251</td>
</tr>
<tr>
<td>UH-SAS1.12</td>
<td>5/251</td>
<td></td>
</tr>
<tr>
<td>UH-SAS1.13</td>
<td>0/245</td>
<td>3/251</td>
</tr>
</tbody>
</table>

\(^1\) Number of times a phage clone was identified/total number of clones screened

### 4.3.2 Sequence analysis of enriched phage clones

Sequence analysis was performed to obtain the identity of the selected antigenic targets. Homology searches performed at the nucleotide level revealed 100% similarity with the DNA sequence of known genes for 12 clones while similarity to an expressed sequence tag (EST) was found for 1 other clone (UH-SAS1.9) (table 4.3). cDNA sequences were translated according to the open reading frame of the vector (pSPVIA, pSPVIB or pSPVIC) in which the corresponding peptide/protein is displayed. The length of the translated sequences ranged from 4 to 66 AA. At the amino acid level homology to both known proteins like mitogen activated protein kinase (MAPK) 7 (UH-SAS1.8) and novel peptides resulting from expression of 3’ untranslated regions of known genes and out of frame sequences was found as depicted in table 4.3.
## Table 4.3: Sequence analysis of 13 enriched phage clones

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>Source</th>
<th>cDNA identity* (Accession No.)</th>
<th>Orientation</th>
<th>Translated cDNA product</th>
<th>Size*</th>
<th>BLASTp homology* (Accession No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-SAS1.1</td>
<td>CSF</td>
<td>Solute carrier C (NM_015865.2)</td>
<td>out of frame</td>
<td>YLLV*</td>
<td>4</td>
<td>100% (4/4) brain apoptosis-associated tyrosine kinase (XP_001714134.1)</td>
</tr>
<tr>
<td>UH-SAS1.2</td>
<td>CSF</td>
<td>Cordon-blue homolog (NM_015198.2)</td>
<td>3' UTR</td>
<td>PTKLLKINYYHPENSPT*</td>
<td>17</td>
<td>100% (7/7) hypothetical protein (XP_001127293)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>69% (9/13) eukaryotic translation initiation factor 3 (EAW56805)</td>
</tr>
<tr>
<td>UH-SAS1.3</td>
<td>CSF/serum</td>
<td>Calcium binding protein39 (NM_016289.2)</td>
<td>3' UTR</td>
<td>KITTN*</td>
<td>6</td>
<td>83% (5/6) kinase anchor protein 9 (EAW76856)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Viral: 83% (5/6) orf FR3 human herpesvirus 6 (AA819785)</td>
</tr>
<tr>
<td>UH-SAS1.4</td>
<td>CSF/serum</td>
<td>CCAAT/enhancer binding protein alpha (NM_004364.2)</td>
<td>3' UTR</td>
<td>PLVGEQDLEIWLWGGHG*</td>
<td>18</td>
<td>80% (8/10) mannosidase (EAX02122)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Viral: 100% (5/5) and 66% (4/6) ORF1 human parvovirus (AVB71710)</td>
</tr>
<tr>
<td>UH-SAS1.5</td>
<td>CSF/serum</td>
<td>WWC family member 3 (NM_015691.2)</td>
<td>out of frame</td>
<td>PSLF*</td>
<td>4</td>
<td>100% (4/4) hypothetical protein (XP_001722527)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Viral: 100% (4/4) large protein, measles virus (ACA09727)</td>
</tr>
<tr>
<td>UH-SAS1.6</td>
<td>CSF/serum</td>
<td>Apurinic/apyrimidinic endonuclease 1 (NM_001641.2)</td>
<td>out of frame</td>
<td>GFEPGLRRKDQIG*</td>
<td>13</td>
<td>77% (7/9) caspase recruitment domain protein 12 (AAK38730)</td>
</tr>
<tr>
<td>UH-SAS1.7</td>
<td>CSF/serum</td>
<td>Contactin associated-like protein 3 (NM_033655.2)</td>
<td>out of frame</td>
<td>RYILKFDQF*</td>
<td>9</td>
<td>85% (6/7) potassium channel tetramerisation domain containing 12 (NP_612453)</td>
</tr>
</tbody>
</table>
## Table 4.3 (continued): Sequence analysis of 13 enriched phage clones

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>Source</th>
<th>cDNA identity* (Accession No.)</th>
<th>Orientation</th>
<th>Translated cDNA product</th>
<th>Sizeb</th>
<th>BLASTp homologyc (Accession No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-SAS1.8</td>
<td>CSF/serum</td>
<td>Mitogen activated protein kinase 7 (NM_139032.1)</td>
<td>in frame</td>
<td>LNQSFDMGVADGPQDQQADSAASLS ASLLADWLEWHGMNPADIESLQREI QMDSPMLIALPDLPQDP*</td>
<td>66</td>
<td>100% (66/66) MAPK7 (Q13164)</td>
</tr>
<tr>
<td>UH-SAS1.9</td>
<td>serum</td>
<td>NIH_MGC_181 (CD245920) ESTd</td>
<td>ESTd</td>
<td>RSVVNL*</td>
<td>6</td>
<td>83% (5/6) Cdc2-related kinase (AAI50266.1)</td>
</tr>
<tr>
<td>UH-SAS1.10</td>
<td>serum</td>
<td>Putative small membrane protein NID67 (NM_032947.3)</td>
<td>3' UTR</td>
<td>PHRDVRRT*</td>
<td>8</td>
<td>100% (6/6) SLIT and NTRK-like 3 family (AAI14622)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Viral: 100% (5/5) ORF66, human herpesvirus 8 type P (ABD28921)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UH-SAS1.11</td>
<td>serum</td>
<td>RAB6 interacting protein (NM_015213.2)</td>
<td>3' UTR</td>
<td>RKLEDFWNK*</td>
<td>9</td>
<td>100% (6/6) transposase-like protein (AAF18452)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Viral: 87% (7/8) polyprotein, hepatitis C virus subtype 6i (AB136973.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UH-SAS1.12</td>
<td>serum</td>
<td>Heat shock protein 90AB1 (NM_007355.2)</td>
<td>out of frame</td>
<td>EHLRLDSQYGHADHESPTSQQLHH GLYDQKAPGDQP*</td>
<td>37</td>
<td>72% (21/29) Hsp90AB6P (pseudogene)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(AAX38254.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UH-SAS1.13</td>
<td>serum</td>
<td>Anaphase promoting complex subunit (NM_015391.1)</td>
<td>3' UTR</td>
<td>YRDRTPGQWRTPPGSDLGPKIFR LTVGSL*</td>
<td>32</td>
<td>41% (12/29) KIAA1407 (Q8NCU4)</td>
</tr>
</tbody>
</table>

* Sequence similarity on nucleotide level as determined by nucleotide BLAST  
* Size of protein product in amino acids fused to pIV coat protein  
* Indicates a stop codon  
* Sequence similarity at amino acid level as determined by protein BLAST  
  
EST: expressed sequence tag
4.3.3 Immunoreactivity towards enriched phage clones

Immunoreactivity towards 8 of 13 identified phage clones (UH-SAS1.2 - UH-SAS1.8, UH-SAS1.13) was confirmed in the CSF or serum of the patient used in the selection procedure by phage ELISA (figure 4.1). The level of immunoreactivity in CSF and serum was highest for the phage clones selected with both CSF and serum antibodies which is consistent with the amount of enrichment as identified with PCR and restriction digestion. Immunoreactivity towards 5 other enriched phage clones could not be detected by ELISA in CSF or serum. An explanation could be the lower level of enrichment of these clones due to a low concentration of specific antibodies in combination with the lower sensitivity of the phage ELISA compared with the affinity selections.

![Graph showing immunoreactivity of enriched phage clones on paired CSF and serum](image)

Figure 4.1: Immunoreactivity of enriched phage clones on paired CSF and serum used for affinity selections

Total IgG concentration in CSF and serum used in the ELISA was diluted to 600 pg. Immunoreactivity was confirmed for 7 CSF-derived phage clones (UH-SAS1.2 - UH-SAS1.8) and 1 serum-derived phage clone (UH-SAS1.13). No significant difference in the level of immunoreactivity in CSF and serum could be detected. Immunoreactivity against each phage clone (OD sample) was measured in relation to empty phage (OD background) that was used as an internal control. A signal was considered to be positive when the ratio \( \text{OD}_{\text{sample}} / \text{OD}_{\text{background}} > 1.5 \) while a ratio \( >1.2 \) was considered to be borderline positive. Upper and lower dashed lines represent the cut off of 1.5 and 1.2, respectively.
4.3.4 Role for the MAPK-pathway in the pathogenesis of MS?

Identifying antibody reactivity may provide further insights into the disease mechanism of MS. An interesting finding in this study was the identification of a member of the MAPK-family, namely MAPK7 or ERK5 which was encoded by UH-SAS1.8 (table 4.2). UH-SAS1.8 was highly enriched in both CSF and serum of the patient used in the affinity selection and displayed a consistent high immunoreactivity in paired CSF and serum. In addition, a previous study, where the SAS-procedure was applied to pooled CSF of 10 RR MS patients, resulted in the identification of another member of the same family (see chapter 3 and table 3.7). UH-SAS.CP2 was selected multiple times by antibodies present in pooled MS CSF and encoded the last 26 AA of MAPK3, also called ERK1.

Because 2 different protein members of the same family were identified with antibodies present in the CSF and serum of different MS patients, we decided to analyze the expression patterns of the activated (phosphorylated) form of these proteins in an MS brain lesion using immunohistochemistry. Intense p-ERK1/2 staining was seen in cells throughout the brain parenchyma and around blood vessels (figure 4.2, A-B). Dependent on the brain region, approximately 50% of the cells expressed activated ERK1/2. p-ERK5 reactivity was observed in cells in close proximity of blood vessels (figure 4.2, C-D) and in cells with a glial-like cell morphology (figure 4.2, E). Because several of the p-ERK5 and p-ERK1/2 positive cells in proximity of blood vessels resembled infiltrating macrophages and these cells are important contributors to MS lesion pathology\(^9\), double staining with anti-CD68 antibody was performed. CD68 staining revealed a high number of infiltrating macrophages throughout the tissue of which some were p-ERK1/2 (figure 4.2, F-G) and p-ERK5 positive (figure 4.2, H-I), reflecting the inflammatory process in the brain.
Figure 4.2: Expression of activated ERK1/2 and ERK5 in an MS brain plaque
p-ERK1/2 and p-ERK5 expression was assessed in MS brain slices. p-ERK-reactivity was visualized with DAB (brown staining) whereas double staining was performed with anti-CD68 antibody (red staining). Nuclear counterstaining was performed with haematoxylin (blue). p-ERK1/2 staining was observed in cells throughout the brain parenchyma (A, arrow, magnified in B) whereas p-ERK5 activity was mainly visible in cells in close proximity of blood vessels (C, arrow, magnified in D) and in cells with a glial-like appearance (E). Some macrophages expressed p-ERK1/2 (F, arrow, magnified in G) and p-ERK5 (H, arrow, magnified in I). No immunostaining was observed when primary antibody was omitted (data not shown).

4.3.4 Detailed serological analysis of enriched phage clones
Next, we examined whether immunoreactivity detected towards our panel of enriched phage clones could also be found in other MS patients. Therefore 8 immunoreactive phage clones were selected for a detailed serological analysis in serum of 22 HC and paired CSF and serum of 22 MS patients and 19 control patients. The results of the serological analysis are presented in table 4.3. The highest frequency of MS-specific antibody responses was detected in CSF
Analysis of antibody reactivity against UH-SAS1.2, UH-SAS1.3 and UH-SAS1.6: 22% (5/23) of the MS patients displayed immunoreactivity against one of these 3 clones in comparison to 5% (1/19) of the control group. When all phage clones are considered, no difference was found in antibody positivity between the MS group (30%, 7/23 patients) and the control group (26%, 5/19 patients). In serum no difference in immunoreactivity could be detected between the MS group and the control group. No statistical differences were observed in clinical parameters between antibody-positive and antibody-negative patients.

### Table 4.4: Detailed serological analysis of enriched phage clones

<table>
<thead>
<tr>
<th>Phage clone</th>
<th>MS $^{1}$</th>
<th>HC</th>
<th>OND $^{3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSF$^{2}$</td>
<td>Serum$^{3}$</td>
<td>CSF$^{2}$</td>
</tr>
<tr>
<td>UH-SAS1.2</td>
<td>2/23</td>
<td>1/23</td>
<td>0/19</td>
</tr>
<tr>
<td>UH-SAS1.3</td>
<td>4/23</td>
<td>1/23</td>
<td>0/22</td>
</tr>
<tr>
<td>UH-SAS1.4</td>
<td>3/23</td>
<td>3/23</td>
<td>1/19</td>
</tr>
<tr>
<td>UH-SAS1.5</td>
<td>2/23</td>
<td>1/23</td>
<td>2/22</td>
</tr>
<tr>
<td>UH-SAS1.6</td>
<td>3/23</td>
<td>1/23</td>
<td>0/19</td>
</tr>
<tr>
<td>UH-SAS1.7</td>
<td>1/23</td>
<td>2/23</td>
<td>0/22</td>
</tr>
<tr>
<td>UH-SAS1.8</td>
<td>1/23</td>
<td>1/23</td>
<td>0/19</td>
</tr>
<tr>
<td>UH-SAS1.13</td>
<td>2/23</td>
<td>8/23</td>
<td>6/22</td>
</tr>
</tbody>
</table>

$^{1}$ MS patients including UH-SAS1

$^{2}$ Cut off: average ratio OD sample/OD background CSF OND + 3xSTDEV

$^{3}$ Cut off: average ratio OD sample/OD background serum OND + 3xSTDEV
4.4 Discussion

In this study, serological antigen selection was applied to affinity select an MS cDNA phage display library with antibodies present in paired CSF and serum of a single RR MS patient. This resulted in the enrichment and identification of 13 candidate MS-related antigenic targets. Seven phage clones were selected by CSF or serum antibodies alone whereas the remaining 6 phage clones were shown to be recognized by both CSF and serum antibodies. The enrichment of phage clones by CSF and serum antibodies reflected the presence of identical OCB in serum and CSF of the MS patient used in the SAS-procedure. Since this patient had no signs of additional infections, the presence of identical OCB in CSF and serum suggests a peripheral involvement of the humoral immune system in the disease process of this patient. This finding is in line with a previous study identifying higher numbers of IgG-, IgA- and IgM-secreting B cells both in bone marrow and peripheral blood of MS patients without any signs of concurrent infections compared with healthy controls, indicating a systemic involvement of the humoral immune response in MS. This systemic B cell response was also present in patients with other inflammatory neurological diseases.236 On the other hand, the identification of CSF-specific phage clones in this study points towards the existence of an additional intrathecal B cell repertoire in this patient which is supported by the presence of unique OCB in the CSF35.

An interesting finding in this study was the identification of a phage clone (UH-SAS1.8) that encoded the C-terminal domain of MAPK7 or ERK5. Another member of the same family, namely MAPK3 or ERK1, was already identified in a previous study using pooled MS CSF (chapter 3). Diverse extracellular stimuli, including oxidative stress, antigen-receptor engagement, growth factors and cytokines induce activation of the MAPK pathway237-239 suggesting an involvement of ERK5 and ERK1 in the inflammatory process in the CNS of MS patients. In this study activated ERK1/2 staining was observed in approximately
half the cells present in the MS lesion area. ERK5 staining was observed in cells associated with blood vessels and in cells with a glial-like morphology. Ongoing inflammation in the brain plaque was further supported by a high number of infiltrating macrophages of which several expressed p-ERK1 and p-ERK5. This is consistent with previous studies demonstrating the involvement of ERK5 and ERK1/2 activation in TNF-production by macrophages240,241 and activation of glial cells in the CNS242-245. However, in this study antibody reactivity towards ERK5 was found in paired CSF and serum of a RR MS patient. Antibody reactivity towards intracellular proteins has been documented before in MS246 and other autoimmune diseases including rheumatoid arthritis (RA)247 and systemic lupus erythematosus (SLE)248. Autoantibody production against ERK5 can be induced by breaking immune tolerance which could have been achieved by an upregulation of p-ERK5 or by an ineffective immune clearance as described in SLE248,249. However, whether these antibodies have pathological relevance or are a result of a secondary immune response still needs to be determined. In addition, determining the expression profile of ERK1/2 and ERK5 in control brain derived from healthy controls and neurological controls can provide further insight into the role of these proteins in inflammatory processes involved in MS.

Antibody reactivity towards 8 selected phage clones was confirmed in the CSF and serum of the patient used in the selection procedure. A preliminary screening in a larger patient population indicated that there was no difference in immunoreactivity towards these 8 immunoreactive clones in serum of MS patients and control patients. However, a higher number of immunoreactive MS patients displaying antibody reactivity in CSF could be found (22%) compared with the control group (5%). In the future, a more extensive screening has to be performed to confirm these preliminary findings.

In summary, serological antigen selection has proven to be an excellent technique to identify and compare reactivity of antibodies present in both CSF and serum of this MS patient. Further studies are needed to assess if immunoreactivity towards the identified antigens of this patient can also be found in CSF and serum of a larger patient group. This may lead to the
identification of disease-related biomarkers that can be used in diagnosis, prognosis or subtyping of patients.
The occurrence of frameshifting in the gene VI cDNA phage display system

Based on:
Evidence for frameshifting in the pVI cDNA phage display system
Govarts, C.; Somers, K.; Medaer R.; Stinissen, P.; Somers, V.
Submitted
Chapter 5

Abstract

Phage display has been used to select targets displayed as a fusion protein to one of the phage coat proteins. By means of a repeated process of affinity selections, specific enrichment of displayed targets will occur. However, uncommon translation events like frameshifting can result in the occasional specific enrichment of targets not containing an open reading frame. These events have already been described in studies using N-terminal fusion of targets to minor coat protein 3 and major coat protein 8 of filamentous phage M13. In this study, we provide evidence for the occurrence of frameshifting when using C-terminal fusion of cDNA to minor coat protein 6.
5.1 Introduction

Phage display is a high-throughput molecular technique that has been used successfully to select targets displayed as a fusion protein to one of the phage coat proteins\textsuperscript{191}. Because of its physical link between genotype and phenotype, filamentous phage clones that display a relevant polypeptide will be retained during affinity selections on binding ligands followed by easy identification of the polypeptide\textsuperscript{191}. The most commonly used phage coat proteins are minor coat protein 3 (p3) and major coat protein 8 (p8)\textsuperscript{192,232}. When using these coat proteins, N-terminal fusion of the ligand is mandatory for successful phage propagation. Recently, we reported the use of minor coat protein 6 (p6)\textsuperscript{212,224,250}. By using the p6-display system, C-terminal fusion of ligands is possible which enables cDNA libraries to be displayed on the surface of the phage. This is not possible in an N-terminal display system due to inherent stop codons present in the cDNA. By constructing a cDNA display library derived from multiple sclerosis (MS) brain plaques and performing subsequent affinity selections with MS cerebrospinal fluid (CSF) and sera, our group was able to successfully identify MS-specific antigenic targets that have not been associated with MS before\textsuperscript{224,250}.

During affinity selections, antibodies present in CSF and sera of patients interact with their antigenic targets displayed on the phage surface. After several rounds of affinity selection, this interaction results in a specific enrichment of relevant phage clones which is reflected by the multiple selection of phage clones containing the same antigenic target. In some of these enriched phage clones a stop codon was observed immediately after the gene VI (gVI) sequence which prevented the display the peptide. However, for correct display of the corresponding peptide on the surface of the phage a shift to a different reading frame is required. Frameshifting was documented previously in the field of phage display and bacterial expression systems\textsuperscript{251-253}. Because in these studies N-terminal fusion to p3 or p8 was used, frameshifting had to occur in order to
express the coat proteins and enable phage propagation. However, C-terminal fusion to p6 does not require expression of the inserted DNA sequence for successful phage propagation\textsuperscript{194}. In this study, we investigated whether frameshifting could be demonstrated in the p6-display system. We focused on a phage clone that was selected multiple times during affinity selections on MS sera. Although the cDNA insert of the phage clone was identical to the Apolipoprotein E (ApoE) gene, no protein was expected to be displayed due to a \textsuperscript{-1} shift of the original reading frame which resulted in an early stop codon. However, when expressed in the correct reading frame, antibody reactivity against the ApoE clone was observed in the sera of MS patients used in the selection procedure indicating that enrichment resulted from specific interactions between serum antibodies and the displayed ApoE protein. An ELISA-format in which expression of the ApoE protein, coupled to E-tag signal detection, was developed to demonstrate the occurrence of frameshifting.
5.2 Materials and methods

5.2.1 Serological antigen selection of an MS cDNA phage display library on patient serum
Serological antigen selection (SAS) was applied on an MS cDNA phage display library as described in 2.1.3. Affinity selections on pooled serum from 10 MS patients resulted in specific enrichment of MS-related phage clones. A cDNA phage clone UH-FS (short for University Hasselt-frameshifting) was selected multiple times during affinity selection and was chosen for analysis of frameshifting. The cDNA of UH-FS was identical to the nucleotide sequence of the ApoE sequence (from nucleotide position -50 to 389). However, after translation of the nucleotide sequence an early stop codon occurs due to out of frame insertion of the cDNA which jeopardizes the display of the protein (figure 5.1A). A +1 shift in the reading frame of UH-FS would result in the correct expression of the ApoE protein (figure 5.2A-B).

5.2.2 Generation of E-tag constructs
An ApoE - E-tag construct was generated by C-terminal fusion of an E-tag (GAPVPYPDPLEPR) and NotI restriction site (GC/GGCC) to the ApoE sequence of UH-FS using the ApoE forward and the E-tag reverse primer (Figure 5.2A and table 5.1). A negative control (NC) for E-tag display was generated by an out of frame fusion of the E-tag to the 3'-end of gVI, resulting in an early stop codon that prevents the display of the E-tag. This was done using the overlapping ApoE forward and the NC reverse primer (table 5.1). A positive control (PC) for E-tag display was generated by using the overlapping ApoE forward en PC reverse primer. Addition of an extra nucleotide (shaded in grey, table 5.1) to the PC reverse primer resulted in an in frame fusion of the E-tag at the 3'-end of gVI.
Chapter 5

(A) UH-FS: ApoE in pSPVIB vector

(B) Addition of E-tag and NolI restriction site 3’ of the ApoE sequence

(C) Subcloning of different constructs in pCR2.1 TOPO vector

(D) BglII and NolI digestion of TOPO vector and pSPVIA, pSPVIB, and pSPVIC vector

(E) Detection of immunoreactivity in sera of MS patients

(F) Detection of E-tag expression
Figure 5.1: Outline of the study

(A) The ApoE sequence in the pSPVIB vector as identified during the SAS-procedure on pooled MS sera. The top line represents the cDNA sequence and translated amino acids are depicted in the bottom line. The reading frame is determined by the gene VI (gVI) sequence. The linker refers to a glycine and serine-rich region between gVI and the ApoE sequence. The first nucleotide of the ApoE insert is encircled. The start codon of ApoE is boxed and a stop codon is presented by *.

(B) An E-tag and NotI restriction site were added at the 3'-end of the ApoE sequence resulting in the ApoE - E-tag construct. Positive and negative controls were generated by fusing the E-tag sequence in and out of frame, respectively with the 3'-end of gVI. (C) After subcloning the ApoE - E-tag construct in the pCR2.1 Topo vector and transformation in Top10 cells, the constructs were excised using BgIII and NotI and (D) ligated in the pSPVIA, pSPVIB and pSPVIC vector resulting in phage clones UH-FSE\(^+\)\(_{pSPA}\), UH-FSE\(^+\)\(_{pSPB}\) and UH-FSE\(^+\)\(_{pSPC}\), respectively. Positive and negative control constructs were ligated in the pSPVIB vector resulting in UH-PC and UH-NC, respectively. (E) After purification of phage clones, immunoreactivity towards the correctly expressed ApoE protein (UH-FSE\(^+\)\(_{pSPA}\)) was analyzed in serum of MS patients originally used in the SAS procedure. (F) ApoE expression and concomitant E-tag expression was detected with an anti-E-tag antibody.


All primers were obtained from Eurogentec (Seraing, Belgium). PCR products were purified using the GFX gel band purification kit (GE Healthcare, Diegem, Belgium), ligated into the pCR2.1 TOPO vector and transformed into E. coli Top10 cells according to manufacturer's instructions (TopoTA cloning kit, Invitrogen, Merelbeke, Belgium). Insert sequences were determined using M13 forward and reverse primer (table 5.1). After BgIII and NotI digestion, the ApoE - E-tag constructs were ligated into cDNA phage display vectors pSPVIA, pSPVIB and pSPVIC digested with the same restriction enzymes. The positive and negative control constructs were ligated into pSPVIB. Ligation mixtures were used to transform E. coli TG1 cells by electroporation to obtain UH-FSE\(^+\)\(_{pSPA}\) (University Hasselt-frameshifting clone with ApoE - E-tag construct in pSPVIA), UH-FSE\(^+\)\(_{pSPB}\), UH-FSE\(^+\)\(_{pSPC}\), UH-PC (University Hasselt-positive control) and UH-NC (University Hasselt-negative control). Resulting colonies were picked and sequence was confirmed with gVI forward primer (table 5.1). Phage were purified as described in 2.1.2. Purified phage clones were used in ELISA to
analyze immunoreactivity towards the displayed ApoE protein on the one hand and the level of E-tag expression on the other hand.

Table 5.1: Primers used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GGATCCGGTGGAGGCTCAGGCGGAGGGCCAAGTGGGCCAGATCTCTTAGAGAATT</td>
<td>ApoE forward primer</td>
</tr>
<tr>
<td>2</td>
<td>GCGGCGCAGCGGGTTTCAGCGGATCCCGGATACGGCACCCGCACCACGTCCCAT</td>
<td>E-tag reverse primer</td>
</tr>
<tr>
<td>3</td>
<td>GCGGCGCAGCGGGTTTCAGCGGATCCCGGATACGGCACCCGCACCACGTCCCAT</td>
<td>NC reverse primer</td>
</tr>
<tr>
<td>4</td>
<td>GCGGCGCAGCGGGTTTCAGCGGATCCCGGATACGGCACCCGCACCACGTCCCAT</td>
<td>PC reverse primer</td>
</tr>
<tr>
<td>5</td>
<td>CAGGAAAAACAGCTATGAC</td>
<td>M13 forward primer</td>
</tr>
<tr>
<td>6</td>
<td>GTAAAAACGACGGCCAG</td>
<td>M13 reverse primer</td>
</tr>
<tr>
<td>7</td>
<td>TTACCCTCTGACTTTTGTTCA</td>
<td>gVI forward primer</td>
</tr>
</tbody>
</table>

All primers are listed from 5’ to 3’. Overlapping nucleotides are indicated in bold. The underlined bases represent the E-tag sequence while grey text represents NotI restriction site. The added nucleotide in the PC reverse primer is encircled.
Figure 5.2: ApoE – E-tag construct ligated into the pSPVI vector in 3 different reading frames

After cloning the E-tag at the 3'-end of the ApoE sequence, the construct is ligated into the pSPVIA, pSPVIB and pSPVIC vector. (A) The ApoE sequence was originally identified in the pSPVIB vector. By cloning the E-tag sequence in frame with the ApoE sequence, E-tag expression will only occur after +1 frameshifting. ApoE FW: ApoE forward primer; E-tag REV: E-tag reverse primer. (B) The pSPVIA vector represents the correct reading frame for expression of the ApoE protein and E-tag. Due to amber suppression of TG1 E. coli, the TAG-stop codon in pSPVIA will be translated into glutamine (Q). (C) In the pSPVIC vector no E-tag expression will occur due to an early stop codon. The top line represents the cDNA sequence and translated amino acids are depicted in the bottom line. The first nucleotide of the ApoE insert is encircled. The start codon of the ApoE sequence in each vector is boxed while * represents a stop codon. Linker represents a glycine and serine-rich linker sequence between gene VI and the cDNA insert.
5.2.3 Analysis of E-tag display and serum immunoreactivity via ELISA

E-tag-display on purified phage clones UH-FSE+PSA, UH-FSE+PSB, UH-FSE+PSC, UH-PC and UH-NC was measured by ELISA. Briefly, 96-well flat-bottomed microtiter plates (Falcon/BD, Erembodegem, Belgium) were coated overnight at 4°C with 200 µl of anti-E-tag antibody (GE Healthcare), 10 µg/ml in coating buffer (0.1 M NaHCO3 pH 9.6) and blocked with 200 µl of 2 % MPBS (2 % (w/v) skimmed milk powder in PBS, 750 mM NaCl, 40 mM Na2HPO4, 7.8 mM KH₂PO₄, pH 7.4) for 1h at room temperature (RT). PEG (20% polyethylene glycol (PEG) - 2.5 M NaCl) - purified phage (10¹⁰ phage/well) were preblocked with 2 % MPBS in a 96-well round-bottomed plate (Nunc, Roskilde, Denmark) for 1h at 37°C, followed by 30 min shaking at RT. After washing with 0.1 % (v/v) PBS/Tween 20 (PBST) and PBS, the preblocked phage were transferred to the anti-E-tag antibody-coated plate and incubated for 1h at 37°C and 30 min shaking at RT. After washing the plate with 0.1 % PBST and PBS, 150 µl of a peroxidase-conjugated anti-M13 monoclonal antibody (Amersham/Pharmacia/Biotech, Diegem, Belgium), diluted 1:5,000 in 2 % MPBS was incubated for 1h shaking at RT. After washing the plate with 0.1 % PBST and PBS, 130 µl of a 3,3',5,5' tetramethyl-benzidine dihydrochloride chromogen solution (10 mg/ml) was added. Colour development was stopped with 65 µl/well 2 M H₂SO₄. The plates were read at 450 nm in a Bio-Rad Benchmark microplate reader (Bio-Rad, Nazareth Eke, Belgium).

Detection of antibody reactivity of individual serum samples of MS patients against the different phage clones displaying the ApoE protein was measured by phage ELISA as described in 2.1.7. Serum samples tested showed equivalent IgG concentrations.
5.3 Results

5.3.1 Generation of E-tag constructs

The cDNA sequence of UH-FS encoded part of the signal sequence in addition to the first 130 AA of the ApoE protein (figure 5.1A and figure 5.2A). However, due to out of frame insertion of the ApoE gene into the pSPVIB vector a stop codon was observed immediately after p6 preventing the display of the protein. A +1 shift in the reading frame of pSPVIB, which could be achieved by frameshifting, would result in the correct expression of the ApoE protein. To detect frameshifting, an E-tag sequence was cloned in frame with the 3’-end of the ApoE sequence resulting in simultaneous expression of the ApoE protein and the E-tag as depicted in the schematic overview in figure 5.1. A positive control was constructed by cloning the E-tag sequence in frame with the 3’-end of gVI while a negative control was created by a -1 shift in the reading frame of the E-tag sequence which prevented the display of the E-tag. After subcloning in the pCR2.1 TOPO vector (figure 5.1C) the ApoE – E-tag fragment was ligated into 3 cDNA phage display vectors (pSPVIA, pSPVIB, pSPVIC) each encoding a different reading frame to obtain phage clones UH-FSE\textsuperscript{+}_{spa}, UH-FSE\textsuperscript{+}_{spb} and UH-FSE\textsuperscript{+}_{spc}, respectively (figure 5.1D and figure 5.2). UH-FSE\textsuperscript{+}_{spa} expresses the ApoE protein and E-tag from the pSPVIA vector which corresponds to the correct reading frame of ApoE due to a +1 nucleotide shift in comparison with the pSPVIB vector. When the ApoE – E-tag construct is expressed from the pSPVIC vector (UH-FSE\textsuperscript{+}_{spc}) no ApoE protein or E-tag is expected to be displayed since the pSPVIC vector represents a -1 shift in the reading frame compared to pSPVIB. UH-FSE\textsuperscript{+}_{spb} represents the original reading frame in which the ApoE protein was initially identified. This phage clone was used to study frameshifting. UH-PC and UH-NC were created by ligation of the positive and negative control constructs in the pSPVIB vector resulting in respectively, a high and no E-tag expression.
5.3.2 Immunoreactivity towards the ApoE protein

Because UH-FS was originally selected during affinity selections on MS sera and ApoE has already been implicated in the pathogenesis of MS, it would be relevant to examine the level of immunoreactivity towards the selected ApoE protein in the individual serum samples of the 10 MS patients used in the SAS procedure. However, due to the early stop codon, immunoreactivity towards UH-FS could not be detected. Therefore, immunoreactivity towards the correctly expressed ApoE protein (UH-FSE$^{+}_{pSPA}$), together with UH-FSE$^{+}_{pSPB}$ was examined in the individual MS sera. UH-PC and empty phage, not displaying any protein, were used as negative controls. Immunoreactivity towards UH-PC and empty phage was comparable in all serum samples (data not shown). As shown in figure 5.3A, immunoreactivity against UH-FSE$^{+}_{pSPA}$ was detected in the serum of 2 MS patients (MS3 and MS9). Antibody reactivity against the E-tag alone (UH-PC) was not detected in the serum of these patients which indicated that the observed immunoreactivity was not directed towards the displayed E-tag (data not shown). Serum of 2 other patients (MS1 and MS5) showed a nonsignificantly increased immunoreactivity towards UH-FSE$^{+}_{pSPA}$. By demonstrating immunoreactivity towards the correctly expressed ApoE protein in MS serum used in the SAS procedure, specific enrichment of UH-FS could be confirmed thereby again pointing towards the occurrence of frameshifting.

5.3.2 Simultaneous expression of ApoE and E-tag

By cloning an E-tag in frame with the ApoE sequence, simultaneous expression of the ApoE protein and E-tag could be measured by ELISA. Besides UH-PC and UH-NC, empty phage was included as an additional negative control. As shown in figure 5.3B both UH-PC and UH-FSE$^{+}_{pSPA}$ displayed a high E-tag signal while no signal could be detected for UH-FSE$^{+}_{pSPB}$. Of note, the E-tag signal of UH-FSE$^{+}_{pSPA}$ was shown to be almost 10-fold lower than UH-PC. Even when phage concentration of UH-PC was reduced to $10^7$ colony forming units (cfu) (10,000-fold dilution) the signal was shown to be almost 3x higher than background values (figure 5.3C). In contrast, the E-tag signal of UH-FSE$^{+}_{pSPA}$ was already undetectable when $10^9$ cfu were used (1000-fold dilution) indicating a low level of E-tag expression.
Figure 5.3: ELISA of ApoE – E-tag-displaying phage
(A) Antibody reactivity towards the ApoE protein displayed on the phage surface was measured in the sera of 10 MS patients used in the SAS-procedure. Antibody reactivity towards UH-FSE<sup>pSPA</sup> and UH-FSE<sup>pSPV</sup> is shown. Empty phage, containing an empty pSPVI vector, and UH-PC were used as negative controls (data not shown). Dashed line represents the threshold value which is determined by ratio OD<sub>450</sub> UH- FSE<sup>pSPA</sup>/OD<sub>450</sub> empty phage + 3xSTDEV (B) E-tag expression of the different phage clones (1x10<sup>10</sup> colony forming units (cfu)) was measured with an anti-E-tag antibody. (C) Comparison of E-tag expression between UH-PC and UH-FSE<sup>pSPA</sup>. Empty phage was used as a negative control. Different concentrations of phage particles were used including 1x10<sup>10</sup> cfu, 2x10<sup>9</sup> cfu, 1x10<sup>8</sup> cfu, 1x10<sup>7</sup> cfu and 1x10<sup>6</sup> cfu. Values are represented as a ratio of OD<sub>450nm</sub> specific phage/OD<sub>450nm</sub> empty phage.
5.4 Discussion

The goal of this study was to investigate whether frameshifting could be demonstrated in the p6-display system. Therefore, we focused on a phage clone, UH-FS that was selected multiple times during affinity selections on MS sera. Due to a shift in the reading frame an early stop codon prevented the display of the ApoE protein. However, a +1 shift in the reading frame would result in the correct expression of the ApoE protein allowing the selection of UH-FS by antibodies present in MS sera. This is supported by detection of immunoreactivity in the serum of MS patients towards the correctly expressed ApoE protein. These results are a confirmation that selection of UH-FS was the result of specific interactions between antibodies present in MS sera and the ApoE protein displayed on the surface of the phage and they provide further support for the frameshifting hypothesis.

However, when the E-tag signal of the different constructs was measured, a strong signal could only be measured when ApoE was expressed in the correct reading frame (UH-FSE\textsuperscript{pSPA}) and in the positive control. No signal could be detected when ApoE was expressed in the pSPVIB vector in which it was originally identified. The significant decrease in E-tag expression of UH-FSE\textsuperscript{pSPA} compared to the positive control can have several reasons. Expression of a human protein in a bacterial expression system can pose problems concerning the stability or the folding of the displayed polypeptide which would result in an increased degradation and thus a lower expression. Also, the lipophilic nature of the ApoE protein can cause a problematic display of the protein. Finally, it could be possible that the correctly displayed ApoE was not present in the MS cDNA display library or was lost during the first selection round, resulting in the selection of the out of frame variant. The repeated selection and amplification of phage clones during the selection procedure results in a high sensitivity that cannot be reached with the ELISA. This can explain, in combination with a low
Evidence for frameshifting frequency, why detection of frameshifting using a standard ELISA may be difficult.

The question remains why this unusual clone was retrieved by our selection procedure. To accomplish correct expression of the ApoE sequence of UH-FS, a +1 translational shift is required. This type of frameshifting is also seen between the GAG3 and POL3 genes of the retrotransposon Ty3 of yeast\textsuperscript{256,257}. A +1 frameshift occurs within the sequence GCG AGU U. The first codon (GCG) is located at the P-site of the ribosome while the A-site AGU codon is vacant. In the case of Ty3, slippage would be promoted by the relative sparse presence of tRNA decoding the 0 frame A-site codon (AGU) in combination with an abundance of the tRNA decoding its overlapping +1 frame codon (GUU). Due to the paucity of the 0 frame tRNA, a translation pause is induced which is essential for slippage\textsuperscript{256}. A comparable situation could occur in UH-FS within the sequence GCA CGA GGG. The arginine codon CGA, which is located 3 nucleotides before the early stop codon TGA in the pSPVIB vector, is a sparsely used codon in \textit{E. coli}. When the first codon (GCA) is in the P-site the relative paucity of the tRNA decoding the arginine codon CGA could induce a translational pause. This can in combination with the more abundantly present +1 tRNA, that encodes the GAG anti-codon, promote frameshifting at that position. Indeed, studies have recently shown that +1 frameshifting in \textit{E. coli} occurred after sparse codons like CGA\textsuperscript{258}. In addition, secondary signals consisting of primary mRNA sequences and secondary stem-loop structures can augment the occurrence of frameshifting\textsuperscript{259}.

Previous studies using N-terminal display of proteins reported frameshifting frequencies ranging from 0 to 90\%, dependent on the ligand used\textsuperscript{253}. Although we have no conclusive data on the frequency of frameshifting in the pVI display system, the phenomenon may have implications for affinity selection procedures applied on complex cDNA libraries since frameshift mutations can revert display phenotypes on phage in the presence of a suitable selection pressure. As a result, a single phage clone could encode different antigenic targets. Although this can benefit the complexity of the library, deducing the correct peptide sequence that has been selected by the selector molecule can be difficult,
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implying the need to verify the selection specificity in a phage-free format (i.e. purified peptides or recombinant proteins).

In conclusion we can state that the concept of frameshifting provides an explanation for the enrichment of UH-FS during affinity selections on MS sera. The enrichment seems to be the result of specific interactions of serum antibodies and the displayed ApoE protein, although an early stop codon would prevent this display. This is supported by the confirmed antibody reactivity towards the correctly expressed form of ApoE in the serum of MS patients used in the SAS procedure. Although frameshifting could not be detected using our E-tag ELISA format, the confirmation of immunoreactivity combined with the required sequence conditions to induce a shift in the reading frame, strongly points to the occurrence of frameshifting in the p6-display system.
Summary and general discussion
Chapter 6

Antibody reactivity can be studied to obtain more information about MS-related antigenic targets and ongoing disease mechanisms in MS. On the other hand, antibodies can also function as potential disease biomarkers which can be used for either prognostic purposes, monitoring disease progression or classification of immunopathogenic subtypes of MS.

Phage display has proven its potential as an invaluable tool to study antibody reactivity in body fluids of patients. This thesis describes the application of cDNA phage display to study antibody repertoires in CSF and serum of MS patients. cDNA phage display can be achieved by a C-terminal fusion of the cDNA-encoded protein to minor coat protein 6 of filamentous M13 phage. In this study, a cDNA phage display library derived from MS CNS lesions with varying degrees of inflammation and demyelination of an MS patient was used for affinity selections on MS CSF and serum to identify antigens that evoke an antibody response. In addition, the p6-display system itself was analyzed in more detail to study the occurrence of frameshifting during the selection procedure. In this chapter, the main results of this thesis will be summarized and discussed.

1. Searching for new biomarkers in multiple sclerosis

In chapter 3 we describe the optimization of the SAS-procedure by introducing an alternative selection procedure in which selection rounds on MS sera or CSF were alternated with selection rounds on HC sera resulting in a diminishment of aspecific phage clones. This approach resulted in the identification of 5 enriched candidate MS-related phage clones derived from selections on MS sera whereas selection rounds on MS CSF resulted in the identification of 12 enriched candidate MS-related phage clones. Although the identified peptides/proteins may represent mimotopes, significant homology with MAPK proteins, a heat shock protein, hypothetical proteins and viral proteins was found. Of note, homology with the EBV-derived protein EBNA-1 was found strengthening a possible role of EBV in the pathogenesis of MS\textsuperscript{260}.

Although no difference in immunoreactivity was found between MS patients and controls towards 3 CSF-derived phage clones, 83 % (5/6) of antibody-positive
MS patients were in a progressive disease stage. This could point towards an association of this panel with a progressive disease course. On the other hand, since the number of antibody reactive MS patients is small, no unambiguous conclusion can be drawn before more patients are implemented in the screening procedure. Immunoreactivity towards the phage clones derived from selection on MS sera could be found in a subgroup of MS patients. However, no correlation was found with clinical parameters.

Our results demonstrate that the alternating selection procedure was successful in diminishing the occurrence of aspecific phage clones. This approach resulted in the identification of a panel of candidate MS-related phage clones to which antibody reactivity could be identified, especially in MS sera. However, more patients representing different disease subtypes of MS need to be tested before any conclusions can be made regarding the potential of the identified phage clones as biomarkers in MS.

### 2. Analysis of antibody reactivity in cerebrospinal fluid and serum of a relapsing remitting multiple sclerosis patient

In chapter 4, we explored the possibility to apply SAS as a tool to analyze in parallel the antigen reactivity of antibodies in CSF and serum of a patient with MS. Therefore, the SAS-procedure was applied to paired CSF and serum of a single RR MS patient.

Affinity selections on paired CSF and serum of a RR MS patient resulted in the enrichment of 13 different candidate MS-related antigenic targets. We demonstrated the existence of both unique and common antibody reactivities in paired CSF and serum which corresponded well with the presence of OCB in serum and CSF of the MS patient used in the SAS-procedure. This may indicate the presence of a systemic activation of the humoral immune response in addition to an intrathecal antibody production.

Homology search analysis of the identified phage clones revealed the identification of MAPK7, also known as ERK5. Another member of the same
family, namely MAPK3 or ERK1, was identified using pooled MS CSF (chapter 3). Immunohistochemical analysis of an MS brain plaque confirmed the presence of phosphorylated ERK1 and ERK5-positive cells throughout the MS lesion. Double staining revealed many ERK1 or ERK5-positive macrophages which reflect ongoing inflammation in the brain. The MAPK pathway is regulated by a wide variety of cell-stressing and proliferative agents and has a role in several physiological responses including cell proliferation, differentiation and survival. Many of these activation signals are also implicated in the pathogenesis of MS. Together with our data this could indicate that ERK5 and ERK1 expression in infiltrating immune cells and residual glial cells may play a role in the inflammatory processes in the MS brain. However, more studies are needed to determine if the MAPK-directed antibodies have pathological relevance or if they are a result of a secondary immune response.

Immunoreactivity towards 8 out of 13 enriched clones was confirmed in the CSF or serum of the MS patient used in the selection procedure. To determine if antibody reactivity towards our panel of 8 immunoreactive clones could also be found in other patients, an initial screening was performed in which immunoreactivity was analyzed in CSF and serum of a larger group of MS patients and control patients. The highest frequency of antibody responses was detected towards 3 clones from our panel in CSF of 22% of MS patients compared to 5% of control patients. In serum, no difference in immunoreactivity could be detected between MS patients and control patients.

In summary, SAS is a suitable technique for the identification and comparison of antibody reactivities present in CSF and serum of MS patients. Further studies have to assess if immunoreactivity towards the identified antigens of this patient can also be found in CSF and serum of a larger group of patients.
Summary and discussion

3. Frameshifting in the gene VI cDNA phage display system

Uncommon translation events like frameshifting can, during affinity selections, result in the occasional enrichment of phage clones not containing an in frame open reading frame. In chapter 5, the focus was to investigate whether frameshifting could be demonstrated in the p6-display system.

In chapter 5 we focused on an enriched phage clone, UH-FS, encoding the ApoE protein. Due to an out of frame insertion which resulted in an early stop codon, no ApoE protein was expected to be displayed at the phage surface. By cloning an E-tag at the C-terminal end of the cDNA insert expression of an ApoE protein fused to an E-tag was achieved. After expression of the ApoE – E-tag construct in 3 different reading frames, it was possible to examine antibody reactivity to the in frame expressed ApoE protein on the one hand and E-tag expression from the different constructs on the other hand.

Immunoreactivity in the serum of MS patients used in the selection procedure, directed towards an in frame expressed ApoE protein, suggested that enrichment of UH-FS was the result of specific interactions between antibodies present in MS sera and the ApoE protein displayed on the phage surface. However, E-tag expression could only be measured in the positive control and when the ApoE protein was expressed in the correct reading frame. Several causes, such as improper folding or the lipophilic properties of the ApoE protein can explain this reduction in signal. Furthermore, the in frame ApoE clone could be absent in the original MS cDNA display library or could be lost in the first selection round. The lower sensitivity of the ELISA procedure can explain why the out of frame ApoE clone was selected during affinity selections but could not be detected in the ELISA.

In summary, the concept of frameshifting provides a suitable explanation for the enrichment of UH-FS during affinity selections on MS sera. Confirmed antibody reactivity in MS sera used in the SAS-procedure suggests that enrichment of UH-FS is the result of specific interactions of serum antibodies and the displayed
Chapter 6

ApoE protein. Although frameshifted phage clones could not be detected using our E-tag ELISA format, the confirmation of immunoreactivity combined with the required sequence conditions to induce a shift in the reading frame, strongly points to the occurrence of frameshifting in the p6-display system.

4. General discussion

Given the heterogeneity of MS, it will be highly unlikely that a single biomarker will be able to cover the complex disease process of MS. Nevertheless, as indicated in table 1.1 (chapter 1) most of the biomarker studies have been limited to one or a few biomarkers at a time. Analysis of the general (auto)antibody repertoire can provide more information concerning the different disease processes in MS. Indeed, when serum antibody reactivity towards brain tissue was analyzed via Western blotting, specific antibody patterns towards 16 brain antigens were identified that were able to discriminate MS patients and healthy controls\(^{172,262}\). In addition, specific IgG patterns allowed to distinguish between RR, SP and PP MS patients with an excellent degree of concordance with clinical data. Similar results were obtained using antigen microarray analysis\(^{173}\). Specific serum antibody profiles towards a panel of CNS proteins, heat shock proteins and lipid autoantigens were identified that distinguished the different subtypes of MS from both healthy controls and other neurologic or autoimmune diseases such as Alzheimer’s disease and systemic lupus erythematosus (SLE). These results clearly indicate the distortion of antibody profiles in serum as a result of the MS pathogenesis.

In this study, we aimed to investigate whether cDNA phage display could be used for the identification of antibody reactivity in CSF and serum of MS patients and to subsequently use the identified antigenic targets as biomarkers in MS.

Affinity selections on MS CSF have previously been applied by our group which has resulted in the identification of 8 MS-related antigenic targets with 86% sensitivity and 45% specificity in discriminating MS patients and controls\(^{224}\). Since these antigenic targets were derived from CSF selections, we wanted to explore the antibody profile in serum as well. Therefore a new pool of 10 RR MS
patients was composed of which both CSF and serum was used for affinity selections of the MS cDNA phage display library. Our group was not the first to use phage display to identify biomarkers for MS. Most of the phage display studies used random peptide libraries for the identification of antibody specificity in CSF or serum of MS patients. Jolivet-Reynaud and colleagues identified 4 CSF-specific motifs which displayed an increased immunoreactivity in MS patients compared to controls. Others only retrieved patient-specific antigenic targets or did not perform an extensive screening in other MS patients and controls. cDNA lambda-phage expression libraries were also used. Screening of a cDNA library derived from an oligodendrocyte-precursor cell line with pooled MS CSF resulted in the identification of an Alu-repeat to which 44% of the screened MS patients displayed CSF or serum antibody reactivity whereas screening of a cDNA library prepared from MS brain plaques with MS CSF did not retrieve MS-related antigenic targets. Our study differs from the previously performed studies in the use of pooled patient samples in addition with the use of a cDNA library expressed on filamentous phage. This approach resulted in a panel of 3 CSF and 3 serum-derived phage clones to which immunoreactivity was further determined in a group of MS patients and controls. Immunoreactivity towards the identified antigenic targets was rather low when evaluated in a larger group of patients. Nevertheless, our results indicate that immunoreactivity towards our CSF panel was mostly found in progressive MS patients whereas immunoreactivity towards our serum panel could be found in a subgroup of MS patients although no correlation could be found with available clinical parameters. Therefore, the ELISA screening has to be extended in a larger patient group comprising all different subtypes of MS to confirm these findings and validate the potential of our antigenic targets as biomarkers for MS.

Homology search analysis of the identified antigenic targets revealed that many of the selected cDNA sequences encoded polypeptides derived from expression of untranslated regions and out of frame sequences. Part of these peptides may originate from alternative splicing of mRNA products. Alternative splicing allows one pre-mRNA to be processed into many different mature forms allowing a tissue-specific or temporal expression of a novel gene product. It has been
suggested that alternative splicing potentially modulates the immunogenicity of autoantigens by the generation of novel tolerance-breaking epitopes within the autoantigen\textsuperscript{229}. Consistent with this theory is the association of alternative splicing with disease phenotypes\textsuperscript{264,265} and autoimmunity\textsuperscript{266-268} although it remains difficult to distinguish whether the increased prevalence of alternative splicing is a cause or consequence of disease.

Some of the identified sequences probably represent mimotopes which are peptides that mimic the functional binding properties of natural occurring proteins. The identification of mimotopes is a common finding in cDNA phage display\textsuperscript{212,213,223,224}. Homology search analysis revealed significant homology with brain-derived proteins, heat shock proteins, intracellular proteins and hypothetical proteins. In line with several other reports we identified epitopes displaying significant homology with viral proteins from human T-lymphotrophic virus (HTLV) 1, hepatitis B and C virus, measles virus and several herpes viruses including EBV\textsuperscript{47,48,217-219,263}. Viruses have long been considered as possible culprits in the activation of autoreactive immune cells and breaking of immune tolerance via molecular mimicry\textsuperscript{28}. Studies have shown that antibodies directed against brain-specific protein and nuclear proteins cross-reacted with herpes simplex virus 1 and HTLV, respectively\textsuperscript{263,269}. In line with previous reports, we identified antibody-directed epitopes showing homology with EBV proteins\textsuperscript{47,48,219}. The role of EBV in the pathogenesis of MS is subject of a longstanding debate. Epidemiological studies have found a strong association between late EBV-infection and development of MS\textsuperscript{30,270}. Furthermore, MS patients display higher EBV titres in serum which can be detected years before onset of disease\textsuperscript{43,260}. EBV reactivation has been associated with disease activity\textsuperscript{46,271} whereas elevated EBV DNA levels have been found in the blood of MS patients during relapses\textsuperscript{272}. Although these results implicate an involvement of EBV in the pathogenesis of MS, it is highly unlikely that EBV would be a causal factor in MS. However, EBV could play an indirect role in breaking immune tolerance through the induction of molecular mimicry or through the permanent infection of B-cells which would induce proliferation and increased antibody production\textsuperscript{231}. 
An interesting finding in this study was the identification of 2 different members of the MAPK-family in separate selection procedures. MAP kinases are a family of highly conserved serine/threonine protein kinases that are involved in various cellular functions, including cell growth, proliferation, differentiation, migration, survival, immunity and development (reviewed in \(^{273,274}\)). MAPKs are part of a three kinase signaling module composed of a MAPK, a MAPK kinase (MAPKK or MKK) and a MAPK kinase kinase (MAPKKK or MKKK). MAPKKKs phosphorylate and activate MAPKKs, which in turn phosphorylate and activate MAPKs as indicated in figure 6.1\(^{275}\). The specificity of the MAPK response is guided by scaffolding proteins. Dependent on the activation signal, scaffolding proteins provide docking sites for binding and organizing multiple members of the MAPK cascade to different cellular compartments. The duration of the stimulus is an additional factor in dictating the biological response of the MAPK pathway (reviewed in \(^{273}\)). Fourteen MAPK isoforms have been identified in mammalian cells which can be grouped into 4 main subfamilies: the classical MAPKs (ERK1 and ERK2, also called ERK1/2), c-Jun amino-terminal kinase (JNK), the p38 stress-activated kinase and the atypical MAPKs including ERK5, ERK3 and ERK8\(^{276,277}\). In this study ERK1 and ERK5 were identified as antigenic targets of antibodies present in MS CSF or serum. ERK1 and ERK2 are highly conserved and ubiquitously expressed proteins for which distinct roles have not yet been fully elucidated. Studies in knockout animals have shown that they are only partially redundant since ERK2 is able to maintain most physiological functions in the absence of ERK1 whereas deletion of ERK2 is embryonically lethal\(^{278}\). The ERK1/2 pathway is mainly involved in cell motility, proliferation and differentiation (reviewed in \(^{279}\)). ERK5 on the other hand is a unique member of the MAPK family. The N-terminus of ERK5 is similar to that of ERK1/2 and contains the kinase domain and the TEY-activation motif whereas the unique C-terminal tail is involved in nucleocytoplasmatic shuttling of ERK5 and in activating transcriptional activity of target molecules\(^{280-282}\). ERK5 is involved in the development of the brain, maintaining of vascular integrity and in survival pathways of developing neurons (reviewed in \(^{283}\)).
Figure 6.1: Schematic representation of the ERK1/2 and ERK5 signaling pathway

M KKs are activated and phosphorylated by a diverse repertoire of extracellular stimuli. The ERK5 kinase module is composed of MEK kinase (MEKK) 2 or MEKK3 that phosphorylates MAPK/ERK kinase (MEK) 5 which is the upstream dual-specificity kinase of ERK5. In the ERK1/2 pathway Raf isoforms are the primary M KK which activate MEK1/2 that in turn phosphorylates ERK1/2. Phosphorylation of the MAPK protein results in translocation to the nucleus and the activation of transcription factors that subsequently trigger a specific biological function such as proliferation, differentiation, cell growth and apoptosis.

Since many of the activation signals of the MAPK pathway, including reactive oxygen species (ROS), cytokines and growth factors, are also implicated in inflammation, the MAPK pathway could be involved in the inflammatory process ongoing in an MS brain lesion. This hypothesis is supported by our study in which the presence of activated ERK1/2 and ERK5 in an MS lesion was
demonstrated, both in residual CNS cells and in infiltrating macrophages. Expression in these cell types is consistent with other studies demonstrating an involvement of ERK1/2 and ERK5 in TNF-production by macrophages \cite{240,241} and activation of glial cells in the CNS \cite{242,243,244,245}. Studies in EAE have already demonstrated an increased expression of activated ERK1/2 in the spinal cord during the peak of the disease compared to control animals\cite{284}. Others have shown that inflammation in spinal cord induces ERK5 expression\cite{285,286} providing further evidence for a role of ERK1/2 and ERK5 in the inflammatory process in MS. However, it has been shown that both ERK1/2 and ERK5 pathways are involved in neurotrophin-mediated protection of neurons indicating that these pathways could also have beneficial effects in the MS brain\cite{287,288,289,290}. In addition, ERK1/2 has also been shown to stimulate proliferation, process extension and survival of oligodendrocytes\cite{292}. Together, these results support a possible involvement of both ERK1/2 and ERK5 in different disease processes of MS including inflammation and neuroprotection.

We must keep in mind that in this study antibody reactivity towards ERK1/2 and ERK5 has been found. Antibody reactivity towards intracellular proteins has been documented before in MS\cite{246} and other autoimmune diseases including rheumatoid arthritis (RA)\cite{247} and SLE\cite{248}. However, how these antibodies arise is currently not known. It is possible that through the mechanism of bystander activation immune cells of any specificity are attracted and activated in the inflamed lesion, leading to a secondary immune response towards these proteins. Another possibility is that inflammation in the brain leads to an enhanced antigen processing and presentation. In addition, many cells will undergo cell damage or apoptosis. Apoptosis-related post-translational modifications of autoantigens can create novel antigens to which no immunological tolerance has been established\cite{293,294}. Together with the enhanced antigen presentation, these modified antigens could evoke an immune response that, via epitope spreading, can lead to a polyclonal autoantibody response against the whole protein. This process can be facilitated by the inflammatory environment already present in the CNS\cite{295}. The mechanism of apoptosis-induced modification of autoantigens has already been reported for other autoimmune diseases including RA and SLE\cite{247,296}.
Chapter 6

In the last part of this thesis the mechanism of frameshifting was further studied. Enrichment of phage clones not displaying a protein could be the result of aspecific binding during the selection procedure. However, the chance of selecting a phage clone containing the same cDNA insert multiple times while no protein is displayed on its surface is very small. Furthermore, immunoreactivity towards the correctly expressed protein could be found in MS serum samples originally used in the selection procedure favoring the theory of specific enrichment of the phage clone. Another explanation for the enrichment of this phage clone could be translational frameshifting. During standard translation, DNA codons do not overlap and are not separated by any nucleotides. However, during translation of certain mRNAs, a proportion of ribosomes shift frame at a particular site, and a second product is synthesized. This -1 or +1 shift in the reading frame can be stimulated by certain mRNA sequences and secondary stem-loop structures such as mRNA pseudoknots\textsuperscript{259,297}. In our study, a +1 frameshift would have to take place for correct expression of the cDNA insert as is observed between the GAG3 and POL3 genes of the retrotransposon Ty3 of yeast\textsuperscript{256,257}. In addition, sequence conditions favoring frameshifting are similar in the case of Ty3 and our enriched phage clone. However, our detection format based on E-tag expression was not suitable for the detection of frameshifting. Previous studies have shown that the level of protein display as a fusion to p6 is 100 times less compared to display at p3\textsuperscript{194,210}. In combination with a low frameshifting frequency, this suggests that our assay was not sensitive enough. In the future, more sensitive detection assays must be used to assess the occurrence of frameshifting in the p6-display system. This could be achieved via sensitive enzymatic reporter assays based on luciferase activity. These assays have already been successfully used in the past to detect frameshifting\textsuperscript{298-300}.

Previous phage display studies using N-terminal display of proteins showed that frameshifting was highly dependent on the type of ligand used and varied between 0-90\%\textsuperscript{251-253}. Frameshifting could result in a single DNA sequence encoding different polypeptides, thereby increasing the complexity of the original diversity of the library. Although in our study no exact quantification was performed, our results suggest that frameshifting is not a dominant event. This was also shown by previous results from our group in which immunoreactivity
towards certain phage clones was confirmed using synthetically produced peptides of the displayed antigenic target, thereby establishing the correct peptide sequences of the selected phage clones\textsuperscript{224}. This indicates that frameshifting, at least in our study, does not have a major impact on the selection of antigenic targets by antibodies in CSF or serum.

Phage display allows the efficient handling of large diversity libraries in a small volume. In this way, protein-protein interactions can be identified and characterized. Binding partners with either low or high affinity can be selected, dependent on the specific selection conditions which can be controlled. In addition, the use of an MS cDNA library in combination with antibodies present in MS serum and CSF has the advantage of identifying disease-related antigenic targets without knowing the identity of the antigens in advance. However, all display technologies based on biological systems will suffer from the constraints limiting the system itself. When using a bacterial expression system, the biased codon usage, lack of post-translational modifications and potential toxicity of some gene products will influence the expression of gene products. Furthermore, despite the use of a cDNA display library, many of the identified clones in this study encoded mimotopes. The question remains why our selection procedure has revealed these polypeptides. First of all, our library is composed of MS brain plaques that contained varying degrees of inflammation and demyelination. Previous sequencing of this library revealed known and also many novel proteins indicating the complexity of our library\textsuperscript{301}. Secondly, MS serum and CSF were used to affinity select our library. These body fluids contain a highly diverse repertoire of antibodies which adds to the complexity of our selection system. Third, in our selection procedure only high-affinity interactions are selected as a result of monovalent display of the insert in combination with stringent selection conditions. Therefore it may well be that naturally occurring proteins are selected preferentially but, if such clones are not available for more difficult targets, bio-panning may lead to selection of more unusual clones resulting from 3'UTR-regions or out of frame expression. However, despite these limitations our group, as well as other groups, has demonstrated the efficiency of p6 display through the successful identification of serine protease inhibitors\textsuperscript{210}, peroxisomal proteins\textsuperscript{302,303}, candidate tumor antigens in colorectal cancer\textsuperscript{212}, antigenic
targets associated with the presence of ruptured peripheral atherosclerotic lesions$^{213}$ and MS-related antigenic targets$^{224,250}$.

**5. Concluding remarks and future perspectives**

In this study, cDNA phage display was used to explore the antibody profile in MS patients. By performing affinity selections of an MS cDNA display library against MS CSF and serum, we intended to identify disease-related antibody biomarkers on the one hand and antibody reactivity on the other hand.

From the biomarker study we can conclude that, based on the identified immunoreactivity towards our panel of phage clones, these antigenic targets are not suitable as MS-related biomarkers. A larger number of MS patients representing different disease subtypes needs to be tested before a definite conclusion can be made concerning the potential use of our selected phage clones as biomarkers in MS. In addition, more phage clones could be implemented in future screening processes in order to obtain a panel of phage clones representing different disease processes. The existence of different clinical and immunopathological disease subtypes in MS makes it difficult to find a panel of antibody biomarkers specific for all disease types. More likely, patient subgroups reactive for certain biomarkers can be defined. Therefore, a large patient population is needed for the screening of antibody reactivity in which each clinical subtype (CIS – RR MS – SP MS – PP MS – PR MS) has to be represented to optimally correlate antibody reactivity with a specific disease subtype or disease mechanism. A clinically well defined patient group is therefore indispensable. Protein arrays carrying recombinantly produced antigenic targets would be suitable for the sensitive screening of more patient samples. In this way, a large number of phage clones and patients can be implemented in the screening process. Furthermore, less CSF/serum would be needed thereby increasing the number of assays that can be performed by a single serum or CSF sample.

When antibody profiles in paired CSF and serum were compared, a pattern of enrichment was identified that corresponded well with the presence of unique
OCB in CSF and common OCB in CSF and serum. This study showed that cDNA phage display was an excellent technique for the identification of antibody reactivity in CSF and serum. However, we have not yet confirmed if these OCB are also reactive with our selected antigenic targets. In the future, it would be relevant to assess if antibody reactivity towards our selected antigens can also be confirmed with OCB-derived antibodies of the patient used for the affinity selection since the intrathecal antibody response is thought to originate from a specific B-cell response targeting disease-related antigens\(^{112}\).

In this thesis, the nature of the identified mimotopes was not further studied. In the future, it will be necessary to retrieve the identity of the natural antigens to obtain more information regarding the disease process of MS. This can be achieved in several ways. Firstly, identified proteins can be produced recombinantly whereas shorter peptides can be synthetically generated. These peptides/proteins can then be used in the construction of sensitive protein arrays to easily detect antibody reactivity in CSF or serum of patients. Secondly, in vitro studies can be used to study the effect of the peptide/protein on proliferation, activation, survival and viability of different cell populations involved in the MS pathogenesis. Thirdly, animal studies can be implemented to study the overall effect on disease onset or progression. By administering the peptide/protein before, during or after disease onset, a possible effect on disease can be assessed. Finally, recombinant peptides/proteins can also be used for the production of monoclonal antibodies (mAb). mAb have a wide range of applications to identify their specific target. Immunostaining and immunoblotting can reveal more information concerning expression in certain tissues or cell types whereas protein arrays can be used to identify antibody specificity. Since B and T cells work together in a coordinated humoral and cellular response, it would be interesting to investigate whether T-cell reactivity towards the identified peptides/proteins can be found. T cell responses can be measured via in vitro proliferation assays whereas the phenotype can be determined by studying surface markers and cytokine profiles. This can provide more information about a possible role of the selected antigenic targets in the pathogenesis of MS.
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Chapter 7

Multiple sclerose (MS) is een autoimmune aandoening van het centrale zenuwstelsel. Deze neurologische aandoening treft voornamelijk jonge volwassenen tussen de 20 en 40 jaar en komt tweemaal zoveel voor bij vrouwen dan bij mannen. Wanneer het immuunsysteem zich richt tegen het lichaamseigen myeline ontstaat een inflammatoire reactie waardoor het myeline wordt afgebroken. Dit leidt tot de vorming van meerdere plaques of lesies in het centrale zenuwstelsel. Naargelang de plaats van de lesies in de hersenen ontstaan verschillende klinische symptomen waaronder coördinatieproblemen, visuele stoornissen en verlamming. Aangezien er nog steeds geen diagnostische test bestaat wordt de diagnose van MS gesteld aan de hand van klinische symptomen, magnetic resonance imaging (MRI) en de analyse van ruggenmergvocht.

Hoewel de etiologie van MS nog steeds onbekend is, heeft onderzoek aangetoond dat zowel genetische als omgevingsfactoren een rol spelen in het ontstaan van MS. Wat de precieze oorzaak is van de ontsporing van het eigen immuunsysteem is niet duidelijk maar de gangbare hypothese stelt dat autoreactieve T-cellen geactiveerd worden in de periferie, mogelijk door kruisreactiviteit met virale antigenen. Deze geactiveerde T-cellen dringen door de bloed-hersen barrière waar ze opnieuw geactiveerd worden door hun specifieke antigen dat aanwezig is in het centrale zenuwstelsel. De resulterende inflammatoire reactie trekt andere cellen van het immuunsysteem aan wat uiteindelijk leidt tot de afbraak van het myeline. Intensief onderzoek heeft aangetoond dat ook B-cellen een uitermate belangrijke rol spelen in de pathogenese van MS. B-cellen zijn uiterst efficiënte antigen-presenterende cellen en zorgen op die manier voor de activatie van autoreactieve T-cellen. Verder produceren ze cytokines die de immuunrespons moduleren en de door B-cellen geproduceerde antilichamen zorgen voor complementactivatie, opsonisatie, weefselschade en zelfs remyelinisatie.

Antilichamen in MS kunnen aangewend worden om meer informatie te verkrijgen over MS-gerelateerde antigenen en op die manier ook over pathogene mechanismen. Ze kunnen eveneens gebruikt worden als biomarkers die kunnen zorgen voor een efficiëntere diagnose, prognose en subclassificatie van
patiënten naargelang hun immuunpathogene profiel. Er bestaan verschillende technieken om de specificiteit van antilichamen te achterhalen. In dit doctoraat wordt de toepassing van serologische antigen selectie (SAS) beschreven om het antilichaam repertoire in CSV en serum van MS-patiënten te karakteriseren. SAS is een cDNA-faad display-gebaseerde techniek die gebruik maakt van antilichamen aanwezig in CSV of serum om antigenen te identificeren die een antilichaam respons uitlokken. In deze studie werd een cDNA bibliotheek, gemaakt van MS- hersenplaques met verschillende stadia van inflammatoire en demyelinisatie, tot expressie gebracht op het oppervlak van filamentouze M13 fagen als een fusie met mantelproteine 6 (p6). De expressie bibliotheek die op die manier ontstond werd vervolgens geïncubeerd met CSV of serum van MS-patiënten. De antilichamen aanwezig in deze lichaamsvloeistoffen kunnen vervolgens binden aan hun specifieke antigenen die tot expressie werden gebracht op het faagoppervlak. De gebonden fagen werden vervolgens geïsoleerd, geamplificeerd en gebruikt voor een volgende selectieronde met de MS cDNA expressie bibliotheek. Na een aantal selectierondes werden de geselecteerde antigenen geïdentificeerd en verder gevalideerd. Daarnaast werd het p6-display systeem zelf geanalyseerd door het mechanisme van frameshifting in meer detail te bekijken. In dit hoofdstuk worden de belangrijkste resultaten van dit doctoraat samengevat en vervolgens bediscussieerd.
1. Identificatie van moleculaire markers voor multiple sclerose

Om biomarkers in CSV en serum van MS-patiënten te identificeren, werd SAS toegepast op gepoold CSV en serum van 10 MS-patiënten. In het eerste deel van hoofdstuk 3 werd een alternatieve selectieprocedure geoptimaliseerd waarbij gebruik gemaakt werd van serum van gezonde personen om het voorkomen van niet-relevante faagklonen te doen afnemen. Deze depletiemethode resulteerde in een afname van aspecifieke faagklonen. Bovendien resulteerde deze aanpak in de identificatie van 5 aangrijkte kandidaat MS antigenen na selectierondes met serum-antilichamen. De selectierondes met CSV-antilichamen resulteerden in de identificatie van 12 aangrijkte kandidaat MS antigenen. De geïdentificeerde faagklonen bevatten epitopen die sterke homologie vertoonden met verscheidene leden van de MAPK-familie, heat shock proteïnen, hypothetische proteïnen en een aantal virale sequenties. Opmerkelijk is de homologie die gevonden werd met een viraal eiwit van het Epstein – Barr virus (EBV). EBV is één van de virussen die reeds lange tijd in verband gebracht worden met MS.

Wanneer immuunreactiviteit tegen een panel van 3 CSV-afgeleide faagklonen bepaald werd in CSV van een grotere groep MS-patiënten en controles, werd er geen verschil gedetecteerd tussen beide groepen. Opvallend was wel dat 83% van de MS-patiënten die een antilichaamrespons vertoonden tegen 1 van de 3 geselecteerde faagklonen een progressief ziekteverloop kenden ten opzichte van 30% van de MS-patiënten die geen antilichaamreactiviteit vertoonden. Dit kan er op wijzen dat deze faagklonen gerelateerd zijn aan ziektemechanismen die verbonden zijn met een progressief ziekteverloop. Maar aangezien het hier slechts om een kleine patiëntengroep gaat, is het noodzakelijk om meer patiënten te analyseren om deze hypothese te testen. Immuunreactiviteit tegen de 3 faagklonen geselecteerd door serum antilichamen werd gedetecteerd in het serum van een subgroep van MS-patiënten. Er werd echter geen correlatie gevonden tussen antilichaamreactiviteit en klinische parameters.

Uit deze resultaten kan besloten worden dat door het invoeren van een alternatieve selectieprocedure gebaseerd op het gebruik van controle serum, het
voorkomen van een aantal dominante en aspecifieke faagklonen kon verminderd worden. Deze aanpak resulteerde in de identificatie van een panel van kandidaat MS-gerateerde faagklonen. Immunoreactiviteit tegen deze klonen moet verder gevalideerd worden in een grotere groep van MS-patiënten waarin alle subtypes vertegenwoordigd zijn vooraleer een duidelijke conclusie kan genomen worden betreffende het gebruik van dit panel als biomarkers in MS.

2. Analyse van antilichaamprofielen in gepaard CSV en serum van één multiple sclerose patiënt

De precieze antigen-reactiviteit van MS-antilichamen in serum en CSV is ondanks intensief onderzoek nog steeds niet gekend. Dit zou echter meer inzicht kunnen verschaffen in het ziekteproces van MS. In hoofdstuk 4 werd onderzocht of de SAS-procedure gebruikt kon worden om antilichaamreactiviteit te bepalen in gepaard CSV en serum van één MS-patiënt. Het bekomen antilichaamprofiel in het CSV werd vervolgens vergeleken met dat aanwezig in het serum.


De cDNA-sequentie van één van de uitgesproken aangerijkte klonen codeerde voor MAPK7, ook wel ERK5 genoemd. Dit was opmerkelijk aangezien een ander lid van dezelfde familie, namelijk MAPK3 of ERK1, eerder was geïdentificeerd in de selectierondes met gepoold CSV (hoofdstuk 3). De MAPK-familie is betrokken bij verschillende fysiologische responsen zoals celproliferatie, -differentiatie en -overleving en wordt daarbij gereguleerd door een verscheidenheid aan proliferatieve en stress-geïnduceerde signalen. Veel van deze signalen zijn ook betrokken in het ontstekingsproces van MS wat erop kan wijzen dat de MAPK-
familie betrokken is bij MS-gerelateerde ziekteprocessen zoals inflammatie. Dit werd eveneens gesuggereerd door onze resultaten die de aanwezigheid van ERK5- en ERK1/2-positieve cellen aantonen in een inflammatoire MS-plaque. Bovendien blijken verschillende infiltrerende macrofagen positief te zijn voor geactiveerd ERK5 of ERK1/2. Er zijn echter meer studies nodig om te bepalen of de MAPK-specifieke antilichamen een pathologische rol hebben of het resultaat zijn van een secundaire immuunrespons.

Immuunreactiviteit tegen 8 van de 13 aangerijkte klonen werd bevestigd in het CSV of serum van de MS-patiënt gebruikt in de SAS-procedure. Om te bepalen of antilichaamreactiviteit tegen het panel van 8 faagklonen ook gevonden kon worden in andere patiënten werd in een initiële screening de immuunreactiviteit tegen het panel bepaald in het CSV en serum van een grotere groep van MS-patiënten en controles. De hoogste antilichaamrespons werd gevonden in het CSV tegen 3 faagklonen van ons panel. In de MS-groep vertoonde 22% van de patiënten antilichaamreactiviteit tegen minstens één van de 3 klonen ten opzichte van 5% van de controlepatiënten. In het serum werd geen verschil in immuunreactiviteit gevonden tussen de MS-groep en de controle-groep.

Samenvattend kunnen we stellen dat de SAS-procedure een geschikte techniek is om antilichaamprofielen te vergelijken in gepaard CSV en serum. Verdere studies moeten bevestigen of de antigenen geselecteerd door antilichamen van deze patiënt ook reactiv zijn in CSV of serum van een grote groep MS-patiënten en controles.

3. **Frameshifting in het p6-display systeem**

Faag display wordt gebruikt om verschillende soorten antigene targets tot expressie te brengen op het faagoppervlak als een fusieproteïne met één van de M13 faagmantelproteinen. Het herhaalde proces van selectie en amplificatie zorgt voor een specifieke aanrijking van geselecteerde liganden. Niettemin kunnen ongewone translationele gebeurtenissen zoals frameshifting soms leiden tot de aanrijking van liganden die geen open leesraam bevatten in het proteïne-

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coderende deel van het DNA. In hoofdstuk 5 werd onderzocht of frameshifting ook aangetoond kon worden in het p6-display systeem.


Door het ApoE - E-tag construct in 3 verschillende leesramen tot expressie te brengen in onze cDNA-faad display vectoren, is het mogelijk om immuunreactiviteit te bepalen tegen het correct tot expressie gebrachte ApoE eiwit in het serum van de 10 MS-patiënten gebruikt in de SAS-procedure. Het aantonen van immuunreactiviteit in het serum van MS-patiënten gebruikt in de selectieprocedure is een sterke aanwijzing dat de aanrijking van de ApoE-kloon tijdens de selectierondes een resultaat was van specifieke interacties tussen antilichamen aanwezig in het serum en het proteïne op het faagoppervlak. Echter, E-tag expressie kon alleen gedetecteerd worden met de positieve controle en wanneer het ApoE eiwit correct tot expressie gebracht werd.

In deze studie werd een faag kloon meerdere keren geselecteerd door antilichamen aanwezig in MS-serum, ondanks het feit dat een vroeg stopcodon de expressie van een eiwit zou verhinderen. Echter, antilichaamreactiviteit tegen het correct geëxpreseerde eiwit is een duidelijke aanwijzing dat deze aanrijking het gevolg was van specifieke interacties. Ondanks dat frameshifting niet rechtstreeks kon aangetoond worden met behulp van ELISA, wijzen de bevestigde immuunreactiviteit in MS serum in combinatie met de geschikte sequentiecondities die een verschuiving van het leesraam stimuleren sterk op het voorkomen van frameshifting in het p6-display systeem.
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4. Eindconclusie en toekomstperspectieven

In deze studie werd cDNA-faag display gebruikt om antilichaamprofielen in MS te onderzoeken. Het doel was om via het uitvoeren van affiniteitsselecties met een MS cDNA-faag display bibliotheek en CSV en serum van MS-patiënten, zowel antilichaamreactiviteit als ziekte-gerelateerde biomarkers te identificeren.

Uit de biomarkerstudie kunnen we besluiten dat op basis van de gevonden immuunreactiviteit, we momenteel geen MS-gerelateerd biomarker panel geïdentificeerd hebben. Daarom is het noodzakelijk om de kandidaat biomarkers in een grote patiëntgroep te testen waarin alle subtypes van MS in voldoende mate vertegenwoordigd zijn vooraleer er een definitieve conclusie kan genomen worden. Bovendien zou een uitgebreider panel van faagklonen kunnen leiden tot een betere representatie van de verschillende ziekteprocessen in MS waardoor de sensitiviteit en specificiteit verhoogd kan worden.

De aanwezigheid van verschillende klinische en immuunpathologische subtypes van MS maakt het vrijwel onmogelijk om een panel van biomarkers te identificeren die specifiek zijn voor elk subtype. Een biomarker panel zal eerder specifiek zijn voor een bepaald subtype of ziektemechanisme van MS. Daarom is het noodzakelijk om kandidaatmerkers in een grote patiëntgroep te valideren om een mogelijke correlatie tussen antilichaampositiviteit en bepaalde ziektemechanismen te identificeren. Proteïne-arrays met daarop de recombinant geproduceerde kandidaat biomarkers zijn uitermate geschikt voor deze screening procedure. Deze techniek is gevoelig en zorgt ervoor dat een groot aantal kandidaat biomarkers snel en efficiënt in meerdere patiënten getest kan worden.

Tijdens de vergelijking van antilichaamprofielen in gepaard CSV en serum werd een aanrijkingspatroon gevonden dat goed overeenkomt met de aanrijking van OCB in CSV en serum. Deze studie toonde aan dat cDNA-faag display een uitstekende techniek is om antilichaamreactiviteit in CSV en serum te bepalen. We hebben echter nog niet bevestigd of OCB in CSV en serum van deze patiënt reactiviteit vertonen tegen de geselecteerde antigenne targets. In de toekomst
zal dit nog uitgevoerd moeten worden aangezien aangenomen wordt dat een deel van de OCB het resultaat zijn van een specifieke en antigen-gedreven immuunrespons.

van de geïdentificeerde peptiden/proteïnen. De T-cel respons kan bepaald worden aan de hand van *in vitro* proliferatie-assays terwijl oppervlaktemarkers en cytokineprofielen meer informatie kunnen verschaffen over het fenotype. Deze resultaten kunnen meer inzicht verschaffen over mogelijke rol van de geïdentificeerde antigenen in het ziekteproces van MS.
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“Profiling the autoantibody repertoire by serological antigen selection”
V. Somers, R. Hupperts, C. Govarts, N. Hellings and P. Stinissen

“Exploring cDNA phage display for autoantibody profiling in the serum of multiple sclerosis patients: Optimization of the selection procedure”
C. Govarts, K. Somers, R. Hupperts, P. Stinissen and V. Somers

“Autoantibody profiling in multiple sclerosis reveals novel antigenic candidates”
V. Somers, C. Govarts, K. Somers, R. Hupperts, R. Medaer and P. Stinissen

“Multiplexing approaches for autoantibody profiling in multiple sclerosis”
K. Somers, C. Govarts, P. Stinissen, V. Somers
Autoimmun. rev., in press

“Evidence for frameshifting in the pVI cDNA phage display system”
C. Govarts, K. Somers, R. Medaer, P. Stinissen, V. Somers
Submitted

“Analysis of antibody reactivity in paired cerebrospinal fluid and serum of a relapsing remitting multiple sclerosis patient”
C. Govarts, K. Somers, R. Hupperts, P. Stinissen, V. Somers
Submitted
Published abstracts

“A panel of novel candidate antigens in multiple sclerosis revealed by phage cDNA display”
V. Somers, C. Govarts, J. Raus and P. Stinissen

“Anti-ergotypic T cell responses in healthy controls and multiple sclerosis patients”
C. Govarts, J. Raus, N. Hellings, and P. Stinissen

“Novel candidate markers for multiple sclerosis using phage cDNA display”
V. Somers, C. Govarts, K. Somers and P. Stinissen

“Autoantibody profiling in multiple sclerosis reveals novel antigenic candidates”
V. Somers, C. Govarts, K. Somers R. Hupperts, R. Medaer and P. Stinissen

“Identification of novel biomarkers for multiple sclerosis based on the humoral immune response”
V. Somers, K. Somers, C. Govarts and P. Stinissen

Oral presentations at international meetings

“Identification of new molecular markers for MS using phage cDNA display”
C. Govarts, V. Somers and P. Stinissen
MS research days
Amsterdam, the Netherlands, 22 December 2004
Curriculum Vitae

"Exploring cDNA phage display to identify new molecular markers for autoimmune diseases"
**C. Govarts**, K. Somers, J. Robben, P. Stinissen and V. Somers
NUBIN symposium
Amsterdam, the Netherlands, 19-20 June 2006

"Profiling the antibody responses of multiple sclerosis patients using phage cDNA display"
**C. Govarts**, K. Somers, P. Stinissen, V. Somers
International congress of autoimmunity
Sorrento, Italy, 29 November – 3 December 2006

"Analysis of autoantibody profiles in CSF and serum of a RR MS patient with active disease"
**C. Govarts**, K. Somers, R. Hupperts, P. Jongen, P. Stinissen and V. Somers
MS Research days 2008
Oegstgeest, The Netherlands, 13-14 November 2008

**Poster presentations**

"The search for novel molecular markers in Multiple Sclerosis using phage cDNA display"
**V. Somers**, **C. Govarts**, J. Bleus, J. Raus and P. Stinissen
- FOCIS 4th Annual Conference, Montreal, Quebec, Canada, 18 – 23 July 2004

"Identification of new molecular markers for multiple sclerosis using phage cDNA display"
**C. Govarts**, P. Stinissen en V. Somers
- Annual BIS, Brussel, 26 November 2004
- Bioforum, Luik, 7 December 2004
- 189th meeting of the Belgian Society of Biochemistry and Molecular Biology,
"Signaling in the brain", 18 March 2005
"Study of anti-ergotypic T-cells in multiple sclerosis: role of candidate ergotopes IL-2 receptor and heat shock protein 60"
K. Pannemans, N. Hellings, C. Govarts and P. Stinissen
- 9th Maastricht Medical Students Research Conference, Maastricht, The Netherlands, 23 March 2005

"The search for novel molecular markers in Multiple Sclerosis using phage cDNA display"
C. Govarts, V. Somers, P. Stinissen
- MS research days, Amsterdam, The Netherlands, 24-25 November 2005

"Identification of novel biomarkers for Multiple Sclerosis based on the humoral immune response"V. Somers, C. Govarts, K. Somers, and P. Stinissen
- FOCIS, San Francisco, California, USA, 1-5 June 2006
- ISNI, Nagoya, Japan, 15-19 October 2006

"Exploring cDNA phage display to identify new molecular markers for autoimmune disease"V. Somers, C. Govarts, K. Somers, and P. Stinissen
- PEGS, Boston, USA, 24-28 April 2006

"Identification of novel biomarkers for Multiple Sclerosis based on the humoral immune response"C. Govarts, K. Somers, P. Stinissen en V. Somers
- Bioforum, Luik, Belgium, 17 May 2006

"Exploring cDNA phage display to identify new molecular markers for autoimmune diseases"
C. Govarts, K. Somers, J. Robben, P. Stinissen and V. Somers
- Biomedica, Aken, Duitsland, 21-22 March 2007
Curriculum Vitae

“Analysis of CSF and serum autoantibody profiles in a RR MS patient with active disease using phage cDNA display”

C. Govarts, K. Somers, R. Hupperts, P. Stinissen and V. Somers
- MS research days, Hasselt, Belgium, 15-16 November 2007
- Biomedica, Maastricht, The Netherlands, 16-17 April 2008
- Knowledge for growth, Gent, Belgium, 6 June 2008
- International congres of autoimmunity, Porto, Portugal, 10-14 September 2008
- ISNI, Fort Worth, Texas, USA, 26-30 October 2008

Awards

Poster award: “Biomedical Innovation” Award (Dyax)
Bioforum 2004
Luik, Belgium 7 December 2004

Poster award
Knowledge for growth
Gent, Belgium, 6 June 2008

Poster award
MS research days
Hasselt, Belgium, 15-16 November 2007
Dankwoord

Nu mijn boekje geschreven is, kom ik aan het moeilijkste maar ook het meest dankbare stuk: het bedanken van al de mensen die mij tijdens mijn doctoraat op één of andere manier hebben bijgestaan. Want tijdens de afgelopen jaren heb ik wel geleerd dat een doctoraat niet iets is dat je alleen doet, ik heb heel wat hulp en inspiratie gehad!

Allereerst wil ik mijn promotor Piet bedanken. Je kritische vragen en commentaren zorgden altijd voor een nieuwe insteek of bijsturing van een experiment of artikel. Je helder inzicht zorgde voor nieuwe mogelijkheden op momenten dat het allemaal even vastliep. En ook al heb je een drukke agenda, ik weet dat je deur openstaat wanneer het nodig is. Bedankt voor de grappige momenten op congres en natuurlijk voor je medewerking aan de talloze filmpjes die we hebben gemaakt als er een trouwfeest of dergelijke was. Je presentatietalent is meermaals van pas gekomen om onze filmpjes aaneen te praten! 😊

Veerle, dank je wel voor de steun en hulp de afgelopen 4 jaar. Ik kan me al niet meer herinneren hoe dikwijls ik je bureau ben binnengestormd met één of andere vraag of een update van de laatste resultaten. Je was even enthousiast als mij als er iets gelukt was en als het tegendeel, hielp je zoeken naar oplossingen. Meerdere malen gaf je me weer net dat duwtje dat ik nodig had om weer door te gaan als het even wat tegenzat. Ook bedankt voor de hulp met de laatste (zware) loodjes, dit doctoraat was er zeker niet geweest zonder jou!

Niels, jou wil ik ook zeker bedanken! Als mijn stagebegeleider was jij de eerste die mij de kiepzjes van het vak leerde. Jouw bureau werd toen evenzeer regelmatig platgelopen! Daar is het natuurlijk niet bij gebleven, tijdens mijn doctoraat ben ik nog regelmatig komen aankloppen voor raad. Ook buiten het werk hebben we veel gelachen en plezier gemaakt. Congressen, housewarmings, ’s middags uit eten, gangconferenties, vrijdagnamiddag-drink, ... het is altijd een leuke bedoeling. En ook al heb ik het nooit zo gezegd maar je welgemeende ‘ça va?’ (zeker tijdens de megaCindy-periode) werd en wordt zeker op prijs gesteld!
Dankwoord

De juryleden wil ik bedanken voor de kritische evaluatie van dit doctoraatsproefschrift en hun constructieve commentaren en suggesties. Deze hebben zeker bijgedragen aan de kwaliteit van dit doctoraatsproefschrift.

Onderzoek kan nooit uitgevoerd worden zonder de noodzakelijke financiële middelen. Daarom graag een woord van dank aan het ‘Bijzonder Onderzoeksfonds (BOF)’ van de Universiteit Hasselt voor hun financiële ondersteuning aan dit project.

Tijdens dit onderzoek zijn er veel bloed, CSF en weefselstalen van patiënten en controlepersonen verzameld en verwerkt. Ik zou daarom heel graag een woord van dank willen richten aan al deze mensen die dit mogelijk gemaakt hebben, zowel artsen, verpleegkundig personeel als patiënten. Zonder hun zou ik niet veel onderzoek hebben kunnen uitvoeren! Mijn dank aan de Nederlandse Hersenbank voor het ter beschikking stellen van het patiëntennateriaal. Mijn oprechte dank aan Dr. Hupperts, Dr. Medaer, Dr. Jongen, Dr. Zwanikken, Dr. Rummens, het personeel van de klinische biologie van het Virga Jesse ziekenhuis, het aZM en het MS centrum Nijmegen, Marcel Verbeek, Riet Schmitz, Bertine Timmermans, Riny Wieërs, Bianca Pulinx, Anne Bogaers en Marleen Misotten.

Dat ik graag naar Biomed kwam, had niet alleen te maken met het onderzoek maar natuurlijk ook heel veel met de ongelooflijk toffe sfeer en leuke collega’s! Mijn voorgangers: Loes, Joris, Debbie, Marielle en Koen, van jullie heb ik geleerd dat er naast het serieuze labowork altijd wel tijd is om te lachen en grappen uit te halen! Dank je wel voor de leuke middagen in de KHLIM, de gezellige housewarmings die samengingen met het oprakelen van allerlei leuke anekdotes en talloze bulderlachsalvo’s, onvergetelijke congressen, bureaubabbels, feestjes en bijhorende voorbereidingen en nog zoveel meer! De huidige lichting is al even plezant als uitgebreid! Annelies, Bieke, Evi, Jeroen, Jerome, Judith, Kim, Karolien, Klaartje, Kurt, Leentje, Myrthe, Ruth, Sofie, Tom en Veronique, bedankt voor de leuke sfeer, de gezellige babbels en vooral de vele
Dankwoord

smoutebollen- en taartfestijnen! We zitten ondertussen wat meer verspreid maar tijd en plaats voor een gezellige babbel is er nog altijd!

Nog een speciaal woord van dank aan ‘de faag-meisjes’. Klaartje, Igna, Myrthe en Veerle, het was geweldig om met jullie te werken en ik vind dat we een geweldig team vormen. Fagen opgroeien, ELISA’s uitvoeren, prutsen met western blots en kleuringen, delen van frustraties, zoeken naar patiëntstalen in de -80°C, verhuizen van labo’s, Fluffy als huisdier houden en vrijdagnamiddag-nurt-humor... We hebben samen heel wat watertjes doorwormmen zonder dat er daarbij veel stormen zijn ontstaan. We slagen er zelfs in om elkaars neurotische trekken aan te vullen en/of over te nemen! 😊 Dank je wel voor de leuke gesprekken gaande van het opstellen van cut-off’s en bepalen van controlegroepen tot discussies over welke taart nu het lekkerst is (conclusie: als er maar chocolade inzit) en welke TV-serie interessant is om te volgen (Lost natuurlijk, zo een spannend en vooral knap verhaal! 😊). Klaartje, nog veel succes met de laatste loodjes, je gaat het gewoon super doen!


Ariane, Bjorn, Roel en Kelly, jullie zorgden altijd voor momenten van ontspanning en hadden er alle begrip voor als de zaterdagavond-‘date’ niet doorging omdat het te druk was. Merci voor de leuke weekendjes en vakanties, uitstapjes naar pretparken of gewoon de avondjes thuis met een film of een
Dankwoord

gezelschapsspel. In de toekomst hoop ik nog vele leuke (en kindvriendelijke!) weekendjes en uitstapjes te beleven met de hele bende. Roel, jou wil ik nog eens extra bedanken voor al de computerhulp! Ik ben zo blij dat jij als persoonlijke helpdesk wou fungeren! Jouw ingebakken drang om uit te zoeken waar het misgaat en vooral hoe het op te lossen heeft mij meermaals van een zenuwinzinking gered!

Lou, Rik, Frans, Chris en Bianca, ondanks dat het niet duidelijk was wat ik nu precies deed en doe, toch toonden jullie altijd interesse en kon ik bij jullie terecht. Dank je wel voor de leuke familiefeesten en de gezellige sfeer. En natuurlijk voor al de hulp in huis en tuin als het even wat druk werd en we het allemaal niet meer voor elkaar kregen. Ik kan in ieder geval zeggen dat mijn boekje nu WEL eindelijk af is!

De mensen op wie ik mijn hele leven al kan terugvallen: mama, papa, Michael, Angie en Shana. Mama en papa, zonder jullie hulp stond ik hier niet vandaag. Jullie hebben me altijd gesteund om te doen wat ik graag deed, zelfs toen ik als kind aan het smodderen was met potjes en flesjes om te zien wat er zou gebeuren als ik dit en dat zou samengieten (meestal niets tot mijn grote spijt). No matter what, ik weet dat ik op jullie kan terugvallen en dat jullie achter me staan terwijl ik toch ook de vrijheid krijg om mijn eigen leven te leiden. En papa, ook al kon je totaal niet volgen, toch vroeg je altijd hoe het nu ging op het werk en toonde je interesse. En ook al duurde het even, ik ben uiteindelijk dan toch doctor in de bio-dinges geworden! 😊 Mike, Angie en Shana, we vormen samen een vrolijke en luidruchtige bende die ik zeker niet zou kunnen missen. Ondanks de discussies af en toe (die er bij horen) zijn jullie stuk voor stuk schattig!

Kyra, lieve dochter, toen jij kwam werd ons leven helemaal ondersteboven gegoooid. We hebben lang op je gewacht en waren dan ook dolgelukkig toen je je aankondigde. Het was niet altijd makkelijk om deze drukke periode te combineren met een opgroeiende baby maar het was het zo waard. Jou zien opgroeien is het mooiste dat er is en een glimlach van jou maakt elke dag weer goed!
Dankwoord

De persoon op wie ik het hardste gesteund heb de afgelopen maanden is de persoon die het ook het hardste te verduren heeft gekregen. Ben, ik weet dat het voor jou ook zeker niet makkelijk was maar zonder jou was ik de laatste maanden niet doorgekomen. Je zorgde ervoor dat alles bleef draaien thuis, zorgde voor Kyra, offerde je weekends op, luisterde naar mijn klaagzangen en leefde mee met mijn triomfen als er weer een hoofdstuk af was. Jij was diegene die mij tot rust kon brengen na een hectische dag en die mij aan het lachen kon brengen als ik het niet meer zag zitten. Met jou naast mij weet ik dat het allemaal wel gaat lukken. We hebben al een hele geschiedenis samen en ik ben er zeker van dat we samen nog een mooie toekomst kunnen opbouwen! xxx

Cindy
Februari 2009

Nothing great in the world has ever been accomplished without passion

Georg Wilhelm
Addendum
Table 1.1_S: Antibodies against MS-related proteins

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Ig isotype</th>
<th>MS (pt no)</th>
<th>controls (pt no)</th>
<th>Source</th>
<th>Results</th>
<th>Method, Reference</th>
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<tbody>
<tr>
<td><strong>Axonal antigens</strong></td>
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<tr>
<td>NFL, NFH, tubulin</td>
<td>IgG</td>
<td>RR(39), SP(18), PP(10)</td>
<td>OIND(21), NIND(40), HC(12)</td>
<td>CSF, serum</td>
<td>CSF IgG SP, PP&gt;RR, controls</td>
<td>ELISA, immunoblot, immunocytochemistry, 160</td>
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<td>NFL</td>
<td>IgG</td>
<td>RR(69), SP(38), PP(23)</td>
<td>OIND(95), ND(70), NIND(43), HC(94)</td>
<td>CSF, serum</td>
<td>serum IgG PP&gt;SP, OIND, HC; CSF IgG no difference</td>
<td>ELISA, 161</td>
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<tr>
<td>NFL, NFH</td>
<td>IgG</td>
<td>RR(21), SP(20), PP(10)</td>
<td></td>
<td>CSF</td>
<td>CSF IgG correlated with brain atrophy</td>
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</tr>
<tr>
<td>NFL, MBP</td>
<td>IgG</td>
<td>MS(63), NMO(15)</td>
<td>OIND(31), NIND(18), HC(46)</td>
<td>CSF, serum</td>
<td>CSF IgG OIND&gt;MS&gt;NIND, HC; correlated with MBP Ab level</td>
<td>ELISA, 304*</td>
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<tr>
<td>NFL</td>
<td>IgG/IgM</td>
<td>CUS(8), RR(37), SP(15), PP(6)</td>
<td>HC(24), ND(31), controls(49)</td>
<td>CSF, serum</td>
<td>serum IgM controls&gt;MS, HC; serum IgG: ND&gt;MS, HC, controls; CSF IgG: no difference; CSF IgG: MS&gt;controls</td>
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<tr>
<td>NFM</td>
<td>IgG/IgM</td>
<td>RR(29), SP(14), PP(6)</td>
<td>HC(16), controls(21), NIND(14)</td>
<td>CSF, serum</td>
<td>serum IgM: no difference; serum IgG HC, controls&gt;MS; CSF IgG/IgM MS&gt;HC, controls</td>
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### Addendum

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<td><strong>Heat shock proteins</strong></td>
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<tr>
<td>αBC</td>
<td>IgG</td>
<td>MS(30)</td>
<td>OIND(22), NIND(42), HC(23)</td>
<td>serum</td>
<td>serum IgG MS&gt;OIND&gt;NIND, not in HC; correlation severe disease</td>
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<tr>
<td>αBC</td>
<td>IgG/IgM</td>
<td>RR(25), SP(5), PP(3)</td>
<td>NIND(25), OIND(60)</td>
<td>CSF, serum</td>
<td>serum IgM: OIND&gt;MS&gt;NIND; CSF IgG OIND&gt;MS, NIND; CSF IgM/IgG OIND&gt;MS, NIND;</td>
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<td>MS(11)</td>
<td>HC(70)</td>
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<td>serum Ab MS=HC</td>
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<td>Hsp27, MBP</td>
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<td>OIND(22), NIND(42), HC(23)</td>
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<td>serum IgG not detected</td>
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<td>Hsp90β</td>
<td>IgG</td>
<td>RR(33)</td>
<td>NIND(17)</td>
<td>CSF</td>
<td>MS IgG recognize Hsp90 on OPC</td>
<td>immuno-precipitation, immunoblot</td>
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<td>OIND(12), NIND(22)</td>
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<td>CSF IgG RR&gt;OIND, NIND</td>
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<td>Myelin antigens</td>
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<td>MOG, MBP</td>
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<td>MS(40)</td>
<td>HC(47)</td>
<td>serum</td>
<td>serum IgG relapse&gt;remission, controls</td>
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<td>MBP, MOG, oBC</td>
<td>IgG/IgM/IgA</td>
<td>MS(20)</td>
<td>HC(20)</td>
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<td>serum Ab MS&gt;HC</td>
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<td>RBA, Western blot</td>
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<td>MS(3)</td>
<td>NIND(2)</td>
<td>CNS tissue</td>
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<td>immunogold labeling</td>
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<td>RR(27), SP(17)</td>
<td>NIND(15), HC(18)</td>
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<td>HC(20)</td>
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<td>serum Ab MS&gt;HC for 2 MOG-epitopes</td>
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<td>HC(37)</td>
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<td>MS &gt;controls</td>
<td>Western blot</td>
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<td>MOG</td>
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<td>RR(175), SP(44), PP(43), NIND(131), HC(307)</td>
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<td>serum IgG: SP&gt;RR, PP; MS, OND&gt;HC; CSF IgG: no difference</td>
<td>ELISA</td>
<td>317</td>
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<table>
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<tr>
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<tr>
<td>MOG</td>
<td>IgG</td>
<td>CNS(14)</td>
<td>CNS(8)</td>
<td>brain, CSF, serum</td>
<td>brain&gt;CSF, serum</td>
<td>solid-phase assay, RIA</td>
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<td>MOG</td>
<td>IgG/IgM</td>
<td>MS(126)</td>
<td>HC(252)</td>
<td>serum</td>
<td>serum IgG increased risk developing MS</td>
<td>ELISA</td>
<td>318</td>
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<td>MS(72)</td>
<td>serum</td>
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<td>ELISA, Western blot</td>
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<td>MOG</td>
<td>IgG</td>
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<td>NIND(19)</td>
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**rhMOG (1-125) and native MOG**

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<td>CSF</td>
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**Oligoclonal bands**

- **OCB**
  - IgG: CSF, serum
  - OCB in > 95% of MS pt; >2 OCB=positive test for MS
  - IEF, immunoblot (review: 133,137)

- **OCB**
  - IgM: CIS(42), OIND(11), NIND(23)
  - IgG OCB no increased risk in developing MS
  - IEF, immunoblot (151)
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*only abstract available