"Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning."

Albert Einstein

"What's in a name? That which we call a rose

By any other name would smell as sweet."

William Shakespeare

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A.U.	arbitrary units
aa	amino acid
ANOVA	analysis of variance
APC	antigen presenting cell
AUC	area under the curve
BBB	blood brain barrier
CIS	clinically isolated syndrome
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSF	cerebrospinal fluid
DIS	dissemination in space
DIT	dissemination in time
DMT	disease-modifying treatment
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
ELISA	enzyme-linked immunosorbent assay
GFAP	glial fibrillary acidic protein
HC	healthy controls
HLA	human leukocyte antigen
IEF	isoelectric focusing
IFN	interferon
Ig	immunoglobulin
IHC	immunohistochemistry
IL	interleukin
mAbs	monoclonal antibodies
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSSS	multiple sclerosis severity score
NAWM	normal-appearing white matter

NDC	non-demented control
NIND	other inflammatory neurological disease
NIND	non-inflammatory neurological disease
NMO	neuromyelitis optica
NO	nitric oxide
NWM	normal white matter
OCB	oligoclonal band
OD	optical density
ON	optic neuritis
Poly I:C	polyinosinic:polycytidylic acid
PPMS	primary progressive MS
ROC	receiver operating characteristic
ROS	reactive oxygen species
RRMS	relapsing–remitting MS
SAS	serological antigen selection
SPAG16	sperm-associated antigen 16
SPAG16-1	sperm-associated antigen 16 isoform 1
SPAG16-2	sperm-associated antigen 16 isoform 2
SPMS	secondary progressive MS
THIO	thioredoxin

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Introduction and aims

Based on the review:

Targets of the humoral autoimmune response in multiple sclerosis

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1.1 Multiple sclerosis (MS)

1.1.1 Diagnostic criteria and clinical course

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by myelin loss, axonal pathology and progressive neurodegeneration [1, 2]. It is the most common neurological and debilitating disease and typically presents in young adults between the ages of 20-50 years, with a peak occurring at 30 years of age. The prevalence varies among countries and is highest in North America (140 per 100,000) and Europe (108 per 100,000). Furthermore, MS is more common in women than men with a 2:1 ratio [3-5].

The diagnosis of MS is not always straightforward and until now there is no single laboratory test [6]. Although the first description of MS dates back to the 14th century, it was in 1868 that J. Charcot first described "la sclérose en plaque" [7]. Since then many different diagnostic criteria have been proposed for MS [6, 8-10]. The oldest Schumacher criteria (1965) called for 2 clinical relapses separated in time and space in patients aged 10-50 years, with no other alternative explanation for their symptoms [11]. The subsequent Poser criteria (1983) added laboratory or paraclinical parameters to make the diagnosis of MS such as the presence of oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) and abnormal or delayed responses of the visual and auditory evoked potentials [12]. Nowadays, with the great advances in magnetic resonance imaging (MRI), the McDonald criteria (Table 1.1) – initially formulated in 2001 and revised in 2010 – are used for the diagnosis of MS [13, 14]. These criteria focus on the combination of clinical, laboratory and radiographic data to establish a diagnosis of MS. The 2001 McDonald diagnostic criteria require 2 clinical attacks varying in time and space to establish a definite diagnosis of MS [13]. The revised McDonald criteria were formulated with the intent to simplify and allow for a more rapid diagnosis of MS. These criteria require fewer MRI scans to establish the diagnosis of definite MS since after one clinical attack, MS may now be diagnosed even based solely on the baseline MRI, if both enhancing and non-enhancing lesions co-exist [14].

The clinical course of MS is highly variable and different disease subtypes exist [6, 9, 15]. 80–85% of the initial diagnoses of MS are relapsing–remitting MS (RRMS)

with defined attacks (relapses) of new or recurrent neurologic symptoms with recovery (remission) but without disease progression. About 40-50% of RRMS patients develop secondary progressive (SP) MS after 10 years and 90% after 25 years with progressive neurological disease. Approximately 10–15% of MS patients have primary progressive MS (PPMS) with disease progression from onset [6, 9].

Table 1.1: 2010 Revised McDonald MS Diagnostic Criteria [14]

Clinical attacks[a]	**Lesions[b]**	**Additional criteria to make diagnosis**
2 or more	Objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of prior attack	None
2 or more	Objective clinical evidence of 1 lesion	DIS[c] (or await further clinical attack implicating a different CNS site)
1	Objective clinical evidence of ≥2 lesions	DIT[d] (or await a second clinical attack)
1	Objective clinical evidence of 1 lesion	DIS or await further clinical attack implicating a different CNS site and DIT; or await a second clinical attack
0 (Progression from onset)		One year of disease progression (retrospective or prospective) and at least 2 out of 3 of the following criteria: DIS in the brain based on ≥1 T2 lesion in periventricular, juxtacortical or infratentorial regions; DIS in the spinal cord on ≥2 T2 lesions; or positive CSF[e]

a. A clinical attack is defined as patient-reported symptoms or objectively observed signs typical of an acute inflammatory demyelinating event in the CNS. b. Both gadolinium-enhancing and non-enhancing lesions. c. DIS: Dissemination in space (MRI); d. DIT:

Dissemination in time (MRI); e. Positive CSF: isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index

Clinically isolated syndrome (CIS) is the first clinical episode suggestive of MS with inflammatory demyelination (objective clinical evidence of 1 lesion) but does not yet fulfill the criteria of dissemination in time [9, 15]. However, using the 2010 revised McDonald diagnostic criteria allows some patients with a single clinical episode to be diagnosed with MS based on the single scan criterion for dissemination in time and space [14] reducing the number of patients who will be categorized as CIS [15]. Due to the heterogeneity of the MS disease course and affected regions in the brain and spinal cord, a wide variety of symptoms is possible. Common symptoms include numbness, motor weakness, visual disturbances, incoordination, dizziness, fatigue, spasticity, neuropathic pain, urinary urgency or retention, sexual dysfunction, depression or other emotional changes, cognitive dysfunction and more [6, 9, 16].

1.1.2 Genes and environment

The cause of MS is unknown but it is generally assumed that complex interactions between environmental factors and genetic susceptibility are involved.

Studies in first-, second- and third-degree relatives of MS patients have shown that there is a higher risk (3-5%) to develop MS in comparison with the general population (0.1%), a phenomenon known as familial clustering [17, 18]. Furthermore, twin studies have shown higher concordance rates in monozygotic twins (~30%) as compared to ~2% in dizygotic twins, indicating a genetic component in MS susceptibility [19]. Genome-wide association studies have identified several MS-associated risk loci but the human leukocyte antigen (HLA) class II genes, more specifically the HLA-DRB1*15:01 allele is associated with a three times elevated risk of developing MS [20, 21]. Besides the association with HLA-II alleles, many other immune-related risk genes have been found to be associated with MS, including genes encoding the cytokine pathway such as the interleukin 7 receptor (IL7R) and interleukin 2 receptor alpha (IL2RA) and genes involved in costimulation such as CD40 and CD86 [20]. While these associations are weaker than the HLA-II risk alleles, many genes seem to contribute to the genetic susceptibility for MS and underline the complexity of this disease.

Besides genetic susceptibility, the cause of MS is believed to also involve environmental exposure [3]. Many environmental risk factors have been reported including Epstein-Barr virus (EBV) infection [22], smoking [23], and latitude resulting in variations in sunlight exposure and vitamin D levels modulating different immune functions [24, 25]. Studies have suggested the involvement of viruses in MS pathogenesis by either bystander activation of the immune system or via molecular mimicry, with the strongest association with EBV [4, 25, 26]. EBV is widely spread and causes a persistent life-long infection. In young children and babies the infection is asymptomatic and is exchanged by saliva by sharing toys. However, during adolescence when exchange of saliva becomes more direct via kissing, EBV presents as infectious mononucleosis which is associated with a two- to threefold increased risk of MS [26]. There is a striking similarity between the epidemiology of infectious mononucleosis and MS in terms of age, geographical distribution, socioeconomic status and ethnicity. A possible explanation for the higher risk for MS following infectious mononucleosis is that both diseases are linked to good hygiene in childhood, which results in a delayed age at infection with EBV and other infectious agents, which is often referred to as the 'hygiene hypothesis' [26].

1.1.3 Immunopathogenesis of MS

Although the CNS has long been considered as an immune-privileged site due to the presence of the blood brain barrier (BBB), it is now clear that immune cells can migrate to the CNS via multiple routes for immune surveillance under physiological conditions [27, 28]. During pathological conditions, however, leukocyte infiltration is increased and inflammatory reactions are started to clear the insult. The main hallmark of MS is the formation of the sclerotic plaque, which represents the end stage of a process involving (uncontrolled) inflammation, demyelination, remyelination, oligodendrocyte depletion and astrocytosis, and neuronal and axon degeneration. The order and relation of these separate components remain to be fully understood but the end result is irreversible damage to resident brain cells [16]. An overview of the current disease models and key players in the MS pathogenesis is provided below.

The conventional outside-in model versus the alternative inside-out model

According to the conventional outside-in model, MS is a primary autoimmune disease. The disease process starts with an increased migration of autoreactive lymphocytes (e.g. reacting to myelin basic protein (MBP)) from the periphery across the BBB due to regulatory defects in genetically susceptible individuals which allow these cells to set up an immune response within the CNS. Two important factors are thought to be crucial for this process. First, regulatory lymphocytes from people with MS fail to suppress effector cells [29]. This immune tolerance normally eliminates autoreactive cells by inducing anergy or apoptosis. Second, autoreactive lymphocytes can be activated due to nonspecific activation by antigenic targets from bacteria or viruses (bystander activation or molecular mimicry). According to the outside-in model, once inside the CNS, pathogenic T cells become reactivated by myelin and other antigens and an inflammatory cascade is initiated, which ultimately leads to demyelination and tissue damage [30].

The alternative inside-out model proposes that MS is a neurodegenerative disorder and that the initial trigger originates in the CNS. In this model, primary cellular degeneration in the CNS, initially focused on the oligodendrocytes and myelin, leads to the release of cellular debris that is highly autoantigenic. This leads to a secondary autoimmune and inflammatory response in predisposed individuals (i.e. dependent on the host's immune system), causing further damage to the CNS and possibly driving degeneration [31-33].

Key players in MS pathogenesis

MS is a complex disease involving the interplay of many different cell types from the innate and adaptive immune system and the CNS [34], summarized in Fig. 1.1. $CD4^+$ T helper (Th) 1 cells have long been considered to be the most important players in MS pathogenesis since adoptive transfer of myelin-specific Th cells could induce experimental autoimmune encephalomyelitis (EAE; an animal model of MS, see section 1.1.5) [35, 36]. Initially, Th1 (interferon-γ secreting) cells were identified as the inflammation-driving cells in MS. However, recent studies have indicated that interleukin-17 (IL-17) secreting Th17 cells are also essential in MS pathogenesis since IL-17 is able to disrupt the human BBB, allowing

efficient migration of Th17 cells in the CNS where they damage neurons [37-39]. The peripheral activation and subsequent migration of both autoreactive Th1 and Th17 cells into the CNS is believed to be an essential step in MS pathogenesis [40]. Next to $CD4^+$ T cells, $CD8^+$ (cytotoxic) T cells are involved in MS and notably in MS lesions they are even more prominent than $CD4^+$ T cells [41]. Furthermore, MBP-reactive $CD8^+$ T cells were found to be more abundant in MS patients compared with healthy individuals, and these cells are able to attract $CD4^+$ myelin-specific T cells via secreted chemokines [42, 43]. Further damage to the CNS by $CD8^+$ T cells occurs via secretion of cytotoxins (e.g. perforin, granzymes) and cytokines, including IL-17 [39].

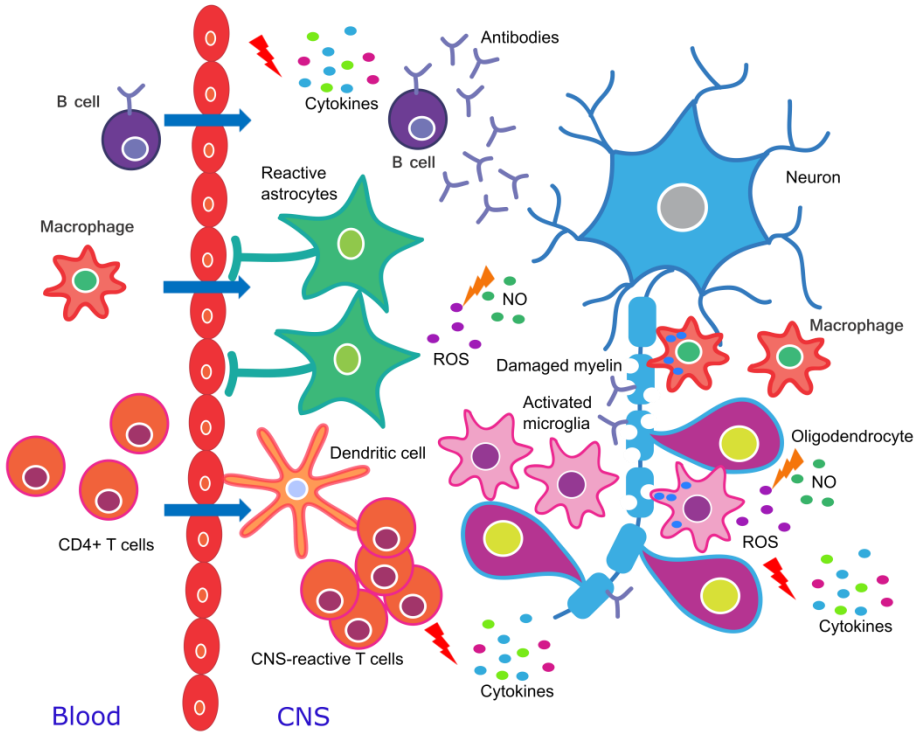

Figure 1.1: A simplified representation of the pathogenesis of MS. T cells are activated in the periphery. In the CNS, T cells become reactivated when antigens are presented by dendritic and microglial cells. Chemokines attract other T cells, B cells and macrophages. The activation of microglia and astrocytes and the secretion of pro-inflammatory cytokines, other inflammatory mediators such as nitric oxide (NO) and

reactive oxygen species (ROS) and antibodies mediate damage to the CNS and myelinated axons. The damaged myelin is phagocytosed by macrophages and activated microglia.

Regulatory T cells (Tregs), in both $CD4^+$ and $CD8^+$ populations, are another T cell type involved in the pathogenesis of MS. Tregs are able to modulate the ongoing immune response by inhibiting proliferation or inducing apoptosis of effector Th cells. Although the Treg frequency is similar between MS patients and healthy controls, MS patients have reduced Treg functionality [44, 45]. In addition to T cells, B cells and antibodies are also key players in the pathogenesis of MS. One of the hallmarks of MS is the identification of OCBs in CSF samples indicating intrathecal IgG production and thus the presence of antibody-secreting cells in the CNS [46, 47]. Furthermore, clinical trials using the B cell depleting antibodies rituximab and ocrelizumab yielded encouraging results for MS patients and indicated that B cells are crucial in MS pathogenesis [48-50]. See subsection 1.2 for more details regarding the different roles of B cells in MS and potential targets of the autoantibodies.

Besides the adaptive immune system, the innate immune system – not limited to – myeloid cells such as macrophages, mast cells, microglia and dendritic cells (DCs), plays a role in MS pathogenesis. In MS patients, the perivascular cuffs consist predominantly of infiltrating monocytes which later differentiate into macrophages after extravasation [51, 52]. Furthermore, activated microglia, which are the resident macrophages of the CNS, can already be found very early in the disease. These "preactive lesions" in normal-appearing white matter (NAWM) are believed to be the earliest stage of MS lesion development [53, 54]. Macrophages/microglia in MS lesions are filled with myelin debris, resulting in the "foamy" appearance of these cells [55]. Macrophages/microglia can have a proinflammatory phenotype due to the proinflammatory cytokines in the MS lesion environment. Furthermore, whilst these cells can mediate myelin destruction and cell damage (e.g. by production of pro-inflammatory cytokines and reactive oxygen species (ROS) and nitric oxide (NO)) and engulf intact myelin (pathogenic), they can also be protective by scavenging myelin debris and inducing remyelination. Hence, macrophages/microglia consisting of a very heterogeneous cell population, are thought to play a dual role in MS [56].

Dendritic cells are professional antigen presenting cells (APCs) that play a critical dual role in MS pathogenesis as well. Two main subsets are recognized; myeloid

DCs (also called conventional DCs) and plasmacytoid DCs [57]. DCs are crucial in determining the fate of $CD4^+$ T cells which can either differentiate into effector T cells (e.g. Th1, Th17), or $CD8^+$ T cells or Tregs, thus affecting the adaptive immune response [58-61]. In MS patients, DCs are found in MS lesions and MS CSF where they contribute to local activation and expansion of T cells and epitope spreading [62-65]. DCs can be described as 'gatekeepers' that control T cell entry to the CNS, presenting self-antigen in situ to effector T cells but also inducing the Treg cell activity that reduces inflammation [66].

Another cell type involved in MS pathogenesis – which has gained much interest in recent years – is the astrocyte. Astrocytes are the most abundant cell type in the CNS and play a broader role in MS than previously thought and not only cause astrogliosis. Besides providing support for neurons, they are involved in a variety of functions including blood flow regulation, ion and water homeostasis, regulation of BBB function, myelination, neurotransmission and higher cognitive functions [67, 68]. Moreover, astrocytes are CNS-resident innate immune cells, closely associated with the BBB, and modulate CNS inflammation by either promoting or inhibiting inflammation via secretion of cytokines and chemokines [69, 70]. This way, astrocytes are actively involved in T cell activation and secrete detrimental factors like NO and ROS. On the other hand, depending on many different factors, astrocytes also have beneficial roles such as limiting T cell proliferation and promoting repair in MS lesions [71, 72].

1.1.4 Lesion pathology

As mentioned previously, the pathological hallmark of MS is focal demyelination of regions called MS plaques or lesions, with variable degrees of inflammation, demyelination, gliosis, and axonal injury. MS lesions can be subdivided as active lesions, characterized by numerous infiltrated macrophages with myelin debris, lymphocytes and astrocytes. Chronic active MS lesions contain few inflammatory cells in the center, but are clearly delineated with macrophages at the lesion edge. Chronic inactive lesions are sharply demarcated and hypocellular with no evidence of active demyelination [73].

Lucchinetti et al. described four distinct demyelination patterns in MS lesions in biopsy and autopsy brain material from MS patients with short disease duration, often with acute onset [74]. Pattern I is characterized by active demyelination,

associated with T cell and macrophage infiltration. Pattern II lesions are characterized by a prominent deposition of immunoglobulins (Igs; mainly IgG) and complement C9neo antigen at sites of active myelin destruction. On the other hand, patterns III and IV are characterized by a pronounced loss of oligodendrocytes with additional loss of myelin-associated glycoprotein (MAG) in pattern III. Lucchinetti et al. observed a heterogeneity of lesion patterns between patients but not within patients, implicating heterogeneity of MS pathogenesis [74]. However, Breij and colleagues demonstrated in 2008 in an unselected collection of autopsy material from patients with established MS, that immunopathological appearance of active demyelinating lesions in established MS is homogeneous. They concluded that the initial heterogeneity of demyelinating lesions in early MS lesion formation may disappear over time as different pathways converge in one general mechanism of demyelination. The authors found consistent presence of complement, antibodies, and Fc- receptors in phagocytic macrophages, suggesting that antibody- and complement-mediated myelin phagocytosis is the dominant mechanism of demyelination in established MS [75].

In MS, whilst white matter lesions in the brain and spinal cord are usually investigated in much detail, grey matter lesions (e.g. in the cortex) have long been neglected. However, grey matter demyelination can be very extensive in MS, especially in the chronic phase of the disease [76, 77]. Histopathologically, grey matter lesions are different from white matter lesions with less lymphocyte infiltration, complement deposition, astrogliosis or disruption of the BBB [78, 79]. Although grey matter involvement is often associated with chronic MS, it is now evident that grey matter involvement can be detected even in the earliest stages of MS [80].

1.1.5 Animal models

Many of our current understanding of the mechanisms, role of the different immune cells and disease processes in MS comes from studies in animal models. The most widely used mouse model of MS is experimental autoimmune encephalomyelitis (EAE) in which immunization with CNS antigens (e.g myelin oligodendrocyte glycoprotein (MOG)) induces neurological and pathological signs of disease in mice [81, 82]. Depending on the research question, different mouse

strains or other animals and immunization strategies are used [81]. EAE induction can be achieved by active immunization of animals with myelin proteins or peptides, passive transfer of autoreactive T cells or transgenic technologies are used to study specific pathways [82, 83]. While B cells and antibodies play an important role in MS (see section 1.2), results from EAE models have yielded many conflicting results. Lyons et al. demonstrated that B cells are critical to induction of EAE by recombinant MOG but not by the encephalitogenic $MOG_{35–55}$ peptide (T cell-mediated EAE). However, injection of a monoclonal antibody against MOG exacerbates T cell-mediated EAE disease in mice and rats [84 85]. The recombinant MOG_{1-125} model is now used as an EAE model that is sensitive to B cell targeting [86]. EAE disease is characterized by an ascending hind limb paralysis that begins in the tail and spreads to the hind limbs and forelimbs. The disease is graded on a 0–5 scale. Since EAE mimics many of the clinical and immunological aspects of MS, it is extensively used as an animal model to study MS pathogenesis and to test new therapeutic strategies. Unfortunately, these EAE animal models are not perfect and only allow the study of certain aspects of the disease and obtained results in animal models cannot always be extrapolated to the human disease [81].

1.1.6 Therapies

There is no cure for MS but treatments are available for RRMS patients to modify the disease course (disease-modifying treatments, DMTs), treat relapses and manage symptoms to enhance patients' comfort and quality of life. There are no DMTs available for progressive MS patients with the exception of interferon beta-1b (Betaseron) for SPMS and novel clinical trials with ocrelizumab and anti-LINGO-1 are promising. However, there is still a great need to find novel treatments that could halt further progression and help patients with SPMS and PPMS [50, 87, 88].

Currently, 13 different treatments with 10 different active components are licensed in the European Union and the United States for the treatment of MS. These treatments are approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). The currently available DMTs are generally assigned as first-line or second-line treatment but no universal guidelines exist that help to decide when and how exactly a transition from first-

line to second-line therapy should be initiated. Further, the concept of first and second-line therapies is constantly evolving and an additional group, the third-line therapies is added for completeness [89]. Table 1.2 provides an overview of the first-, second- and third-line treatment options in RRMS with a short description of primary mode of action, although for most compounds this is not exactly known [89-91].

Table 1.2: Treatment options in RRMS

Name (*Brand*)	Treatment type	Target	Mode of action
First-line treatments			
Interferon-β 1a (*Avonex, Rebif)* Interferon-β 1b *(Betaferon, Betaseron)*	Injectable	Interferon-β receptor	Increases anti-inflammatory cytokines, decreases pro-inflammatory cytokines and reduces cell trafficking across BBB
Glatiramer acetate (*Copaxone*)	Injectable	MHC	Induces tolerogenic T cell immune responses and $CD4^+$ and $CD8^+$ regulatory T cells due to mimicry of MBP.
Dimethylfumarate (*Tecfidera*)	Oral	Nrf2	Interferes in the citric acid cycle and activates the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, resulting in immune-modulatory and neuroprotective effects.
Teriflunomide (*Aubagio*)	Oral	Dihydro-orotate dehydro-genase	Inhibits de novo pyrimidine synthesis by blocking the enzyme dihydroorotate Dehydrogenase resulting in inhibition of the function of T- and B-lymphocytes.
Second-line treatments			
Fingolimod (*Gilenya*)	Oral	Sphingosine-1-phosphate receptor (S1PR)	Modulates the migration of lymphocytes from secondary lymphoid organs.
Natalizumab (*Tysabri*)	IV infusion	VLA-4 (α4-integrin)	Reduces inflammatory cell migration across the BBB.
Third-line treatments			
Mitoxantrone (*Ralenova*)	IV infusion	Type II topoiso-merase	Anti-cancer treatment that suppresses the activity of T- and B-lymphocytes and macrophages.
Alemtuzumab (*Lemtrada*)	IV infusion	CD52	Induces cytolysis of CD52-positive lymphocytes.
Azathioprine (*Imuran*)	Oral	Rac1	Immunosuppressant

MBP: myelin basic protein, IV: intravenous

Besides these treatment options, several clinical trials with other compounds have been performed. For example, rituximab – a monoclonal anti-CD20 antibody – causes B cell depletion. In a preliminary randomized trial of 104 patients with RRMS, treatment with intravenous rituximab was associated with a significant reduction in both total and new gadolinium-enhancing lesions on brain MRI at 24 weeks when compared with placebo [49].

1.2 The humoral immune response in MS and novel autoantibody targets

1.2.1 B cells in MS

Next to T cells, B cells play a crucial role in the pathogenesis of MS and are mostly known for their production of autoantibodies. Since Rituximab treatment in MS patients did not affect plasma cells and autoantibody production, this demonstrated that antibody-independent B cell functions are important in MS pathogenesis [49]. B cells develop in the bone marrow and upon antigenic recognition, B cells undergo affinity maturation in germinal centers. Next, they differentiate into memory B cells or antibody-producing plasma cells. Memory B cells are important in MS pathogenesis and can perform various functions. First of all, memory B cells can serve as potent antigen presenting cells (APC) to activate T cells in MS and EAE [92, 93]. Furthermore, the expression of co-stimulatory and inhibitory molecules on the plasma membrane of B cells, also allows them to regulate T cell activation. Another way to stimulate and regulate different T cell subsets, including Tregs and inflammatory Th1 and Th17 cells, is via the production of cytokines by B cells [92, 94, 95]. Notably, B cells from MS patients have been shown to produce less of the anti-inflammatory cytokine IL-10 [96]. B cells can also activate T cells by non-antigen specific bystander activation [95]. Finally, memory B cells and other B cell subsets can form meningeal ectopic B cell follicle-like structures with germinal center characteristics suggesting local antigen-driven responses in the CNS and allowing a continuous humoral autoimmune reaction in MS [97, 98].

1.2.2 Why are antibodies good biomarkers?

Autoantibodies play an important role in MS pathogenesis, as indicated by the diagnostic use of OCBs in the CSF of most MS patients [99-101]. During the last decade, much effort has been made to characterize the antibody response present in MS patients in order to find early predictors for diagnosis and disease progression [47, 102]. Furthermore, finding specific biomarkers (Fig. 1.2), namely characteristics that are objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses

to a therapeutic intervention, could help to stage specific MS patients or to predict therapy response [103, 104].

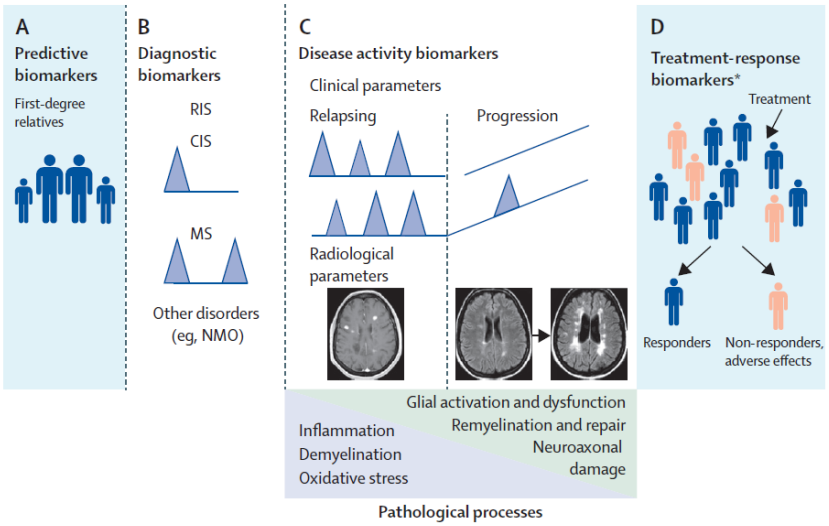

Figure 1.2: Types of biomarkers in MS. (A) Predictive biomarkers comprise biomarkers identified in first-degree relatives of patients with MS. (B) Diagnostic biomarkers comprise those measured in patients with RIS, CIS, MS or other conditions such as NMO. (C) Disease activity biomarkers comprise biomarkers measured in patients with RRMS in whom disease activity can be measured by clinical (presence of relapses and accumulation of disability) or radiological (eg, number of gadolinium-enhancing lesions; see MRI on left) parameters. This group also includes biomarkers measured in patients with progressive disease courses. In these patients, disease activity can be measured by the rate of disability progression or development of brain atrophy (see MRIs on right). In patients with MS, these biomarkers will be associated with the different pathophysiological processes that have been described. (D) Treatment-response biomarkers comprise biomarkers measured in patients who are receiving MS therapies. They can be classified into responders and non-responders according to their clinical or radiological responses to treatments. This group also includes biomarkers that identify patients who develop adverse drug reactions. RIS: radiologically isolated syndrome; CIS: clinically isolated syndrome; NMO: neuromyelitis optica. Reprinted with permission from Comabella et al. Lancet Neurol 2014; 13: 113–26.

Autoantibodies are potential biomarkers in MS since they can reflect the presence, nature and intensity of an immune response. Autoantibodies can thus function as markers of disease activity and severity (if titers correlate with

disease activity or progression), disease classification, and as predictive markers to identify 'risk' patients and responders to a certain treatment [101, 105]. Further, autoantibodies can be found in peripheral blood, allowing easier access to the biomarkers, compared to the lumbar puncture necessary for obtaining CSF samples [106].

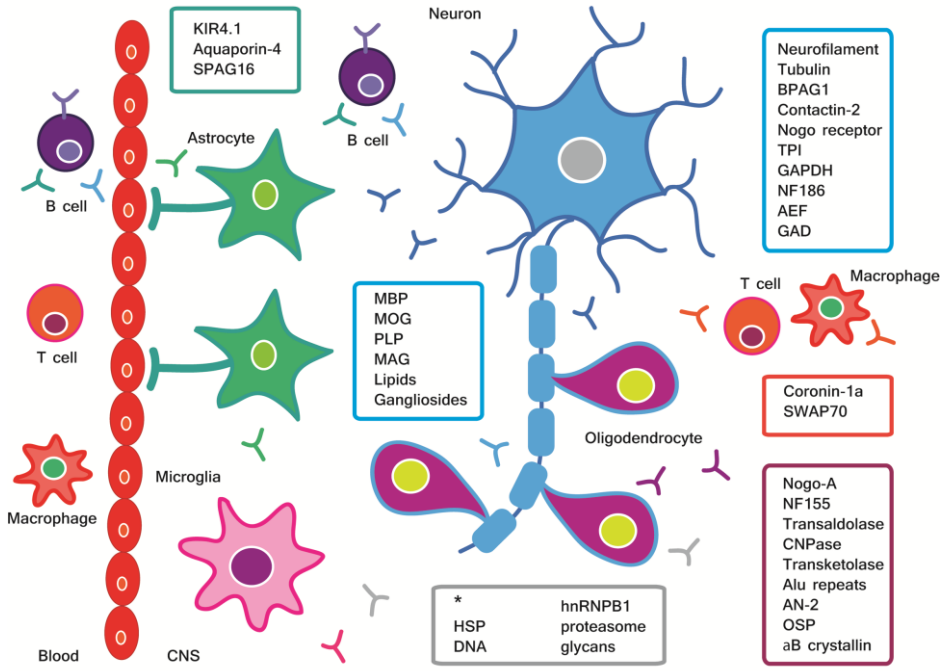

Figure 1.3: Autoantibody targets in MS brain. Autoreactive antibodies in MS target different antigens in myelin, neurons, oligodendrocytes, astrocytes and immune cells. Some ubiquitous antibody targets (*), present in virtually all cells, have been described as well. The color of the text boxes with the identified antigens corresponds with the color of the target cells.

The considerable variation in different autoantibody targets in MS underlines the heterogeneity and complexity of the disease. Furthermore, diagnostic screening for MS should include a panel of antibody specificities, possibly targeting different cell types, in order to increase the specificity and sensitivity of the test. Reprinted with permission from Fraussen et al. Autoimmun Rev. 2014 Nov;13(11):1126-37.

1.2.3 Autoantibody targets in MS

Myelin antigens were longtime considered as the primary targets of the humoral autoimmune response in MS. Recent studies have started to challenge this

concept, as not only myelin but nearly all CNS cells and even immune cells appear to be subject to autoantibody responses. Consequently, humoral autoimmunity in MS is much more variable and widespread throughout the brain than first thought. To illustrate the impact of this response on the MS CNS, an overview is provided on the different CNS cells that are targeted by autoantibodies (Fig. 1.3). Table 1.3 provides a detailed overview of the studies reporting target antigens in MS, including methodology.

Table 1.3: Autoantibody targets in MS

Target	Technique	Serum/CSF	Source
Myelin antigens			
MBP	RIA or ELISA	serum; CSF	[107-110]
MOG	Cell-based FACS, Tetramer or RIA	serum	[111-115]
MOG_{15-40}	ELISA	serum	[110]
MOG_{1-60}	ELISA	serum	[116]
PLP	RIA or Cell-based FACS	serum; CSF	[107, 117]
MAG	RIA	CSF	[118-120]
Lipids, sulfatide, GalC, Phospholopid	Lipid array, OCB blot, ELISA or RIA	serum; CSF	[121-126]
Neuronal & axonal antigens			
NFL	ELISA	serum; CSF	[127-130]
Tubulin	ELISA	serum; CSF	[131, 132]
Gangliosides	Liposome lysis, ELISA, or RIA	serum; CSF	[124, 133-139]
BPAG1	IB	CSF	[140]
Contactin-2	ELISA or Cell-based FACS	serum; CSF	[141, 142]
Nogo receptor	WB	serum	[143]
TPI; GAPDH	ELISA	serum; CSF	[144]
Neurofascin	ELISA	serum	[145]

AEF	ELISA	serum; CSF	[146]
GAD	RIA	serum	[147]
Oligodendrocyte antigens			
CNPase	IB	serum; CSF	[148, 149]
Transaldolase	WB	serum; CSF	[150, 151]
Transketolase	IB	serum; CSF	[149]
Nogo-A	WB or ELISA	serum	[143, 152]
Alu repeats	ELISA	serum	[153]
AN-2 (NG-2)	WB	CSF; serum	[154]
OSP	ELISA	CSF	[155]
α-crystallin	IB or ELISA	serum	[156]
αB-crystallin	WB or ELISA	serum	[157]
Astrocyte antigens			
Aquaporin-4	IIF	serum	[158]
KIR4.1	ELISA	serum; CSF	[159]
SPAG16	ELISA	CSF	[160]
Lymphocyte antigens			
Coronin-1a	ELISA	Serum; CSF	[161, 162]
SWAP70	ELISA	serum	[163]
Ubiquitous antigens			
HSP90β	WB	CSF	[164, 165]
HSP60, HSP70	MA	serum	[166]
HSP70	ELISA	CSF	[167, 168]
DNA	ELISA or IF	CSF/MS brain; serum	[126, 169]
hnRNP B1	WB	CSF	[170]
Proteasome	WB or ELISA	serum; CSF	[171-173]
Glycans	glycan array or ELISA	serum	[174-178]

Abbreviations. MBP: myelin basic protein; MOG: myelin oligodendrocyte glycoprotein; PLP: proteolipid protein; MAG: myelin associated glycoprotein; GalC: galactocerebroside; NFL: neurofilament light chain; BPAG1: bullous pemphigoid antigen 1; TPI: triosephosphate isomerase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; AEF: axolemma-enriched fractions; GAD: glutamate decarboxylase; CNPase: 2′,3′-cyclic nucleotide 3′phosphodiesterase; OSP:

oligodendrocyte-specific protein; SWAP70: switch-associated protein 70; HSP: heat shock protein; CSF: cerebrospinal fluid; ELISA: enzyme-linked immunosorbent assay; FACS: flow cytometry; IB: immunoblot; IF: immunofluorescence; IIF: indirect immunofluorescence; MA: microarray; RIA: radioimmunoassay; WB: Western blot. Reprinted with permission from Fraussen et al. Autoimmun Rev. 2014 Nov;13(11):1126-37.

1.3 Sperm-associated antigen 16 (SPAG16)

1.3.1 SPAG16 as a potential autoantigen in MS

In 2008, using a high-throughput cDNA phage display-based approach, our research group led by Somers et al., identified eight potential autoantibody targets in the CSF of MS patients. One of the identified autoantibody targets was the C-terminus 121 amino acid (aa) sequence of sperm-associated antigen 16 (SPAG16) isoform 2, which is also present in SPAG16 isoform 1 (Fig. 1.4) and had never been linked to MS. Initial phage ELISA screening of CSF samples was performed; 14/73 of MS patients were positive for anti-SPAG16 antibodies (19%), whilst only 7/94 of control samples were positive (7%), indicating that SPAG16 could be a potential autoantigen in MS [160].

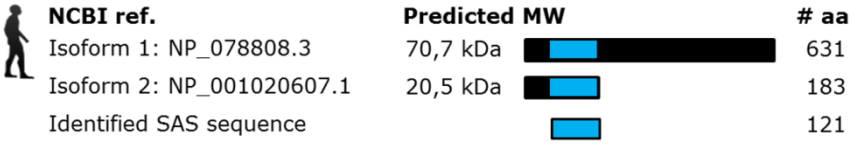

Fig. 1.4: SPAG16 isoforms and location of 121 amino acid (aa) sequence. The 121 aa sequence was identified by serological antigen selection (SAS) [160] and can be found in SPAG16 isoform 1 and 2. MW: molecular weight, NCBI: National Center for Biotechnology Information

1.3.2 SPAG16: an axonemal protein in cilia

SPAG16 is the human orthologue of Chlamydomonas reinhardtii (single-cell green algae) Pf20 [179]. SPAG16 has been mostly studied in the context of fertility, due to its expression in sperm cells. It is interesting to note that 8 of the 15 SPAG genes, namely SPAG2, SPAG4, SPAG5, SPAG6, SPAG8, SPAG15, SPAG16, and SPAG17 have been shown to be important for the function of the centrosome and/or cilia. Several SPAG16 isoforms exist but isoform 1 (SPAG16-1; 71 kDa) and isoform 2 (SPAG16-2; 20.5-kDa) have been experimentally validated. SPAG16-1 contains 7 WD-repeat domains which are known to mediate protein-protein interactions. In sperm cells and motile cilia (e.g. respiratory and oviduct epithelium, brain ventricle ependymal cells) SPAG16-1 is part of the axoneme and plays a role in sperm motility, spermatogenesis and ciliary motility [179, 180]. High SPAG16-1 expression has also been demonstrated in cancers such

colonic and endometrial adenocarcinomas, breast cancer and skin cancer. There is >80% sequence homology between human SPAG16-1 and murine SPAG16L. The function of SPAG16-2 is unknown [179-182]. In sperm cells, SPAG16-1 has been shown to interact with SPAG6 and SPAG17 at the central apparatus of the axoneme [183]. A double knockout mouse model of SPAG6 and SPAG16L showed early mortality in the litters owing to severe phenotypes of hydrocephalus and pneumonia, indicating their role in the proper functioning of mucus and fluid-propelling motile cilia, but not polycystic kidneys or left-right axis defects characteristic to non-motile and nodal ciliopathies [184]. A single SPAG16L knockout mouse model resulted in a milder phenotype without hydrocephalus but was characterized by infertility associated with impaired sperm motility [185]. Besides SPAG6, several other binding partners of SPAG16 have been described (in sperm cells) including meiosis expressed gene 1 (MEIG1) and testis-specific serine kinase 2 (TSSK2)[186]. Until now, there is no evidence of a connection between SPAG16 and the CNS or MS.

1.4 Aims of the study

Our current knowledge of the underlying pathogenesis of MS and the exact antigenic targets – recognized by both pathogenic T cells and autoantibodies – is still incomplete. Consequently, the development of diagnostic tests and effective therapies for MS can still be improved and the research for new candidate autoantigens in MS is warranted. In a previous study aimed at the discovery of novel antigenic targets in MS, a new antibody target was identified by our research group: SPAG16. Our knowledge about the function of SPAG16 and its role in the immunopathology of MS is very limited. Therefore, the goal of this project is to gain a better insight into the relevance of this novel autoantigenic candidate in the disease process of MS and to investigate the biological properties of SPAG16.

AIM 1: INVESTIGATE THE AUTOANTIBODY RESPONSE AGAINST SPAG16 AND ITS BIOMARKER POTENTIAL

The role of SPAG16 has only been studied in detail in the context of male fertility and not much is known about the anti-SPAG16 antibody response in MS and the role of SPAG16 in the CNS. Therefore, we aim to analyze SPAG16 as a novel target of the humoral autoimmune response in MS and investigate its diagnostic value (**chapter 2**). To this end, SPAG16 will be analyzed as a target of OCBs in the CSF. Furthermore, the diagnostic biomarker potential of plasma anti-SPAG16 autoantibodies will be investigated by screening a large cohort of MS patients and control groups. To investigate the pathologic relevance of anti-SPAG16 antibodies, *in vivo* experiments will be performed.

Analyzing SPAG16 as a novel autoantibody target in MS patients further expands our knowledge about the pathologic relevance of SPAG16 and new potential disease mechanisms in MS.

Next, we will investigate whether anti-SPAG16 antibody levels differ between MS subtypes (RR, SP, PP) and if they are associated with clinical characteristics (prognostic value). In **chapter 3**, we aim to compare the different MS subtypes in a large MS patient cohort and investigate whether anti-SPAG16 antibody levels differ to unravel whether they are biomarkers for a certain MS disease course. Furthermore, we will examine whether anti-SPAG16 antibody positivity

(serostatus) is associated with different disease characteristics in MS. Investigating the anti-SPAG16 response in the different MS subtypes will provide further understanding of the immune response against SPAG16 and provide information about the prognostic value of these autoantibodies.

Finally, we will investigate the frequency and prognostic implications of anti-SPAG16 antibodies in patients with optic neuritis (**chapter 4**). Since optic neuritis is often associated with MS and SPAG16 is a novel autoantibody target in MS, we will assess whether anti-SPAG16 antibodies are found in the serum and CSF of optic neuritis patients. Furthermore, we will investigate whether anti-SPAG16 antibody seropositivity in optic neuritis patients can be used to predict the conversion to MS. Investigating the prognostic implications of anti-SPAG16 antibodies in optic neuritis patients will further expand our knowledge about the biomarker potential of these antibodies.

AIM 2: CHARACTERIZATION OF THE BIOLOGICAL ROLE OF SPAG16 IN THE BRAIN AND IN MS DISEASE

Since the role of SPAG16 – a protein mostly known for its function in sperm cell motility – is unknown in the CNS, SPAG16 function will be investigated in more detail. To investigate a potential role of the protein in MS lesion pathology, the expression of SPAG16 will be investigated in MS and control brain tissue (**chapter 2**).

In **chapter 5**, the subcellular localization of SPAG16 will be investigated and the specific SPAG16 isoform expression will be analyzed in brain tissue from MS patients and controls. Finally, SPAG16 expression in different conditions will be analyzed *in vitro* to explore mechanisms that alter SPAG16 expression. Results from this study will increase our current knowledge about SPAG16 – including isoform expression – in another context, namely in neuroinflammatory demyelinating diseases such as MS.

2

Sperm-associated antigen 16 is a novel target of the humoral autoimmune response in multiple sclerosis

Based on:

Sperm-associated antigen 16 is a novel target of the humoral autoimmune response in multiple sclerosis

de Bock L*, Somers K*, Fraussen J, Hendriks JJ, van Horssen J, Rouwette M, Hellings N, Villar LM, Alvarez-Cermeño JC, Espiño M, Hupperts R, Jongen P, Damoiseaux J, Verbeek MM, De Deyn PP, D'hooghe M, Van Wijmeersch B, Stinissen P, Somers V. *Both authors contributed equally to this work.

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ABSTRACT

Background: We have previously identified eight novel autoantibody targets in the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients, including sperm-associated antigen 16 (SPAG16).

Objective: In the current study, we further investigated the autoantibody response against SPAG16 – a protein with unknown function in the CNS – and its expression in MS pathology.

Results: Using isoelectric focusing, we detected SPAG16-specific oligoclonal bands in the CSF of 5 of 23 MS patients (22%). Analysis of the anti-SPAG16 antibody reactivity in the plasma of a total of 531 donors using ELISA demonstrated significantly elevated anti-SPAG16 antibody levels (P= 0.002) in 32 of 153 MS patients (21%) compared to all other control groups with 95% specificity for the disease. To investigate the pathologic relevance of anti-SPAG16 antibodies *in vivo*, anti-SPAG16 antibodies were injected in mice with experimental autoimmune encephalomyelitis (EAE), resulting in a significant disease exacerbation. Finally, we demonstrated a consistent upregulation of SPAG16 in MS brain and EAE spinal cord lesions, more specifically in reactive astrocytes.

Conclusion: We conclude that SPAG16 is a novel autoantibody target in a subgroup of MS patients and in combination with other diagnostic criteria, elevated levels of anti-SPAG16 antibodies could be used as a biomarker for diagnosis. Furthermore, the pathologic relevance of anti-SPAG16 antibodies was shown *in vivo*.

2.1 Introduction

Multiple sclerosis (MS) is a complex chronic inflammatory demyelinating disease of the CNS which causes disability over time in the majority of patients. The disease is characterized by myelin loss, axonal pathology and progressive neurodegeneration, although the exact cause is unknown [187]. Furthermore, MS patients present with a wide spectrum of symptoms and early diagnosis is often difficult. So there is an ongoing need for biomarkers which allow early diagnosis and further prognosis.

The presence of antibodies in MS patients' cerebrospinal fluid (CSF) is one of the inflammatory hallmarks of the disease but the exact targets of this antibody response are largely unknown [188]. Various MS-associated autoantibody targets have been reported including myelin oligodendrocyte glycoprotein (MOG), neurofilament, neurofascin and the potassium channel KIR4.1 [102, 112, 145, 159, 189-191], reflecting the characteristic heterogeneity of the disease.

Using a high-throughput cDNA phage display-based approach, we previously identified eight potential autoantibody targets in the CSF of MS patients and initial phage ELISA screening of CSF samples was performed [160]. One of the identified targets was sperm-associated antigen 16 (SPAG16) isoform 2, which had never been linked to MS. Two SPAG16 isoforms exist; isoform 1 (SPAG16-1; 71 kDa) and isoform 2 (SPAG16-2; 20.4-kDa). In sperm cells SPAG16-1 is part of the axoneme and plays a role in sperm motility. The function of SPAG16-2 is unknown [179, 180]. Most research regarding SPAG16 has focused on its role in male fertility but SPAG16 expression (either isoform) is not restricted to sperm cells. Recent data reveal its presence in many tissues, including other tissues with motile cilia such as the lungs and brain ventricles but also in hematopoietic cells in the bone marrow, fibroblast-like synoviocytes in rheumatoid arthritis and in certain cancers [179, 181, 182, 192].

In the current study, we further investigated SPAG16 – a protein with unknown function in the CNS – in MS pathology. We show that SPAG16 is a protein targeted by the humoral autoimmune response in CSF and plasma of a subgroup MS patients. Isoelectric focusing (IEF) was used to study SPAG16-specific OCB in CSF. To study SPAG16 autoantibody reactivity in plasma, the original phage ELISA was replaced by a recombinant protein ELISA to allow large-scale

screening with increased reproducibility. Besides a role as a possible diagnostic marker in addition to current diagnostic criteria for MS, we have demonstrated that anti-SPAG16 antibodies exacerbate the disease symptoms of mice with experimental autoimmune encephalomyelitis (EAE) – an animal model of MS – and potentially contribute to MS pathology. Furthermore, SPAG16 expression was upregulated in reactive astrocytes, both in MS and EAE lesions.

2.2 Materials and methods

2.2.1 Patient samples

Samples were collected after approval by the Medical Ethical Committee of Hasselt University (Belgium) or Hospital Ramón y Cajal (Spain, IEF experiments) and informed consent from study participants was obtained. Details of patient and control samples are provided in Table 2.1. MS patients were diagnosed according to the McDonald criteria [13]. Clinically isolated syndrome (CIS) patients were clinically followed for 2 years.

Table 2.1: Characteristics of the MS patients and controls used in this study

Diagnosis	N	Age[a]	Gender[b]	EDSS[c]	Disease duration[d]
Paired CSF and plasma samples					
MS	23	40.0±2.3	74%	2.7±0.3	6.4±1.3
Plasma samples					
MS	153	46.4±0.9	67%	3.8±0.2	11.7±0.8
RR-MS[e]	70	42.8±1.1	74%	2.7±0.2	10.6±1.4
PP-MS[f]	25	51.1±2.1	52%	4.1±0.4	8.1±1.3
SP-MS[g]	58	49.3±1.4	64%	4.9±0.3	15.2±1.2
CIS[h]	101	35.5±1.0	79%	1.6±0.1	0.4±0.1
Healthy controls	204	39.5±1.8	65%	N.A.	N.A.
NIND[i]	51	43.8±2.6	73%	N.A.	N.A.
OIND[j]	22	46.7±3.1	59%	N.A.	N.A.

a. Mean age in years ± SEM; b. % females; c. Mean EDSS (*Expanded Disability Status Scale)* score ± SEM; d. Mean disease duration in years ± SEM; e. RR-MS = relapsing-remitting MS; f. PP-MS = primary-progressive MS; g. SP-MS = secondary-progressive MS; h. CIS = clinically isolated syndrome; i. NIND = non-inflammatory neurological disease; j. OIND = other inflammatory neurological disease; N.A. not applicable

2.2.2 Isoelectric focusing (IEF)

Recombinant SPAG16-2 as a fusion protein with thioredoxin (THIO) or THIO alone (control) was expressed and purified using the pBAD/TOPOThioFusion kit

(Invitrogen, Merelbeke, Belgium). Protein purity was assessed by SDS-PAGE and Coomassie Brilliant Blue (Phastgel Blue-R, GE Healthcare, Diegem, Belgium). Two polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Barcelona, Spain) were incubated overnight (4°C; shaking) with either SPAG16 or THIO, 10 µg/ml in PBS. A third membrane was not coated. After incubation, the membranes were washed three times for 30 minutes in 0.2% non-fat milk in PBS (M-PBS) and blocked 3h at room temperature (RT) in 2% M-PBS. Before use, they were rinsed in PBS. To investigate whether oligoclonal IgG recognizes SPAG16, three sets of CSF samples of 23 MS patients were subjected to IEF to separate IgG, as previously described [193]. After IEF, proteins were transferred to the three PVDF membranes prepared previously by placing the membranes on the gel surface, covering them with 25 sheets of dry filter paper, and placing this under a 2-kg weight for 20 minutes. Binding of OCBs was investigated by immunoblotting with a phosphatase alkaline anti-human IgG antibody for 2h30 (Jackson ImmunoResearch, Suffolk, UK). OCB reactivity was then explored as previously described [122]. Patients were considered as negative when they lacked bands in the SPAG16 blot or showed the same pattern in the SPAG16 and THIO (control) membranes. Patients were considered as positive when they exhibited at least one band appearing only in the SPAG16 blot.

2.2.3 ELISA

96-well ELISA plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with 1 µg/ml purified recombinant protein in 0.1 M bicarbonate buffer (pH 9.6). Washing was done using 0.05% PBS-Tween20 (PBS-T). Wells were blocked with 2% M-PBS for 2h at 37°C and then incubated with 100 µl plasma samples (1:100 in M-PBS) for 2h at RT. Antibody binding was detected with HRP-conjugated anti-human IgG (1:2000 in M-PBS; Dako, Heverlee, Belgium) followed by colour development with TMB (3,3',5,5'-Tetramethylbenzidine; Sigma-Aldrich, Diegem, Belgium). The reaction was stopped with 2M H_2SO_4 and read at 450 nm. Background reactivity against a recombinant THIO protein was measured in parallel. The Coefficient of Variation (%CV) was on average ~5% and had to be < 20%. A serially diluted positive sample was included to test for inter-assay variability. The cut-off was determined by receiver operating characteristic (ROC)-analysis.

To analyze the IgG isotype, a representative number of positive MS plasma samples (n=22) were screened using an isotype specific anti-IgG1, -IgG2,-IgG3 (Invitrogen) or -IgG4 (AbD Serotec, Puchheim, Germany) secondary antibody.

2.2.4 Production and specificity of mouse anti-SPAG16 mAbs

mAbs were produced against recombinant SPAG16-2 using the hybridoma technology [194]. Antibodies were purified from culture supernatant by POROS A (Applied Biosystems, Ghent, Belgium) affinity-chromatography for IgG and Protein L (Thermo Fisher Scientific, Erembodegem, Belgium) chromatography for IgM. Antibody isotypes were determined with the mouse mAb isotyping kit (Hycult Biotechnology, Uden, the Netherlands). The specificity of the produced mAbs was confirmed by Western blot and competition protein ELISA with antibody-positive MS plasma samples.

2.2.5 Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded or frozen sections from MS (n=7) and non-demented control (n=5) brain tissue were used (Dutch Brain Bank). Paraffin-embedded sections were deparaffinised and rehydrated. Nonspecific antibody binding was blocked with Protein Block (Dako). Sections were incubated overnight at 4°C with primary antibody (Table 2.2). Bound antibodies were visualized with the Dako-Envision system or HRP-conjugated secondary antibodies followed by DAB colour development (3,3'-Diaminobenzidine; all Dako) and haematoxylin counterstaining (Klinipath, Olen, Belgium). An identically produced isotype control mAb was used as a negative control.

Human and murine frozen tissue sections (5 µm) were fixed in acetone for 10 minutes at RT and washed in PBS-T. After blocking for 30 minutes at RT with Protein Block, sections were incubated overnight at 4°C or 1h at RT with primary antibodies diluted in PBS/0.1% BSA (Table 2.2). Fluorescent secondary antibodies (Invitrogen) were added for 1h at RT. Cell nuclei were labelled with DAPI. Stained tissue sections were evaluated with an Eclipse 80i microscope (Nikon, Brussels, Belgium), using standard objectives and NIS-Elements Basic Research Software (Nikon).

Table 2.2: Primary antibodies used in this study for immunohistochemistry analysis

Primary Abs	Specificity	Concentration/dilution	Source
1F1 and 5F10	SPAG16	30µg/ml	In-house produced
Isotype control	hCG	30 µg/ml	In-house produced
IE7 isotype control	Rubella	30 µg/ml	In-house produced
MAB386	MBP	1:100	Millipore
CR3/43	HLA-DR,DP,DQ	1:100	Dako
EB09826	SPAG16	1:200	Everest Biotech
Clone G-A-5	GFAP	1:500	Sigma-Aldrich
Clone A60	NeuN	1:200	Millipore
CD3-12	CD3	1:100	Serotec
clone Cl:A3-1	F4/80	1:500	Serotec
ab55811	C5b-9	1:500	Abcam

hCG, human chorionic gonadotropin; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei

2.2.6 Western blot

Five µg of healthy mouse spinal cord lysate or 3,75 µg of human testis lysate (Abcam, Cambridge, UK) were separated by 12% SDS-PAGE and blotted onto a PVDF-membrane (Millipore, Overijse, Belgium). The membrane was blocked (1h in 5% MPBS-T) and incubated with anti-SPAG16 mAbs (30 µg/ml) in 2% MPBS-T overnight at 4°C. Bound antibodies were detected with rabbit anti-mouse IgG-HRP (1:1500, Dako) for 1h at RT, followed chemiluminescent detection (Thermo Fisher Scientific). A mouse isotype mAb (IgG1) directed against an irrelevant protein was used as a negative control.

2.2.7 EAE induction and passive antibody transfer

EAE was induced in female C57BL/6J mice (Harlan, Horst, the Netherlands) as previously described [195]. Experiments were approved by the ethical committee for animal experiments of Hasselt University. Animals were weighed and scored daily (blinded) for clinical signs of EAE using a standard 5-point EAE scale. At EAE onset (score 0.5-1), animals were injected i.p. with either 2 mg of the anti-SPAG16 antibody mix (3H5 (IgM), 1F1 (IgG1), 5F10 (IgG1), each 1/3 of the

total injected antibody amount) or isotype control antibody mix (2/3 anti-human chorionic gonadotropin IgG1 and 1/3 anti-treponema pallidum IgM). Mice were sacrificed at day 17 post-antibody transfer (~ day 30 after disease induction). Histological quantification was performed on sections obtained at 3 different spinal cord levels in each animal (Eclipse 80i microscope with NIS-Elements Basic Research Software (Nikon)).

2.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Software Inc.) and SPSS statistical software (version 20). A P-value <0.05 was considered statistically significant. Specific tests and significance levels are described in figure legends.

2.3 Results

2.3.1 SPAG16 is an autoantibody target in CSF and plasma of MS patients

SPAG16 was first identified as a CSF autoantibody target in a subgroup of MS patients using SAS and initial phage ELISA screening of CSF samples was performed. Anti-SPAG16 antibodies were directed against an epitope in the last 121 aa of SPAG16-2 [160]. Using BLAST analysis, this epitope was also found to be present in SPAG16-1 (Fig. 2.1A) and other putative isoforms. Indeed, mAbs directed against SPAG16-2 also bind SPAG16-1, as indicated by Western blot of a testis lysate (Fig. 2.1B).

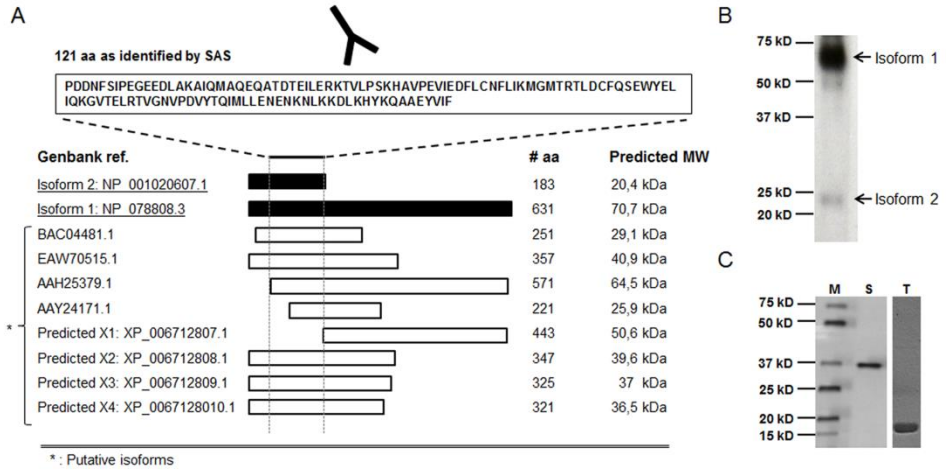

Fig. 2.1: Human SPAG16 isoforms, purity of the recombinant protein and specificity of in-house produced monoclonal anti-SPAG16 antibodies. (A) Different human SPAG16 isoforms in the NCBI database. Isoform 1 and 2 are experimentally validated (black); other putative (*) isoforms are depicted in white. The initially identified autoantibody reactive region of 121 aa using serological antigen selection (SAS) is marked to indicate that the autoreactive antibodies could bind multiple isoforms of the protein. (B) Western blot analysis of our in-house produced mAbs against SPAG16 showed binding to both human isoform 1 (~71 kDa) and 2 (~25 kDa) as confirmed in a testis lysate. (C) The purity of the produced recombinant proteins was verified by Coomassie Brilliant Blue staining. M: protein marker; S: recombinant SPAG16 (~37 kDa; with THIO); T: recombinant THIO (~16 kDa).

After confirming the purity of recombinant SPAG16 or the control protein THIO (Fig. 2.1C), IEF was performed to detect SPAG16-specific OCBs in the CSF of MS patients. We found that 5 out of the 23 MS patients tested, had specific OCBs binding recombinant SPAG16 (Fig. 2.2A, Table 2.3). For these, 4 out of 5 MS patients did not show anti-SPAG16 antibody reactivity in the plasma (measured by recombinant protein ELISA), indicating intrathecal anti-SPAG16 antibody production (Table 2.3). The IgG index was elevated (>0.7) in the MS patients with anti-SPAG16 OCBs. One patient showed OCBs combined with autoantibodies against SPAG16 in the plasma. Interestingly, for these 23 MS patients, we also found that 6 MS patients showed an exclusive anti-SPAG16 autoantibody response in the plasma and no SPAG16-specific OCBs in the CSF.

Next, this deviating humoral immune response was further investigated in a large-scale screening. The original labour-intensive phage ELISA was replaced by a recombinant in-house protein ELISA to screen for anti-SPAG16 immunoreactivity in plasma samples from MS patients and controls. Elevated plasma anti-SPAG16 antibodies were detected in 21% (32/153) of MS patients with a 95% specificity (11/204 healthy controls) for the disease (Fig. 2.2B). Elevated antibody levels against SPAG16 were detected in a significantly larger proportion of MS patients compared to all other groups; namely CIS patients (15/101; $P<0.01$), neurologic controls (non- and other inflammatory neurological diseases; NIND; 3/51; $P<0.01$, OIND; 3/22; $P<0.05$) and healthy controls (11/204; $P<0.001$). Anti-SPAG16 antibodies could be detected in CIS patients, indicating that these antibodies are already present in early disease stages in a subgroup of patients. There was however no correlation between the presence of anti-SPAG16 antibodies and conversion from CIS to MS. The relative frequency of anti-SPAG16 antibody positive MS patients varied between relapsing-remitting (RR)-MS (18/70; 26%), secondary-progressive (SP)-MS (12/58; 21%) and primary-progressive (PP)-MS (2/25; 8%). We observed no correlation between anti-SPAG16 antibody positivity and age, gender, clinical or CSF characteristics, although this study was neither designed nor powered to provide a test for correlation. No differences in anti-SPAG16 antibody levels were found for the majority of MS patients during relapse or remission (n=48; data not shown), indicating continuous anti-SPAG16 antibody production. Upon anti-SPAG16 antibody isotyping (n=22), we observed that most patients had

IgG1, either alone or in combination with IgG3 and to a lesser extent IgG2 or IgG4 (Fig. 2.2C). Since IgG1 and IgG3 are activators of the complement cascade, anti-SPAG16 antibodies could activate the complement system, indicating their pathologic potential.

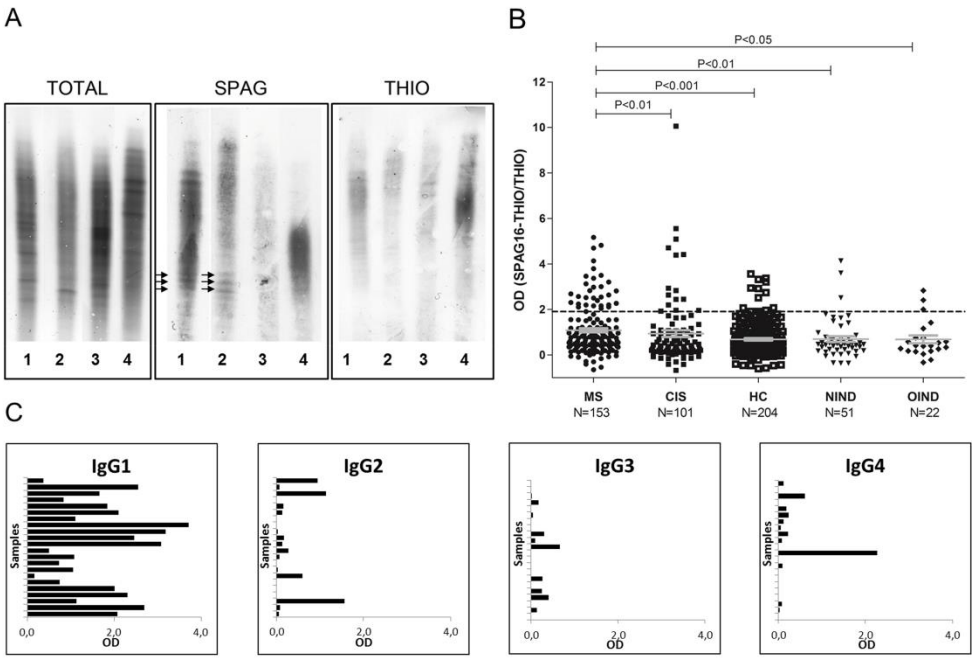

Fig. 2.2: SPAG16 as an autoantibody target in MS patients. (A) IEF was used to separate the CSF IgG of 23 MS patients (4 representative samples are shown; in total 5 MS patients showed reactivity). After separation, affinity-mediated immunoblotting was performed using uncoated (TOTAL; showing total IgG), SPAG16-coated (SPAG) or the control protein thioredoxin-coated (THIO) membranes. SPAG16-specific oligoclonal bands in the CSF are marked with arrows in patient 1 and 2; three additional patients also showed SPAG16-specific oligoclonal bands (not shown; for patient details see Table III). Patient 3 and 4 did not display these specific bands. (B) Anti-SPAG16 antibody levels in plasma were analyzed with recombinant protein ELISA. Background plasma reactivity was measured against recombinant THIO. The cut-off was determined by ROC-analysis (dashed line; P=0.00018). Anti-SPAG16 antibody levels were compared with the Kruskal-Wallis test of One-Way ANOVA with an overall P<0.01. P-values of post-tests between 2 groups (Dunn's multiple comparison test) are depicted in the graph. (C) Analysis of the isotype of the anti-SPAG16 antibodies in 22 seropositive MS patients.

Table 2.3: Characteristics of the MS patients used for anti-SPAG16 OCB testing

Patient	Age	Gender[a]	Type MS	EDSS[b]	Disease duration[c]	IgG index[d]	SPAG16 CSF OCB[e]	Serostatus SPAG16[f]
1	58	M	PP-MS	6.0	N.A.	>0.7	+	-
2	44	F	SP-MS	6.5	16	>0.7	+	+
3	52	F	RR-MS	3.5	9	>0.7	-	+
4	53	M	SP-MS	4.0	6	>0.7	-	+
5	26	F	RR-MS	1.5	0,3	2.89	+	-
6	39	F	RR-MS	1.5	9.0	1.75	-	+
7	34	M	RR-MS	3.5	0.3	1.22	-	-
8	41	F	RR-MS	2.0	23.7	2.88	+	-
9	40	F	RR-MS	2.0	0.3	1.31	-	-
10	43	F	RR-MS	2.0	4.6	1.44	-	-
11	32	F	RR-MS	2.0	0.1	1.23	-	+
12	30	F	RR-MS	0.0	3.7	1.32	+	-
13	27	M	RR-MS	2.0	0.1	1.18	-	-
14	34	F	RR-MS	3.0	10.0	0.78	-	-
15	20	F	RR-MS	1.0	0.2	0.82	-	+
16	28	M	RR-MS	2.0	1.6	0.58	-	+
17	57	F	RR-MS	2.5	9.4	0.62	-	-
18	56	F	RR-MS	3.0	6.4	0.74	-	-
19	29	F	RR-MS	1.5	5.6	0.98	-	-
20	52	M	RR-MS	6.0	3.1	0.93	-	-
21	46	F	RR-MS	1.0	15.5	1.90	-	-
22	42	F	RR-MS	3.0	7.5	2.30	-	-
23	38	F	RR-MS	2.5	8.0	1.26	-	-

a. M = male; F = female; b. EDSS = Expanded Disability Status Scale; c. Disease duration in years; d. IgG index = [CSF IgG/CSF Albumin] / [Serum IgG/Serum Albumin], considered elevated when >0.7; e. Specific OCBs detected against SPAG16 using IEF analysis; f. Autoantibody status against SPAG16 in plasma, measured by recombinant protein ELISA; N.A. = not available

2.3.2 Anti-SPAG16 antibodies exacerbate MOG-peptide induced EAE in mice

To study the pathologic relevance of circulating anti-SPAG16 antibodies, we investigated the effect of passive antibody transfer of anti-SPAG16 mAbs in the chronic MOG_{35-55} peptide-induced EAE model. First, using BLAST analysis we demonstrated that the 121 aa initially identified using SAS are present in murine SPAG16 isoform 1 and 3, but not in isoform 2 (Fig. 2.3A). Next, we confirmed that our in-house produced mouse mAbs against human SPAG16 (binding both human isoform 1 and 2; Fig. 2.1B) also bind murine SPAG16 in the spinal cord. A band of ~ 70 kDa and ~ 47 kDa was identified, corresponding to isoform 1 and 3 in mice, respectively (Fig. 2.3B). Furthermore, mouse anti-SPAG16 mAbs were shown to be representative for the immunoreactivity seen in MS patients, since there was competition between the mouse mAbs and anti-SPAG16 antibody-positive MS plasma (Fig. 2.3C).

Passive mAb transfer via i.p. injection was performed at disease onset (score 0.5-1) allowing for transport of the injected mAbs across a compromised blood-brain barrier. As shown in Fig. 2.3D, anti-SPAG16 mAbs significantly exacerbated EAE, indicated by a higher mean EAE score (overall disease course: repeated measures ANOVA, $P<0.01$) compared to the control group. This was also reflected in the decreased weight of the animals treated with anti-SPAG16 mAbs (Fig. 2.3E). The mean cumulative score ($P<0.01$) and mean number of follow-up days with a disease score of at least 2 ($P<0.01$) were significantly higher in the anti-SPAG16 antibody receiving animals (Table 2.4).

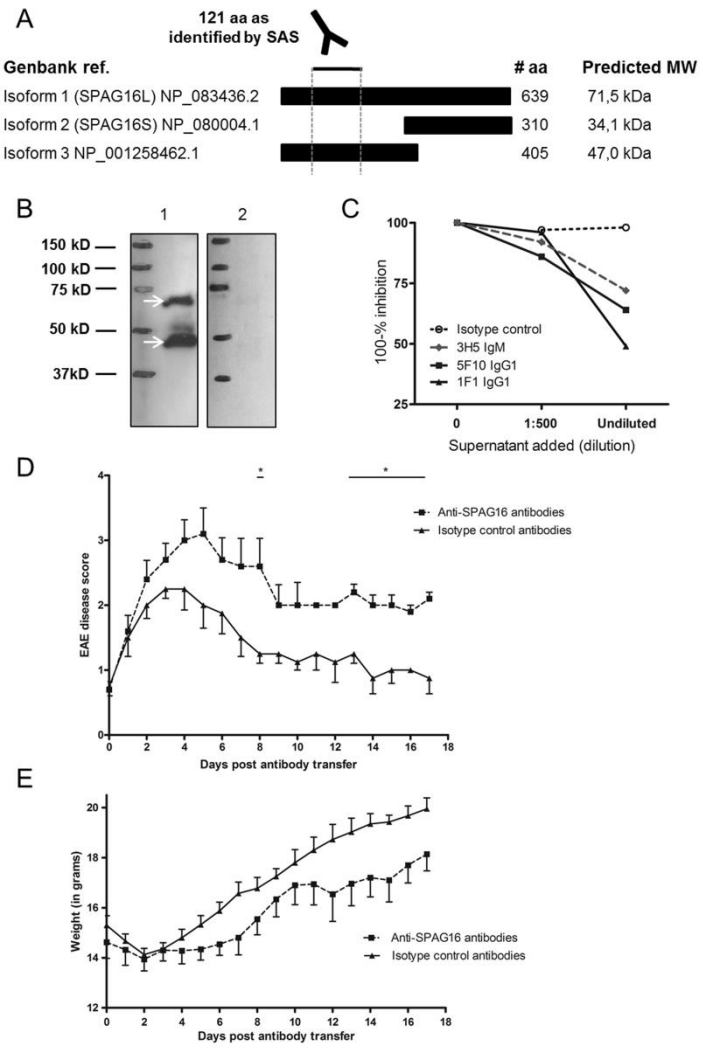

Fig. 2.3: SPAG16 isoforms in mice and anti-SPAG16 antibodies in EAE. (A) Validated murine SPAG16 isoforms in the NCBI database; isoform 1 or SPAG16L (long); isoform 2 or SPAG16S (short). The initially identified autoantibody reactive region of 121 aa is marked to indicate that the autoreactive antibodies could bind multiple SPAG16 isoforms due to sequence homology. (B) Lane 1: mAbs against human SPAG16 bind murine SPAG16 isoform 1 (~71 kDa) and isoform 3 (~ 47 kDa) in healthy mouse spinal cord (indicated by arrows). Lane 2: isotype control. (C) Competition ELISA confirms that the produced mAbs (5F10, 1F1, 3H5) are representative for the detected MS plasma anti-SPAG16 immunoreactivity. (D) EAE score (overall disease course: repeated measures ANOVA, $P<0.01$) and (E) daily weights of mice receiving anti-SPAG16 (--▪--, n=5) or isotype control (-▲-, n=4) mAbs. Representative data from 1 experiment are shown;

n=15 for anti-SPAG16 antibody-treated group and n=16 for isotype control treated group for 2 replicate studies giving equivalent results. Data are expressed as mean ± SEM. * P<0.05 according to Mann-Whitney U testing.

Table 2.4: Clinical features of EAE after passive transfer of anti-SPAG16 or isotype control antibodies

	Disease score[a]	**Maximum score[b]**	**Cumulative score[c]**	**Days with score ≥2[d]**
SPAG16	2.1**	4.0	39.6±2.8**	14.8±1.5**
Isotype	1.3	2.3	25.1±2.9	5.5±1.3

a. Median disease score over the period of follow-up (day 1-17 post-antibody transfer), repeated measures ANOVA; b. Median of the maximum scores; Mann-Whitney U testing; c. Mean of the cumulative scores of all animals in one group. Cumulative score per animal is the sum of the daily clinical scores during follow-up. Groups were compared by Student T-test; d. Mean number of follow-up days with disease score of at least two for all animals in one group. Groups were compared with Student T-test; * <0.05; **, p<0.01

To explore the mechanism by which anti-SPAG16 mAbs mediated EAE exacerbation, IHC staining of spinal cord tissue from mice that received anti-SPAG16 or isotype control mAbs was performed (17 days post-antibody transfer). IHC images were quantified for bound immunoglobulins (Fig. 2.4A-B), C5b-9 complement complex (Fig. 2.4C), inflammatory cell infiltration (Fig. 2.4D-E), demyelination (Fig. 2.4F), and astrogliosis (Fig. 2.4G). Taken together, we observed an enhanced immunoglobulin deposition (P= 0.02 for IgM; P=0.03 for IgG), increased activation of complement and formation of C5b-9 terminal complex (P=0.02) and increased infiltration of macrophages (P=0.03) within the spinal cords of animals injected with anti-SPAG16 mAbs. This indicates that these antibodies could bind their target in the mouse spinal cord, fix complement and mediate enhanced inflammatory cell infiltration. As no obvious effect on myelin basic protein (MBP) loss is seen, the exacerbating effect is most likely not mediated by increased myelin degradation.

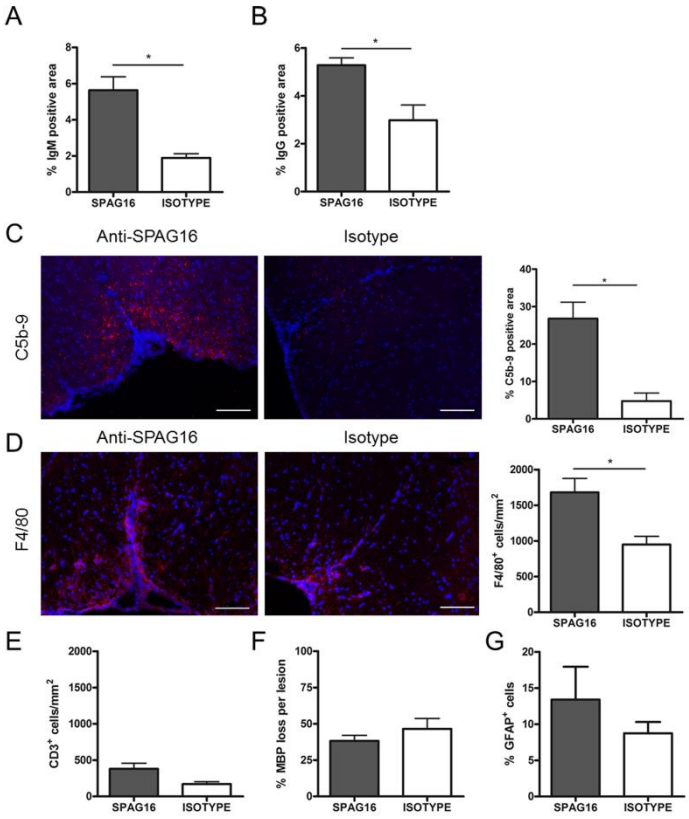

Fig. 2.4: Immunohistochemistry analysis of EAE mouse spinal cord sections. Immunoglobulin deposition in the spinal cord of animals receiving anti-SPAG16 or isotype control mAbs is shown as the percentage of the (A) IgM or (B) IgG positive area. (C) C5b-9 complement staining and (D) F4/80 staining in the spinal cord in mice treated with anti-SPAG16 mAbs or isotype control mAbs. Representative pictures are shown per group. Quantitative (fluorescent) IHC analysis was performed. (E) Numbers of $CD3^+$ T cells in both groups. (F) The extent of demyelination is given by the percentage of MBP loss. (G) The % of $GFAP^+$ astrocytes in the spinal cord is shown. Data were compared with Mann-Whitney test. *, $P<0.05$. Scale bars: (C-D) 100 µm.

2.3.3 SPAG16 is upregulated in astrocytes within MS brain and EAE spinal cord lesions

We performed IHC to determine SPAG16 expression in brain tissue and low expression levels of SPAG16 were detected in the white matter of control brains

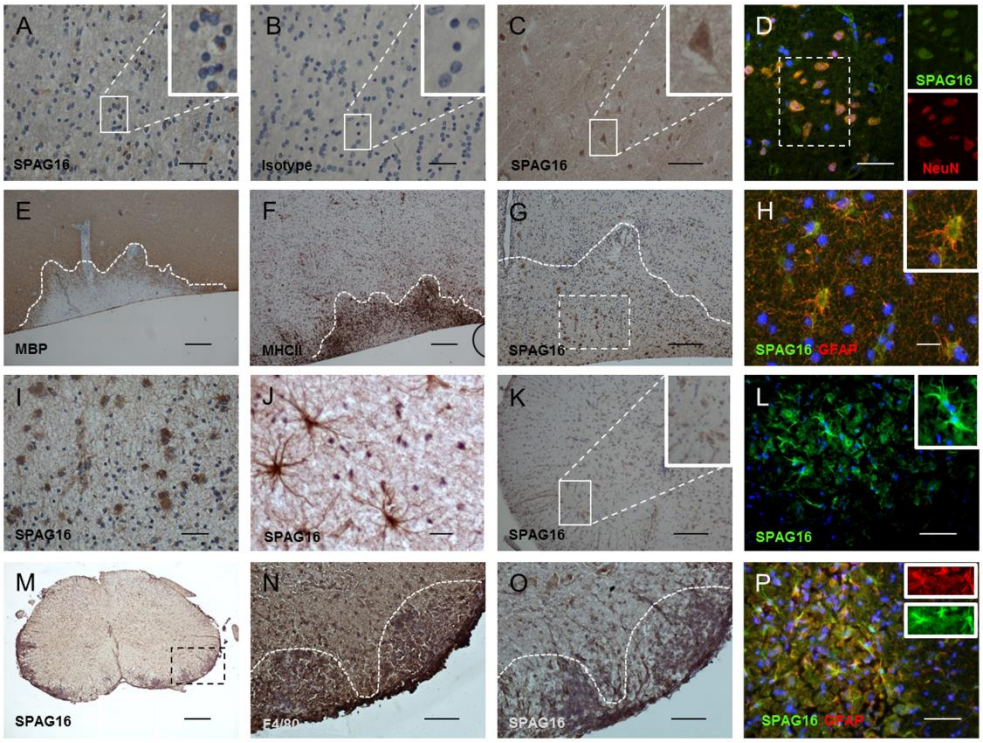

Fig. 2.5: SPAG16 expression in MS brain lesions and EAE spinal cord lesions. Representative IHC image of (A) SPAG16 and (B) isotype control staining in normal white matter (NWM) from control brain tissue (n=5). (C) SPAG16 staining in grey matter from human control brain tissue. Stained neuron is enlarged in top right panel. (D) Double-staining of SPAG16 (green) and neuronal nuclei (NeuN; red) in grey matter from control brain. Single colour images are shown at the right. (E) Delineation of an active MS lesion using MBP staining and (F) HLA-DR/DP/DQ (MHCII) staining to indicate inflammatory cells. (G) Representative SPAG16 staining in delineated MS lesion (brain material from 7 MS patients was analyzed). (H) Double-staining of SPAG16 (green) and an astrocyte marker glial fibrillary acidic protein (GFAP; red) in a MS lesion. (I) 400x magnification of the SPAG16 stained MS lesion in (G). (J) SPAG16 staining in astrocytes in MS lesion. (K) Representative SPAG16 staining in the spinal cord of a healthy mouse (n=4). (L) Immunofluorescence image of SPAG16 expression (green) in astrocytes in EAE spinal cord (n=5). (M) SPAG16 staining at 40x magnification in EAE spinal cord. (N) Macrophage (F4/80) staining indicates the EAE spinal cord infiltrations. (O) SPAG16 staining at 400x magnification in the delineated region in EAE spinal cord in (M). (P) Fluorescent double-staining for SPAG16 (green) and GFAP (red) in EAE spinal cord. Scale bars: (E-F, M) 200 µm; (G, K) 100 µm; (C-D, L, N-P) 50 µm; (A-B, H-I) 20 µm; (J) 10 µm.

(Fig. 2.5A) and in neurons in the grey matter (Fig. 2.5C-D). In MS brain tissue, lesion identification was based on IHC for the absence of myelin (Fig. 2.5E) and activated microglia/macrophages (Fig. 2.5F). In MS lesions, SPAG16 was upregulated (Fig. 2.5G, I) compared with normal appearing white matter tissue of MS patients and normal white and grey matter in controls (Fig. 2.5A, C). Most intense staining was demonstrated in astrocytes in the center of active lesions (Fig. 2.5I-J). At the lesion edge, SPAG16 expression was still detectable in astrocytes (Fig. 2.5G), but less intense. Double-staining for SPAG16 and glial fibrillary acidic protein (GFAP) (Fig. 2.5H) confirmed SPAG16 expression in astrocytes. In controls we found a few, weakly SPAG16-positive astrocytes, which is in accordance with SPAG16 staining in The Human Protein Atlas, where glial cells do not stain positive [181].

Consistent with human control brain tissue, a low level of SPAG16 expression was observed in neurons in healthy mouse spinal cord (Fig. 2.5K). In EAE tissue, spinal cord lesions were identified by staining for infiltrating macrophages (Fig. 2.5N). In accordance to MS lesions, an upregulation of SPAG16 was observed in astrocytes at the EAE spinal cord lesion site (Fig. 2.5L-M, O-P), indicating a similar role for the protein in MS and EAE.

2.4 Discussion

SPAG16 was first discovered as a candidate autoantibody target in MS patient CSF using the SAS technology [160]. Here, we show that SPAG16 is a protein with potential pathologic relevance targeted by the humoral autoimmune response in CSF and plasma of MS patients. Besides a role as a possible diagnostic marker in addition to current diagnostic criteria for MS, a potential pathologic role of anti-SPAG16 antibodies was demonstrated *in vivo*. Furthermore, SPAG16 expression was upregulated in reactive astrocytes, both in MS and EAE lesions.

Specific OCBs against SPAG16 were detected in a subgroup (~22%) of MS CSF, indicating a similar CSF anti-SPAG16 reactivity (~20%) as was found previously by our group using phage ELISA (10). OCBs are an important hallmark of MS CSF and are produced in the context of sustained antigenic stimulation in the intrathecal compartment [196]. Therefore, it is intriguing to find SPAG16-specific OCBs in MS CSF, even when a limited number of patients was tested and particularly since in spite of intensive research, the target antigen(s) recognized by individual OCBs in MS have remained elusive [197]. Furthermore, when paired CSF/plasma samples were compared, we also found that 6/23 MS patients had an exclusive anti-SPAG16 antibody response in the plasma. Though the origin of these anti-SPAG16 autoantibodies remains an important question for further study, Bankoti et al. have shown that in some MS patients B-cell clusters or subclusters corresponding to OCBs are observed exclusively in the peripheral blood. This finding indicates that the periphery represents a site of persistence and that activation of pathogenic B cells in MS can also occur in this compartment [198].

Besides testing for the presence of OCBs against SPAG16 in CSF, the diagnostic potential of anti-SPAG16 antibodies could be reinforced by investigating the anti-SPAG16 antibody response in plasma in a larger-scale screening. Elevated levels of anti-SPAG16 antibodies were present in the plasma of 21% of MS patients with 95% specificity. However, we observed a lower percentage of anti-SPAG16 positive PP-MS patients (8%) compared to RR-MS (26%) and SP-MS (21%), underlining the heterogeneity of the MS disease course in the different subgroups.

Identification of SPAG16 as a target of the B cell responses associated with MS raises two important questions. What mechanisms drive the anti-SPAG16 humoral immune response and how could antibodies against SPAG16 potentially contribute to the pathology of MS? Antibody reactivity towards other intracellular targets like SPAG16 (e.g. neurofilament, tubulin) is not uncommon in MS [131, 199]. There are several hypotheses on the generation of an antibody response towards intracellular proteins [200]. These include a) general dysregulation of the immune system in autoimmune diseases, b) cell death (e.g. neurons which express a low level of SPAG16) leading to exposure of intracellular antigens such as SPAG16 (epitope spreading) or c) molecular mimicry. Moreover, we demonstrated an upregulation of SPAG16 in MS and EAE lesions in reactive astrocytes, indicating a similar role of the protein in MS and EAE. Additionally, such an altered expression pattern could be a mechanism for breaking of immune tolerance as exemplified in systemic sclerosis and cancer, where an altered expression pattern of other sperm-associated antigens elicited an autoantibody response [182, 201].

As for the role of SPAG16, not much is known about the protein in other diseases or tissues, especially the brain and spinal cord, but it has become clear that the expression of SPAG16 isoforms is not restricted to sperm cells. Recent data reveal its presence in many tissues, including disease-relevant fibroblast-like synoviocytes in rheumatoid arthritis [192]. Additionally, the presence of multiple, not yet validated, SPAG16 isoforms increases the complexity. The observation that SPAG16 is upregulated in astrocytes within or near the MS lesion is in part reminiscent of the alpha B-crystallin data. Alpha B-crystallin is a stress-induced heat shock protein that is increased within or surrounding MS lesions [202]. Since an active MS lesion is an area of cellular stress and inflammation, we hypothesize that SPAG16 upregulation in astrocytes is due to local mediators such as pro-inflammatory factors. In these cells, SPAG16 could play a stress-related, structural or motility-associated role, since SPAG16-1 is a known motility protein in sperm cells and motile cilia.

Next, we were interested in how anti-SPAG16 antibodies could contribute to the pathology of MS. We determined the isotype of anti-SPAG16 antibodies in 22 MS patients and mostly found IgG1, which is known to activate the complement system. Furthermore, the pathologic potential of anti-SPAG16 antibodies was

demonstrated by their exacerbating effect on EAE disease symptoms in mice. Immunohistochemistry analysis indicated that this was not due to changes in demyelination but to an increase in immunoglobulin deposition, complement activation and macrophage infiltration at the EAE lesion site in mice that were injected with anti-SPAG16 antibodies.

The present study provides the basis for the involvement of SPAG16 and anti-SPAG16 antibodies in a subpopulation of MS patients. We show specific anti-SPAG16 antibody reactivity in MS which could potentially contribute to the pathology of the disease. Furthermore, we demonstrated the pathologic potential of anti-SPAG16 antibodies in EAE in mice and overexpression of the protein in MS and EAE lesions. Over the past years, many other autoantibody targets have been identified in MS such as MOG, neurofascin and recently KIR 4.1, but rarely these targets showed specific antibody reactivity in MS combined with pathologic relevance *in vivo* [112, 145, 189, 203]. Future studies unravelling the exact role of SPAG16 and anti-SPAG16 autoantibodies in the complex pathology of MS will contribute to a better understanding of SPAG16 autoimmunity in MS.

3

Anti-SPAG16 antibodies in primary progressive multiple sclerosis are associated with an elevated progression index

Based on:

Anti-SPAG16 antibodies in primary progressive multiple sclerosis are associated with an elevated progression index

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ABSTRACT

Background: Sperm-associated antigen 16 (SPAG16), a sperm protein which is upregulated in reactive astrocytes in multiple sclerosis (MS) lesions, has recently been identified as a novel autoantibody target in MS.

Objective: The aim of this study was to investigate whether anti-SPAG16 antibody levels differ between MS subtypes (relapsing-remitting, RR; primary or secondary progressive, PP, SP) and whether antibody positivity is associated with clinical characteristics.

Methods: Plasma anti-SPAG16 antibody levels were determined by recombinant protein enzyme-linked immunosorbent assay (ELISA) in 374 MS patients (274 RRMS, 39 SPMS and 61 PPMS) and 106 healthy controls.

Results: Significantly elevated anti-SPAG16 antibodies were found in 22% of MS patients with 93% specificity. Anti-SPAG16 seropositivity was associated with an increased expanded disability status scale (EDSS) in overall MS. A higher proportion of PPMS patients showed anti-SPAG16 antibody reactivity (34%) compared to RRMS (19%) and SPMS (26%), and presented with higher anti-SPAG16 antibody levels. Seropositive PPMS patients had a significantly increased progression index compared to seronegative patients.

Conclusions: Anti-SPAG16 antibodies are associated with an increased EDSS in overall MS, indicating that they are linked to a worse MS disease outcome. Moreover, the presence of anti-SPAG16 antibodies may be a biomarker for a more severe disease in PPMS patients, as indicated by an increased progression index.

3.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by myelin loss, axonal pathology and progressive neurodegeneration. The cause of the disease is unknown, but it is generally assumed that complex interactions between environmental factors and genetic susceptibility are involved. Different clinical subtypes of MS exist, with 85-90% of patients experiencing relapsing-remitting (RR) disease in which clinical relapses alternate with remission [2]. About 40% of RRMS patients develop secondary progressive (SP) MS with progressive neurological disease. In 10-20% of patients, MS manifests as a primary progressive (PP) disease with a progressive course from the onset [6]. A prevailing view in progressive disease has been that neurodegenerative processes are occurring independent of inflammation. However, this view has been challenged by pathology studies that have shown that inflammation is also present in progressive MS [76, 187]. Since there are no approved therapies for patients with PPMS, there is a great need for biomarkers that identify PPMS patients that could benefit from immune therapy.

Antibody biomarkers are good candidates for diagnosis and prognosis of autoimmune diseases, such as MS, because they reflect the presence, nature and intensity of certain immune responses [105]. In the search for novel antigenic disease markers for MS, we previously identified sperm-associated antigen 16 (SPAG16) as an autoantibody target in the cerebrospinal fluid (CSF) of MS patients [160]. Several SPAG16 isoforms exist, but isoform 1 (SPAG16-1; 71 kDa) and isoform 2 (SPAG16-2; 20.4-kDa) are mostly documented. In sperm cells, SPAG16-1 is part of the axoneme and plays a role in sperm motility and thus fertility. The function of SPAG16-2 and other isoforms is unknown [179]. Recent data reveals the presence of SPAG16 proteins in many tissues, including the lungs and brain ventricles (cilia) but also in hematopoietic cells, fibroblast-like synoviocytes in rheumatoid arthritis and in certain cancers [179, 181, 204].

Since SPAG16 is a novel target of the humoral immune response in MS, with unknown function in the CNS, we further investigated the role of SPAG16 and anti-SPAG16 antibodies in MS pathology. We previously measured anti-SPAG16 antibodies in plasma of MS patients, showing that anti-SPAG16 antibodies were elevated in the plasma of 21% of MS patients with a specificity of 95% [205].

Interestingly, the pathologic relevance of these antibodies was shown by the identification of SPAG16-specific oligoclonal bands in the CSF of 5/23 MS patients. Further, *in vivo* experiments in myelin oligodendrocyte glycoprotein (MOG)-peptide induced experimental autoimmune encephalomyelitis (EAE) mice demonstrated that injection with anti-SPAG16 antibodies resulted in significant disease exacerbation. Finally, we found that SPAG16 was specifically upregulated in reactive astrocytes, both in EAE and MS lesions. These results underline the involvement of SPAG16 and anti-SPAG16 autoantibodies in MS, although their exact role remains to be determined.

In the present study, we aimed to investigate whether anti-SPAG16 antibody levels differed between MS subtypes (RR, SP and PP). Furthermore, since we observed EAE disease exacerbation in mice after injection with anti-SPAG16 antibodies, we examined whether antibody positivity (serostatus) was associated with more severe disease characteristics in MS.

3.2 Materials and methods

3.2.1 Study population and demographics

A total of 374 MS patients and 106 age- and gender matched healthy controls (HC) were included in this study. All MS patients fulfilled diagnostic criteria for MS [14]. Plasma samples (274 RRMS, 39 SPMS and 61 PPMS) were collected after approval by the Medical Ethical Committee of Hasselt University or Cliniques universitaires Saint-Luc (Belgium) or Hospital Ramòn y Cajal (Spain) and informed consent from study participants was obtained. Clinical and demographic data of patients and HC are provided in Table 3.1. Multiple sclerosis severity score (MSSS) was calculated according to Roxburgh *et al.* [206]. Most of the patients (75%) in this cohort received no treatment at time of sampling (9 values missing). Of the treated patients (RRMS or SPMS) at time of sampling, treatments consisted of glatiramer acetate (n=24), natalizumab (n=14) or interferon (n=53).

Table 3.1: Characteristics of the MS patients and HC used in this study

	HC	**MS**	**PPMS**	**SPMS**	**RRMS**
Number	106	374	61	39	274
Age[a]	37.9±14.9	42.1±12.5	53.4±12.1	53.7±9.5	38.0±10.1
Gender[b] F:M	63:40	260:114	34:26	27:12	198:76
(%F)	(61%)	(70%)	(57%)	(69%)	(72%)
EDSS[c]	N.A.	2.6±1.8	4.2±2.0	5.0±1.8	1.9±1.1
Disease duration[a]	N.A.	6.7±7.8	6.8±7.2	17.9±8.9	5.2±6.4

[a] In years, data are presented as mean ± SD. [b] F, female; M, male. [c] Expanded disability status scale (EDSS) data are presented as mean ± SD

3.2.2 ELISA

Recombinant SPAG16 as a fusion protein with thioredoxin (THIO) or THIO alone (control) was expressed and purified using the pBAD/TOPOThioFusion kit (Invitrogen, Merelbeke, Belgium). Recombinant protein ELISA was performed as previously described [205]. The coefficient of variation from duplicate measurements was on average ~5% and had to be <20%. A serially diluted positive sample was included for interassay variability testing and was used as a

calibration curve to calculate arbitrary units (A.U.). A.U. were calculated for both the SPAG16 and THIO optical density (OD) signals and reactivity was defined as SPAG16 (A.U.) - THIO (A.U.; background). A cutoff for seropositivity was determined by ROC analysis based on the anti-SPAG16 reactivity of HC (AUC=0.6433, p-value <0.0001). Samples with values not fulfilling following criteria – OD THIO>mean+2SD of HC and (OD SPAG16 - OD THIO)<0.1 - were excluded from all analyses (n=5 for HC, n=10 for MS).

3.2.3 Statistical analysis

Statistical analysis was performed using JMP® Pro (version 11; SAS Institute Inc. 2014. Cary, NC). A p-value <0.05 was considered statistically significant. Comparisons between different groups of MS patients and controls were performed using the Mann–Whitney U test, Fisher's Exact test (2-tailed), and Pearson's chi square test. The Spearman rank correlation coefficient was used to evaluate the relation between the levels of anti-SPAG16 antibodies and age. Uni- and multivariate logistic regression analysis was performed to identify predictors of anti-SPAG16 seropositivity.

3.3 Results

3.3.1 Anti-SPAG16 antibodies are increased in PPMS patients

Plasma samples of 374 MS patients, including 274 RRMS, 39 SPMS and 61 PPMS subjects, were analyzed for the presence of anti-SPAG16 antibody reactivity and compared with 106 age- and gender matched HC (Fig. 3.1). The levels of anti-SPAG16 antibodies were significantly elevated in MS patients compared with HC ($p<0.0001$, Fig. 3.1), with a sensitivity of 22% and a specificity of 93%. The mean anti-SPAG16 antibody levels (A.U.) were 58.7 in MS and 16.6 in HC (Fig. 3.1).

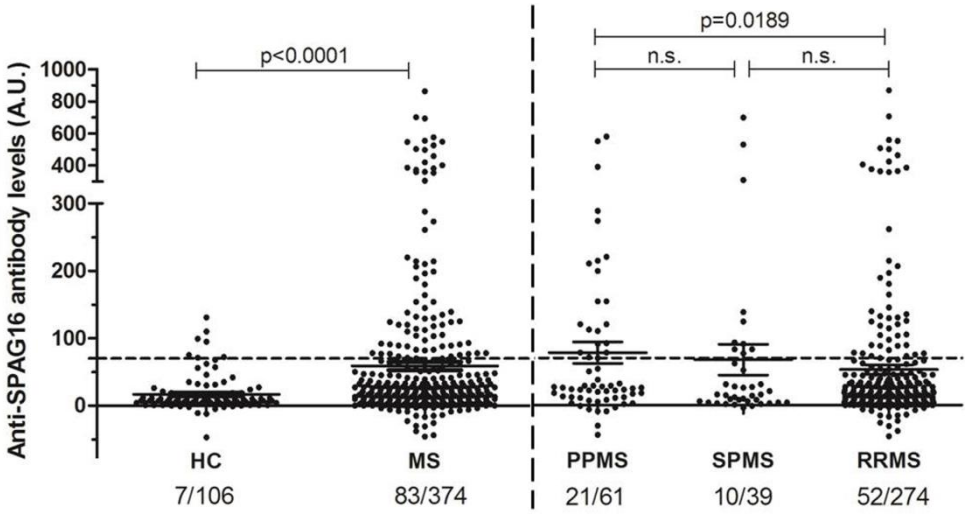

Figure 3.1: Levels of anti-SPAG16 antibodies in MS patients and HC

Plasma samples from MS patients (n=374) or HC (n=106) were examined by recombinant SPAG16 ELISA. Arbitrary Units (A.U.) were calculated for anti-SPAG16 antibody levels using a calibration curve. Anti-SPAG16 antibody levels were compared with Mann-Whitney U test. Analysis of individual groups of MS patients for anti-SPAG16 antibody levels is shown, according to MS subtypes (PPMS (n=61), SPMS (n=39) and RRMS (n=274)). Dots represent individual samples and the mean A.U. values ± SEM are shown. The dashed horizontal line depicts the cutoff for seropositivity, that was set at 70 A.U., based on ROC analysis comparing HC and MS. Anti-SPAG16 antibody levels were compared in the different subtypes with the Kruskal–Wallis test of one-way ANOVA with an overall p=0.0659. The p-values of posttests between 2 groups (Mann-Whitney) are depicted in the graph. n.s. not significant.

When dividing the MS patients according to MS subtype (Fig. 3.1), PPMS patients had significantly higher plasma levels (mean A.U. 77.9) of antibodies to SPAG16 than RRMS (mean A.U. 53.2) patients (p=0.0189). However, this increase in anti-SPAG16 antibody levels is probably due to the significantly increased proportion of PPMS patients (34%) positive for anti-SPAG16 antibodies compared to RRMS patients (19%) (p=0.0084), with a relative risk of 1.81 in the PPMS group (95% confidence interval 1.2-2.8).

Table 3.2: Characteristics of MS patients according to anti-SPAG16 serostatus

	SPAG16 seropositive	**SPAG16 seronegative**	**p-value**
Number	83	291	
Age[a]	**46.0±12.4**	**41.0±12.3**	**0.0014**
Gender[b] F:M (%F)	60:23 (72%)	200:91 (69%)	0.5901
EDSS[c]	**2.9±2.0**	**2.5±1.7**	**0.0425**
Disease duration[a]	7.0±7.0	6.7±8.0	0.7417
Progression index[e]	1.6±3.0	1.2±2.2	0.1807
MSSS[d]	4.8±2.9	4.3±2.6	0.1128
IgG index[f]	1.1±1.1	1.0±0.6	0.4225
Treatment[g] No:Yes (%No)	64:18 (78%)	208:75 (73%)	0.4727

[a] In years, data are presented as mean ± SD. [b] F, female; M, male. [c] Expanded disability status scale (EDSS) data are presented as mean ± SD. [d] Multiple sclerosis severity scale (MSSS) data are presented as mean ± SD. [e] Progression index (EDSS/disease duration) data are presented as mean ± SD. [f] IgG index, data available for 60/83 and 201/291 MS patients (113 values missing). [g] Treatment at time of sampling (9 values missing), No: no treatment, Yes: in seropositive: n=2 glatiramer acetate, n=2 natalizumab, n=13 interferon ; Yes: in seronegative: n=21 glatiramer acetate, n=12 natalizumab, n=40 interferon.

No significant differences were found compared to the SPMS patient group, whose antibody levels (mean A.U. 67.5) and proportion of seropositive patients

(26%) were intermediate between RRMS and PPMS. Anti-SPAG16 antibody levels and age were not correlated in the different groups of MS patients, RRMS (r=0.11, p=0.06), SPMS (r=0.12, p=0.45), PPMS (r=-0.24, p=0.06) or in the HC (r=0.03, p=0.76).

3.3.2 Anti-SPAG16 antibody seropositivity is associated with increased EDSS in overall MS

Eighty-three MS patients (22%) were anti-SPAG16 antibody positive. We compared demographic and clinical characteristics of SPAG16 seropositive and negative MS patients (Table 3.2). SPAG16 seropositive MS patients were older and showed a significantly increased EDSS, indicating a potential pathologic role of the antibodies. Gender, disease duration, MSSS, progression index, IgG index and treatment at time of sampling were not significantly different between groups.

Table 3.3: Characteristics per MS subtype according to anti-SPAG16 serostatus

		PPMS	
	Seropositive	**Seronegative**	**p-value**
Number	21	40	
Age[a]	**48.4±13.8**	**56.0±10.4**	**0.0192**
Gender[b] F:M (%F)	15:6 (71%)	20:20 (50%)	0.1726
EDSS[c]	4.2±1.8	4.1±2.1	0.9034
Disease duration[a]	**4.6±4.1**	**7.9±8.2**	**0.0384**
Progression index[d]	**2.7±4.4**	**1.0±0.8**	**0.0165**
MSSS[e]	7.1±2.1	6.3±2.7	0.2410
		SPMS	
	Seropositive	**Seronegative**	**p-value**
Number	10	29	
Age[a]	57.8±9.0	52.2±9.4	0.1112
Gender[b] F:M (%F)	7:3 (70%)	20:9 (69%)	1.0000
EDSS[c]	4.8±2.3	5.1±1.6	0.6535
Disease duration[a]	17.6±5.3	18.0±10.0	0.9166
Progression index[d]	0.3±0.1	0.5±0.4	0.1991
MSSS[e]	4.7±3.1	5.5±2.4	0.3975

Table 3.3 continued:

	RRMS		
	Seropositive	**Seronegative**	**p-value**
Number	52	222	
Age[a]	**42.7±10.8**	**36.7±9.7**	**0.0002**
Gender[b] F:M (%F)	38:14 (73%)	160:72 (72%)	1.0000
EDSS[c]	2.1±1.4	1.9±1.0	0.1757
Disease duration[a]	5.9±6.4	5.0±6.4	0.3518
Progression index[d]	1.4±2.4	1.4±2.5	0.8787
MSSS[e]	3.9±2.7	3.8±2.4	0.6804

[a] In years, data are presented as mean ± SD. [b] F, female; M, male. [c] Expanded disability status scale (EDSS) data are presented as mean ± SD. [d] Progression index (EDSS/disease duration) data are presented as mean ± SD. [e] Multiple sclerosis severity scale (MSSS) data are presented as mean ± SD

3.3.3 Anti-SPAG16 antibody seropositivity is associated with an increased progression index in PPMS patients

Next, we investigated the clinical characteristics of MS patients according to anti-SPAG16 serostatus per MS subtype (Table 3.3). PPMS patients, in contrast to RRMS and SPMS, were significantly younger when anti-SPAG16 antibodies were detected. Seropositive PPMS patients had a significantly shorter disease duration and comparable EDSS as seronegative PPMS patients. Thus, seropositive PPMS patients obtained this EDSS faster; which is confirmed by the progression index, which was significantly higher in seropositive PPMS patients.

Table 3.4: Logistic regression analysis to identify predictors of anti-SPAG16 positivity in PPMS

		95% CI		
	Odds ratio	**Lower**	**Upper**	**p-value**
Female sex*	3.91	1.15	15.9	0.0285
Progression index	1.78	1.14	3.30	0.0039
EDSS*	0.99	0.74	1.31	0.9320
Disease duration	1.10	1.00	1.25	0.0559
MSSS	0.89	1.01	1.11	0.3343

* Age as covariate

Furthermore, progression index and MSSS were significantly correlated ($r=0.7675$, $p<0.0001$). Logistic regression analysis indicated that both female sex and the progression index were independent predictors of anti-SPAG16 seropositivity (Table 3.4), with an odds ratio of 3.91 and 1.78, respectively. Gender, EDSS, disease duration, progression index and MSSS were not significantly altered between seropositive versus seronegative RR or SPMS.

3.4 Discussion

The present study demonstrates that anti-SPAG16 antibodies are significantly increased in 22% of MS patients. The proportion of MS patients with anti-SPAG16 antibody reactivity is comparable to our previous studies [160, 205] and confirms SPAG16 as an autoantibody target in MS. However, anti-SPAG16 antibodies cannot be used as a single diagnostic biomarker for MS but these antibodies are possibly useful as a prognostic biomarker. Next, we were interested in the different disease characteristics when MS patients were stratified according to anti-SPAG16 serostatus. In the whole MS cohort (n=374), we found that anti-SPAG16 seropositive MS patients had a significantly higher EDSS (~3) compared to those patients that were seronegative for these antibodies (EDSS ~2.5). A score of 2.5 is categorized as still "mild/minimal disability" and a score of 3.0 is categorized as "moderate disability"[207], indicating a clear difference in the clinical status of the patient, and a potential role of anti-SPAG16 antibodies in MS pathology. Furthermore, in our previous *in vivo* EAE experiments we demonstrated a disease exacerbation in mice injected with anti-SPAG16 antibodies, again confirming that these antibodies are related to worse disease outcome [205].

During the last decades, much effort has been made to characterize the autoantibody response in MS patients. Consensus exists on the involvement of multiple autoantibody targets and not a single autoantibody that plays a dominant role in the disease. Various target antigens of these autoantibodies have been described, including MOG, neurofilament, neurofascin, the potassium channel KIR4.1, coronin-1a and SPAG16, reflecting disease heterogeneity [159, 162, 208]. This heterogeneity is also reflected in the different MS lesion types, the different MS subtypes and clinical disease courses [2, 6]. We therefore investigated whether anti-SPAG16 antibodies were related to specific disease characteristics in the different MS subtypes. First, we showed that anti-SPAG16 antibody levels were highest in PPMS patients, followed by SPMS and lowest in the RRMS patients. However, since anti-SPAG16 antibody levels were not correlated to any disease parameters, we focused on serostatus, rather than levels, in further analyses. We found that a significantly increased proportion of PPMS patients (34%) were seropositive for anti-SPAG16 antibodies compared to RRMS patients

(19%) but found no differences compared to the SPMS patient group (26%). This indicates that anti-SPAG16 antibody seropositivity is possibly linked to disease stage and pathologic subtype. As shown by Quintana *et al.*, different antibody reactivities (patterns) are found in the different MS subtypes, indicating that antibodies could be used as biomarkers to monitor disease progression [166].

Next, we stratified each MS subtype according to anti-SPAG16 serostatus and demonstrated that in PPMS, 34% were anti-SPAG16 seropositive (19% and 26% of RRMS and SPMS, respectively) and this was associated with an elevated progression index in PPMS and thus related to a possible worse disease outcome and more aggressive disease. However, future follow-up of seropositive and seronegative PPMS patients is essential to conclude whether the presence of anti-SPAG16 antibodies could be a novel prognostic biomarker in PPMS. It would be interesting to investigate anti-SPAG16 antibodies in relation to neurofilament light levels, which have been shown to be higher in PPMS and can be used as a prognostic marker [209] and investigate whether anti-SPAG16 antibodies can be used as a biomarker for possible treatment monitoring in PPMS. Long-term follow-up (longitudinal studies) of seropositive and negative RRMS patients is needed to investigate the possibility of anti-SPAG16 antibodies as predictors of secondary progression.

The link between the presence of antibodies and progression has been an important research field in MS. It has been shown that an increased CSF B cell to monocyte ratio correlates with disease progression in MS [210] and that the presence of intrathecal IgM synthesis in RRMS is a predicting factor for secondary progression [211]. The presence of CSF IgM OCB may be a biomarker for a subset of PPMS patients with more active inflammatory disease, who may benefit from immune-directed treatments [212]. Furthermore, PPMS patients have increased serum antiganglioside antibody levels compared to those with RRMS and controls. Just as the case with anti-SPAG16 antibodies, the levels in SPMS tend to be intermediate between those in PPMS and RRMS [136]. These findings, and our results of elevated anti-SPAG16 antibodies in PPMS, raise the possibility that disease progression in MS is related to antibody-mediated CNS damage. Moreover, we have previously shown that anti-SPAG16 antibodies in MS patients are mostly IgG1 and IgG3, which can activate complement and in EAE mice injected with anti-SPAG16 antibodies there was more complement deposition

(C5b9) [205], indicating a potential mechanism for the observed exacerbation in EAE disease and worse MS prognosis. Finally, SPAG16 is upregulated in astrocytes in MS lesions [205] and interestingly, astrocytes are becoming increasingly recognized as important players in MS progression and targets of autoantibodies in MS [71, 159, 213, 214]. It is possible that anti-SPAG16 antibodies are formed due to cell damage and death of astrocytes which leads to the exposure of intracellular antigens such as SPAG16 (epitope spreading).

In conclusion, this study shows that anti-SPAG16 seropositivity is associated with an increased EDSS in overall MS, indicating that they are linked to a worse MS disease outcome. Moreover, the presence of anti-SPAG16 antibodies may be a biomarker for a more severe disease in PPMS patients, as indicated by an increased progression index. Future studies, including longitudinal screening of PPMS patients and investigating the role of these antibodies are crucial and might lead to advances in the diagnosis, prognosis and treatment of PPMS.

4

Frequency and prognostic implications of anti-SPAG16 antibodies in patients with optic neuritis

Based on:

Frequency and prognostic implications of anti-SPAG16 antibodies in patients with optic neuritis (in preparation)

de Bock L, Fraussen J, Frederikson J, Stinissen P and Somers V

ABSTRACT

Background: Sperm-associated antigen 16 (SPAG16), a sperm protein which is upregulated in reactive astrocytes in multiple sclerosis (MS) lesions, has been previously identified as a novel autoantibody target in MS. Anti-SPAG16 antibodies are found in 21-22% of patients with MS, a severely disabling demyelinating inflammatory central nervous system (CNS) disorder. Optic neuritis (ON) presents as an inflammatory demyelinating disorder of the optic nerve and is the first presentation of MS in approximately 20-30% of patients.

Objective: The objective of the present study was to investigate whether anti-SPAG16 antibody serostatus is predictive for conversion of ON to MS (prognostic value).

Patients and methods: Anti-SPAG16 antibody levels were determined in paired serum and cerebrospinal fluid (CSF) samples from 85 ON patients using a recombinant protein ELISA assay and the association of anti-SPAG16 serostatus with conversion to MS and other clinical characteristics was investigated.

Results: We found a significant correlation between the levels of anti-SPAG16 antibodies in serum and CSF, indicating that antibody levels measured in the serum are predictive of the levels in the CSF. We also confirmed previous risk factors associated with MS conversion since the percentage of ON patients with an abnormal brain magnetic resonance imaging (MRI), number of MRI brain lesions, percentage of ON patients with oligoclonal bands in the CSF and immunoglobulin G (IgG) index were all significantly elevated in ON patients that converted to MS compared to those that did not convert during the follow-up time. Further, anti-SPAG16 antibodies were present in the serum of 28% of ON patients but anti-SPAG16 antibody seropositivity and levels did not differ between ON patients that did (23%) or did not (31%) convert to MS.

Conclusion: This study did not provide evidence for a link between anti-SPAG16 serostatus and conversion to MS (prognostic value) but ON patients positive for anti-SPAG16 antibodies and other risk factors should be followed up. Further, we

have previously indicated that anti-SPAG16 antibodies are linked to a worse MS disease outcome so future studies investigating the role of these antibodies are crucial and might lead to advances in the prognosis of ON and MS patients.

4.1 Introduction

Optic neuritis (ON) is an inflammation of the optic nerve and the incidence of unilateral ON around the world ranges from 0.94-2.18 per 100,000 per year and more women are affected (in northern European cohorts (1:3)) [215]. Typical ON presents with subacute monocular visual loss associated with pain during eye movement and the visual loss usually develops during hours or days [216]. Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), characterized by myelin loss, axonal pathology and progressive neurodegeneration [2]. ON is a presenting symptom of MS in 25% of ON cases and occurs during the disease in about 70% of MS patients, usually in the relapsing–remitting phase [215, 217]. However, in the absence of other signs of CNS inflammation, ON poses substantial diagnostic challenges to neurologists and ophthalmologists.

We have recently identified sperm-associated antigen 16 (SPAG16) as an autoantibody target in MS [160, 205]. In sperm cells, SPAG16 is part of the axoneme (a microtubular structure also present in other ciliated cells) and plays a role in sperm motility and thus fertility [179, 180] but in MS, SPAG16 is upregulated in reactive astrocytes and its role is still unknown [205]. We have shown that anti-SPAG16 antibodies were elevated in the plasma of 21-22% of MS patients with a specificity of 93-95%. The pathologic relevance of these antibodies was shown *in vivo* in experimental autoimmune encephalomyelitis (EAE) mice where injection with anti-SPAG16 antibodies resulted in significant disease exacerbation [205]. Furthermore, in MS patients, anti-SPAG16 seropositivity was associated with an elevated expanded disability status scale (EDSS) and in primary progressive MS, anti-SPAG16 seropositivity was associated with an elevated progression index [218].

Since ON is often associated with MS and anti-SPAG16 antibodies are found in a subgroup of MS patients, we assessed whether anti-SPAG16 antibodies are found in the serum and cerebrospinal fluid (CSF) of ON patients using our anti-SPAG16 ELISA. Furthermore, we investigated whether anti-SPAG16 antibody seropositivity in ON patients could predict the conversion to MS and if seropositivity was linked to other clinical parameters.

4.2 Materials and methods

4.2.1 Study population and demographics

Paired serum and CSF samples from 85 patients with a history of acute monosymptomatic ON who did not meet the diagnostic criteria for MS [13] or neuromyelitis optica (NMO) [219] or any other established CNS disorder at the time of blood sampling were tested for anti-SPAG16 antibodies. Patient samples were collected after approval by the Medical Ethical Committee (Glostrup - Denmark) and informed consent from study participants was obtained. Clinical and demographic data of the ON patients is given in Table 4.1.

4.2.2 ELISA

Recombinant SPAG16 as a fusion protein with thioredoxin (THIO) or THIO alone (control) was expressed and purified using the pBAD/TOPOThioFusion kit (Invitrogen, Merelbeke, Belgium). Recombinant protein ELISA was performed as previously described [205]. Briefly, 96-well ELISA plates (Greiner Bio-One, Wemmel, Belgium) were coated overnight at 4°C with 1 mg/ml purified recombinant protein in 0.1 M bicarbonate buffer (pH 9.6). Washing was done using 0.05% PBS–Tween 20 (PBS-T). Wells were blocked with PBS containing 2% (w/v) skimmed milk powder (M-PBS) for 2 h at 37°C and then incubated with 100 µl serum samples (1:100 in M-PBS) or 100 µl CSF samples (1:1.33 in M-PBS) for 2h at room temperature. Antibody binding was detected with horseradish peroxidase-conjugated (HRP) anti-human IgG (1:2000 in M-PBS; DakoCytomation, Heverlee, Belgium), followed by color development with 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich, Diegem, Belgium). The reaction was stopped with 2M H_2SO_4 and read at 450 nm. Background reactivity against a recombinant THIO protein was measured in parallel. The coefficient of variation from duplicate measurements was on average ~5% and had to be <20%. A serially diluted positive sample was included to test for interassay variability and was used as a calibration curve to calculate arbitrary units (A.U.). A.U. were calculated for both the SPAG16 and THIO optical density (OD) signals. Anti-SPAG16 antibody reactivity was defined as SPAG16 (A.U.) - THIO (A.U.; background). A cutoff for serum seropositivity was determined previously by performing ROC analysis[205]. A cutoff for CSF positivity was determined by

mean+2xSD. Samples with values not fulfilling following criteria – OD THIO>mean+2SD and (OD SPAG16 – OD THIO)<0.1 - were excluded from all analyses (n=2 for ON).

4.2.3 Statistical analysis

Statistical analysis was performed using JMP® Pro (version 11; SAS Institute Inc. 2014. Cary, NC). One patient was excluded from all analyses after outlier analysis. A p-value <0.05 was considered statistically significant. Comparisons between different groups were performed using the Mann–Whitney U test and Fisher's Exact test (2-tailed unless stated otherwise). The Spearman rank correlation coefficient was used to evaluate the relation between the levels of anti-SPAG16 antibodies in the CSF and serum.

4.3 Results

4.3.1 Anti-SPAG16 serum antibodies are predictive of anti-SPAG16 antibodies in CSF

Anti-SPAG16 antibody levels were determined in paired serum and CSF samples of 85 ON patients. A significant correlation (Spearman ρ = 0.7295, P<0.0001) was found (Fig. 4.1) between the levels of serum and CSF anti-SPAG16 antibodies, indicating that antibody levels measured in the serum are predictive of the levels in the CSF. Furthermore, 4 out of 5 ON patients that were positive for anti-SPAG16 antibodies in the CSF were positive in the serum.

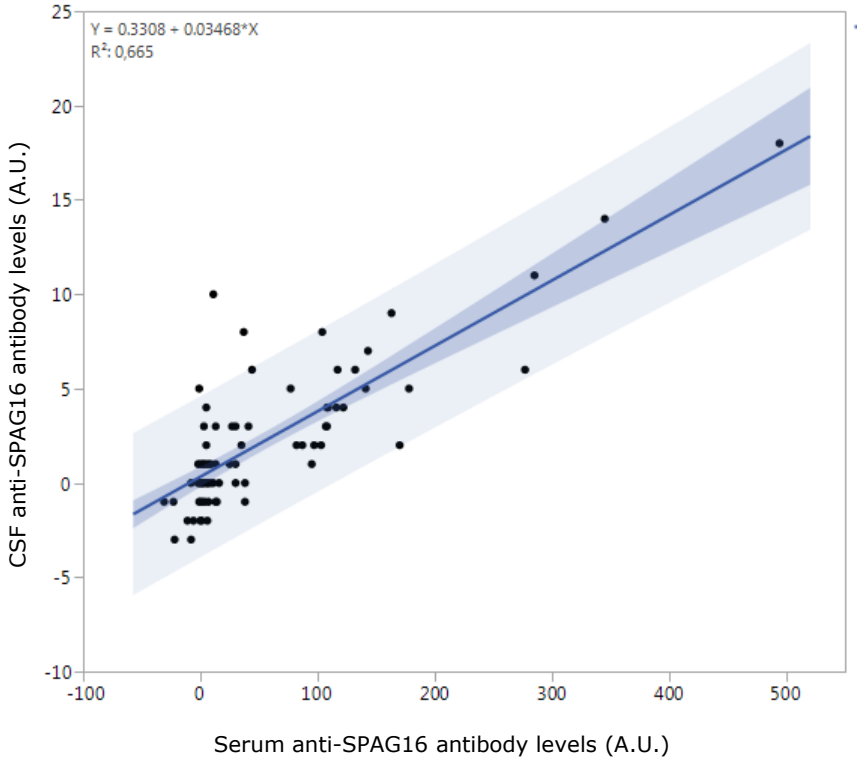

Figure 4.1: Correlation between CSF and serum anti-SPAG16 antibody levels in ON patients

Paired serum and CSF samples from ON patients (n=85) were examined by recombinant SPAG16 ELISA. Arbitrary Units (A.U.) were calculated for anti-SPAG16 antibody levels using a calibration curve. Anti-SPAG16 antibody levels in the CSF were correlated to the levels in the serum. CSF anti-SPAG16 = 0.3308+0.03468*serum anti-SPAG16.

4.3.2 Anti-SPAG16 antibodies are not elevated in ON patients that converted to MS

Table 4.1 shows that the percentage of ON patients presenting with abnormal brain MRIs, number of MRI brain lesions, percentage of ON patients positive for OCB in the CSF and IgG index are all significantly elevated in ON patients that converted to MS compared to those that did not convert during the follow-up time. These results indicate that these characteristics are linked to a certain risk for conversion to MS.

Table 4.1: Characteristics of the ON patients used in this study

	ON total	**ON/MS[e]**	**ON no MS[f]**
Number	85	31	54
Age[a]	35.1±10.8	36.9±11.4	34.1±10.5
Gender[b] F:M (%F)	68:17 (80%)	24:7 (77%)	44:10 (81%)
Follow-up time[a]	3.2±2.5	3.3±1.2	3.2±3.1
Brain MRI (% abnormal[c])	65%	**94%**	**49%*** **
# MRI brain lesions	5.8±9.2	**9.2±10.2**	**3.8±8.0*** **
OCB CSF[d] (% positive)	52%	**74%**	**39%** **
IgG Index	0.72±0.50	**0.97±0.67**	**0.57±0.31*** **

[a] In years, data are presented as mean ± SD. [b] F, female; M, male. [c] Brain MRI is defined as abnormal when MRI lesions are found; whilst normal means without MRI lesions. [d] Oligoclonal bands (OCB) in cerebrospinal fluid (CSF). [e] ON/MS: ON patients that converted to MS during the follow-up time. [f] ON no MS: ON patients that did not convert to MS during the follow-up time. *** P<0.0001; ** P=0.0017

Next, we investigated whether anti-SPAG16 antibodies were present in ON and if they could predict the conversion to MS. Anti-SPAG16 antibodies were present in the serum of 24/85 (28%) patients with ON (Fig. 4.2A), 7/31 (23%) ON patients that converted to MS and 17/54 (31%) of ON patients that did not convert to MS. Anti-SPAG16 antibody levels did not differ between the different groups, indicating that they are not predictive for conversion to MS. Furthermore, anti-SPAG16 antibodies in paired CSF were elevated in 5/85 (6%) ON patients, 1/31 (3%) of ON patients that converted to MS (ON/MS), 4/54 (7%) of ON patients that did not convert to MS (ON no MS) and antibody levels did not differ between the different groups (Fig. 4.2B).

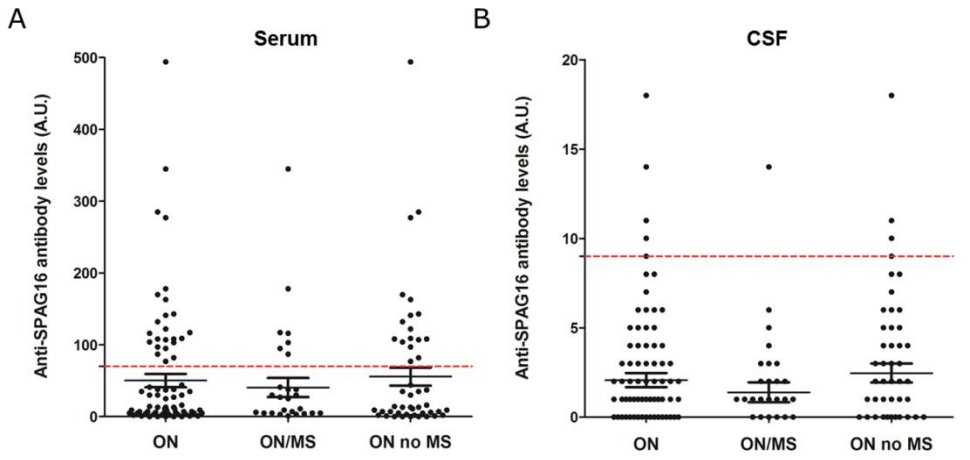

Figure 4.2: Anti-SPAG16 antibody levels in serum and CSF of ON patients

Paired serum (A) and CSF (B) samples of ON patients (n=85) were examined by recombinant SPAG16 ELISA. Arbitrary Units (A.U.) were calculated for anti-SPAG16 antibody levels using a calibration curve. (A) Analysis of individual groups of ON patients (n=85) for anti-SPAG16 antibody serum levels is shown, according to MS conversion (ON/MS (n=31), ON no MS (n=54)). Dots represent individual samples and the mean A.U. values ± SEM are shown. The dashed horizontal line depicts the cutoff for seropositivity. (B) Anti-SPAG16 antibody CSF levels are shown for the total ON patient group (n=85), according to MS conversion ((ON/MS) or (ON no MS)).

4.3.3 Anti-SPAG16 serostatus and ON patient characteristics

We then stratified the ON patients according to anti-SPAG16 serostatus to investigate whether serostatus was linked to specific patients' characteristics (Table 4.2). We demonstrated that anti-SPAG16 antibody levels and the percentage of anti-SPAG16 antibody positive patients in the CSF were significantly higher in anti-SPAG16 seropositive ON patients, confirming the previously reported relation between anti-SPAG16 antibodies in the serum and CSF (Fig. 4.1). Other parameters such as age at onset, gender, conversion to MS, follow-up time, MRI characteristics, OCB in the CSF and IgG index were not significantly different between seropositive and seronegative ON patients. Similar results were obtained when ON patients were subdivided according to conversion to MS status (data not shown).

Table 4.2: Characteristics of ON patients according to anti-SPAG16 serostatus

	SPAG16 seropositive	**SPAG16 seronegative**	**p-value**
Number	24	61	
Age[a] at onset	33.8±9.6	35.6±11.3	0.5382
Gender[b] (%F)	88%	77%	0.3735
Anti-SPAG16 CSF, mean (A.U.)	5.5±4.1	0.71±2.4	**<0.0001**
Anti-SPAG16 CSF status (% positive)	17%	2%	**0.0211**
% converter to MS	29%	39%	0.4580
Follow-up time[a]	3.0±1.3	3.3±2.9	0.8042
Brain MRI (% abnormal[c])	67%	65%	1.0000
# MRI brain lesions	6.6±11.4	5.5±8.2	0.7737
OCB CSF[d] (% positive)	42%	56%	0.3353
IgG Index	0.70±0.45	0.73±0.53	0.6923

[a] In years, data are presented as mean ± SD. [b] F, female; M, male. [c] Brain MRI is defined as abnormal when MRI lesions are found; whilst normal means without MRI lesions. [d] Oligoclonal bands (OCB) in cerebrospinal fluid (CSF).

4.4 Discussion

In the present study we assessed the frequency of antibodies against SPAG16 in paired serum and CSF from 85 ON patients. Our main findings are (i) anti-SPAG16 serum antibodies are predictive of anti-SPAG16 antibodies in CSF; (ii) the percentage of ON patients with abnormal brain MRIs, number of MRI brain lesions, percentage of ON patients positive for OCB in the CSF and IgG index are all significantly elevated in ON patients that converted to MS compared to those that did not convert during the follow-up time; (iii) anti-SPAG16 antibodies are present in the serum of 28% of ON patients but anti-SPAG16 antibody seropositivity and levels did not differ between MS converters and non-converters. Therefore, this study did not provide evidence for a link between anti-SPAG16 serostatus and conversion to MS (prognostic value). With the biomarker potential of anti-SPAG16 antibodies in mind, it is interesting to find a significant positive correlation between the levels of antibodies measured in the CSF and serum, indicating that measuring these antibodies in the serum alone is sufficient.

Next we found several clinical characteristics to be significantly different between ON patients that did or did not convert to MS. This has previously been described with the presence of one or more lesions at baseline brain MRI as the strongest predictor of MS conversion. Other risk factors include the presence of OCB and an elevated IgG index [220-223]. Our results confirm these risk factors.

Subsequently, we found that 28% of ON patients were seropositive for anti-SPAG16 antibodies and we investigated whether anti-SPAG16 antibody serostatus could be an additional predictor in the conversion to MS but found no evidence for the prognostic value of anti-SPAG16 serostatus in ON patients. Of note, the mean follow-up of these ON patients was approximately 3 years and whilst ON is the first presentation of MS in approximately 20% of patients [215, 224], the estimated risk of MS conversion after ON is 30% at 5 years, 38% at 10 years and 50% at 15 years [220]. Therefore, follow-up in the current study is possibly too short and future development of MS in the current non-converters has to be considered; more specifically for the 17/54 ON patients that did not convert to MS but who were seropositive for anti-SPAG16 antibodies. Further, follow-up studies should also assess disease severity parameters (such as EDSS)

in those patients that converted to MS and assess the effect of anti-SPAG16 serostatus. Especially, since we have previously shown that anti-SPAG16 antibodies are related to an elevated EDSS in MS and an elevated progression index in primary progressive MS [218] and in our previous *in vivo* EAE experiments we demonstrated a disease exacerbation in mice injected with anti-SPAG16 antibodies [205], indicating the pathologic potential of anti-SPAG16 antibodies in MS. It should also be investigated whether anti-SPAG16 antibodies play a role in ON disease pathology and if there is a link with aquaporin-4 antibody reactivity as recent evidence suggests that certain forms of ON, mostly aquaporin-4 antibody negative, are associated with anti–myelin oligodendrocyte glycoprotein (MOG) antibodies [225, 226 227].

In conclusion, although we have not confirmed a prognostic value of anti-SPAG16 seropositivity and MS conversion in ON patients, it would be interesting to follow up these patients for a longer period and investigate: (i) the effect of anti-SPAG16 seropositivity during ON and conversion to MS after >5 years follow-up; (ii) the effect of anti-SPAG16 seropositivity on future MS disease. Since we have previously indicated that anti-SPAG16 antibodies are linked to a worse MS disease outcome, future studies investigating the role of these antibodies are crucial and might lead to advances in the prognosis of ON and MS patients. Furthermore, it would be interesting to investigate whether anti-SPAG16 antibodies are related to astrocyte damage in ON, since we have previously shown that SPAG16 is upregulated in reactive astrocytes.

5

Elucidating the role of SPAG16 in astrocytes

ABSTRACT

Background: We have previously identified sperm-associated antigen 16 (SPAG16) as a novel autoantibody target in multiple sclerosis (MS). SPAG16 is a sperm protein and functions as part of the axoneme which is essential for sperm motility and fertility. Besides a role in sperm cells, SPAG16 is expressed in motile cilia from brain ventricle ependymal cells, respiratory and oviduct epithelium. Furthermore, we have recently demonstrated that SPAG16 is also upregulated in reactive astrocytes in MS lesions, but the function of the protein in astrocytes is unknown.

Objective: In the present study we aimed to investigate SPAG16 expression in astrocytes in more detail and obtain more clues about the potential function of this protein in the context of MS.

Methods: Using subcellular protein fractionation we investigated the subcellular localization of SPAG16. Furthermore, specific isoform expression in astrocytes in MS lesions was studied using Western blot. Finally, we analyzed the effect on SPAG16 expression after *in vitro* stimulation of primary human astrocytes.

Results: We found that SPAG16 is a cytoplasmic protein. Additionally, we demonstrated that compared to SPAG16 isoform 2 (SPAG16-2), SPAG16 isoform 1 (SPAG16-1) is predominantly expressed in human astrocytes. Finally, since we hypothesized that the upregulation of SPAG16 in reactive astrocytes in MS lesions is due to 'stress', the effect of *in vitro* stimulation of primary human astrocytes on SPAG16 isoform expression was analyzed. *In vitro* activation of primary human astrocytes resulted in the upregulation of SPAG16-1 after 24h, indicating that the pro-inflammatory environment of MS lesions could be responsible for the previously observed upregulation of SPAG16 in astrocytes in MS lesions.

Conclusion: Future studies investigating the effect of the regulation of SPAG16 in astrocytes are necessary to provide information about the actual function of SPAG16 beyond sperm cells and other ciliated cells.

5.1 Introduction

Multiple sclerosis (MS) is a complex chronic inflammatory demyelinating disease of the central nervous system (CNS), characterized by myelin loss, axonal pathology and progressive neurodegeneration, although the exact cause is unknown [187]. Astrocytes have been identified as reactive components within and surrounding demyelinated lesions in MS. Astrogliosis is one of the pathological hallmarks in MS lesions but until recently, the highly reactive state of astrocytes in MS lesions was considered a secondary response to the primary immune response and followed by demyelination [67, 213]. Now, astrocytes are becoming increasingly recognized as important players in MS progression and targets of autoantibodies in MS [71, 159, 213, 214].

Astrocytes, named for their star-like shape, are the most abundant cell type in the CNS of mammals. They constitute approximately 90% of the human brain and play active and essential roles in brain development and brain function and should not just be considered as supportive cells to the neurons [228]. The ratio of astrocytes per neuron increases considerably among species with increasing brain complexity and size, suggesting that there is an evolutionary advantage in animals with a greater number of astrocytes [229].

Astrocytes can be subdivided according to different classifications but two types of astrocytes in mammals are described; protoplasmic and fibrous. Protoplasmic astrocytes are found in the gray matter and extend abundant ramified branches that contact neuronal surfaces and blood vessels. Whereas, fibrous astrocytes are located next to axon bundles in white matter tracts with longer and thinner processes. Although the morphological and structural differences are obvious, both types appear to exhibit similar functions [67] and play a complex role in the regulation of CNS autoimmunity by their immunomodulatory capacity, regulating the blood-brain barrier function and as a source of chemokines and cytokines [71, 72].

We have previously demonstrated that sperm-associated antigen 16 (SPAG16) is upregulated in reactive astrocytes in MS lesions [205]. SPAG16 is the human orthologue of Chlamydomonas reinhardtii (single-cell green algae) Pf20 and the human gene is located on chromosome 2q34 (Fig. 5.1A) [179]. SPAG16 has been mostly studied in the context of fertility, due to its expression in sperm cells.

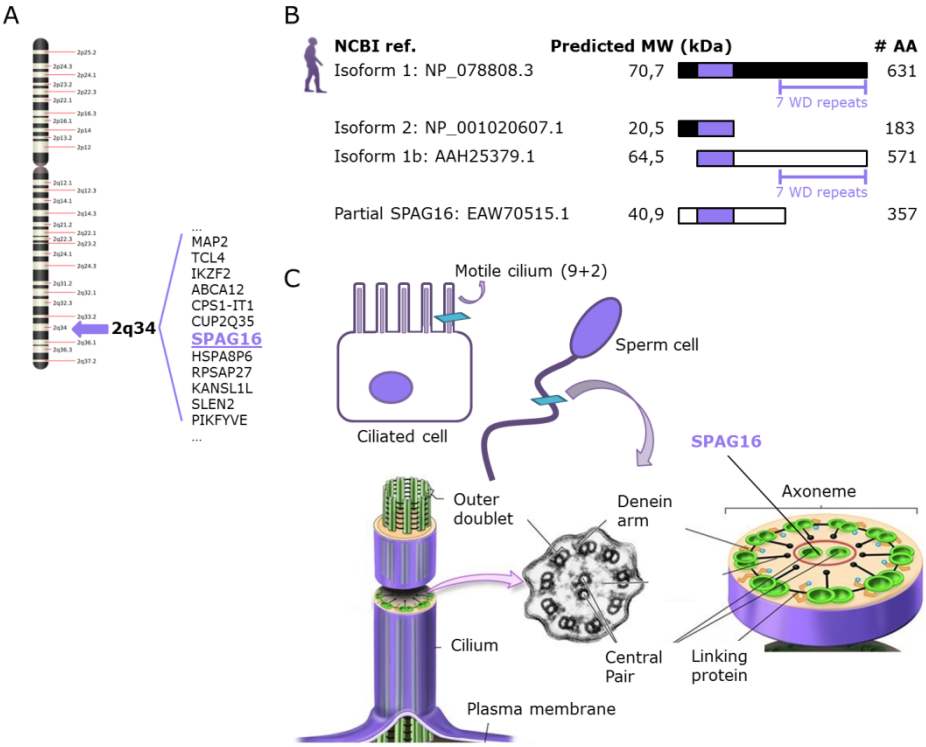

Fig. 5.1: SPAG16 isoforms and function in motile cilia. (A) The SPAG16 gene is located on chromosome 2q34. Nearby genes are summarized: MAP2, microtubule-associated protein 2; TCL4, T-cell leukemia/lymphoma 4; IKZF2, IKAROS family zinc finger 2 (Helios); ABCA12, ATP-binding cassette, sub-family A (ABC1), member 12; CPS1-IT1, CPS1 intronic transcript 1; CUP2Q35, Syndactyly, type I; SPAG16, sperm-associated antigen 16; HSPA8P6, heat shock 70kDa protein 8 pseudogene 6; RPSAP27, ribosomal protein SA pseudogene 27; KANSL1L, KAT8 regulatory NSL complex subunit 1-like; SLEN2, systemic lupus erythematosus with nephritis 2; PIKFYVE, phosphoinositide kinase, FYVE finger containing. (B) According to the National Center for Biotechnology Information (NCBI), there are multiple SPAG16 isoforms (August 2015). Isoform 1 and 2 are validated (black); other isoforms are depicted in white. The initially identified autoantibody reactive region of 121 AA using serological antigen selection (SAS) is marked to indicate that the autoreactive antibodies could bind multiple isoforms of the protein. SPAG16 isoform 1 and 1b contain 7 WD-repeat domains. (C) To illustrate the role of SPAG16 as part of the axoneme in motile cilia, a cross section is shown of a motile cilium/sperm flagellum. Motile cilia are characterized by a "9+2" structure indicated by 9 outer doublet microtubules and the central pair. [Adapted from http://biology-forums.com]

Several human SPAG16 isoforms exist (Fig. 5.1B) and there is >80% sequence homology between human SPAG16 isoform 1 (SPAG16-1) and murine SPAG16L [230]. Both SPAG16-1 and SPAG16-1b contain 7 WD-repeat domains (Fig. 5.1B) ending with tryptophan aspartate (W-D). These regions are highly conserved in eukaryotes and are known to mediate protein-protein interactions by giving rise to a ß–propeller tertiary structure. By interacting with different protein partners, WD-repeat proteins have been shown to play important roles in many cellular activities, including cell division, gene transcription, mRNA modification, regulation of cytoskeletal assembly and transmembrane signaling [231, 232]. SPAG16 has several binding partners (described in sperm cells), namely SPAG6, meiosis expressed gene 1 (MEIG1) and testis-specific serine kinase 2 (TSSK2) [186].

SPAG16 is an important component of the central pair apparatus of motile cilia (9+2 structure; Fig. 5.1C) and is involved in ciliary motility and sperm motility. Cilia and the sperm flagella, are microtubule-based organelles and are responsible for a diverse array of functions. Whilst motile cilia are important for movement and move fluid past cells, for example mucus in the airway, non-motile cilia are important for 'sensing' the environment [233]. Though cilia have long been ignored, it now becomes clear that cilia are important for Hedgehog signaling and can be found in virtually every cell in every tissue, including neurons, brain ventricle ependymal cells where cilia regulate the flow of cerebrospinal fluid (CSF), respiratory and oviduct epithelium. As such, SPAG16 is also present in these tissues containing cells with a ''9+2'' axoneme structure [180, 185, 233, 234]. In the present study, we investigated SPAG16 in more detail by examining the subcellular localization of SPAG16 and specific isoform expression in astrocytes. Moreover, since we hypothesized that the upregulation of SPAG16 in reactive astrocytes in MS lesions is due to 'stress', the effect of *in vitro* stimulation of primary human astrocytes on SPAG16 isoform expression was analyzed.

5.2 Materials and methods

5.2.1 Primary human astrocytes and cell lines

Human Embryonic Kidney 293 (HEK293) cells (ATCC; Manassas, USA) were cultured in dulbecco's modified eagle medium (DMEM high glucose, GIBCO (Invitrogen, Merelbeke, Belgium), supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, Lovendegem, Belgium) and 0.5% Penicillin/Streptomycin (P/S; GIBCO) under optimal growth conditions (37°C, 5% CO_2, humidified air).

The human astrocytoma cell line U373 (ATCC) was cultured in DMEM/F-12 medium (GIBCO), supplemented with 10% FBS and 0.5% P/S at 37°C in a 5% CO_2, humidified incubator. Primary human astrocytes (Sciencell, Uden, The Netherlands) were cultured in astrocyte medium (AM; Sciencell) at 37°C in a 5% CO_2, humidified incubator. For *in vitro* stimulation, primary human astrocytes (80,000 cells/well) were cultured according to supplier's protocol in 24-well plates in AM supplemented with different stimuli (in triplicate) and grown for 2-48h depending on the experiment: 10-50 ng/ml recombinant human ciliary neurotrophic factor (CNTF; Peprotech, Boechout, Belgium), 20 ng/ml recombinant human interleukin-1β (Peprotech) and/or 20 ng/ml recombinant human interferon-γ (IFNγ; eBioscience, Vienna, Austria), 50 µg/ml polyinosinic:polycytidylic acid (Poly I:C; Sigma-Aldrich, Diegem, Belgium) or 100 nM staurosporine (STS; Sigma-Aldrich).

5.2.2 Transfection of HEK293 cells

The pcDNA™3.1/V5-His TOPO® TA Expression Kit (Invitrogen, Ghent, Belgium) was used to transfect HEK293 cells with human SPAG16-2 according to the manufacturer's instructions. Briefly, the SPAG16-2 PCR product was cloned from the MS cDNA library [160] using following primers: SPAG16-2 forward: 5'-ATTATGGCTGCTCAGCGAGGGA-3' and SPAG16-2 reverse: 5'-TTAAAAAATAAC ATACTCAGCTGC-3' and inserted in the pcDNA™3.1/V5-His TOPO® vector. Following analysis and transformation, purified SPAG16-2 plasmids were transfected in HEK293 cells. Geneticin (G418; Invivogen, Toulouse, France) was used for selection of transfected cells (stable transfection). 800 µg/ml G418 was used 48h post-transfection. After 14 days, maintenance medium containing 400 µg/ml G418 was used.

5.2.3 Mass spectrometry

SPAG16-2 transfected cell lysates were separated using electrophoresis on a 12% sodium dodecyl sulfate (SDS) gel and proteins were stained with the Silverquest Silver Staining Kit, according to manufacturer's recommendation (Life technologies). Protein bands were excised, separately trypsinized and resulting peptides were analyzed by nanoliquid chromatography mass spectrometry as previously described [235]. The obtained peptide fragmentation spectra were searched in Proteome Discoverer v1.2 using Sequest v1.2.0.208 and Mascot v2.3.01 against the International Protein Index Human database (v.3.78; 86701 entries). The output of both search engines was validated with Scaffold v3.3.2.

5.2.4 Protein fractionation kit

Protein fractionation was performed using the subcellular protein fractionation kit (Thermo Fisher Scientific, Erembodegem, Belgium) according to the manufacturer's instructions to yield enrichment of proteins from five different cellular compartments, namely the cytoplasmic, membrane, soluble nuclear, chromatin-bound nuclear and cytoskeletal proteins. The Pierce BCA Protein Assay Kit (Thermo Scientific) was used according to kit protocol for protein quantification and protein fractions were stored at -80°C until further analysis.

5.2.5 Protein extraction

Protein extracts were made from primary human astrocytes after *in vitro* stimulation to analyze SPAG16 protein expression. The cells were scraped and lysed in cytoplasmic extraction buffer (CEB; 10 mM Tris-HCl, 60 mM KCl, 1 mM EDTA, 0,1% NP-40; pH: 7,9) and 1% Protease Inhibitor Cocktail (Thermo Scientific) after a 10 min incubation on ice. Lysates were stored at -80°C until further use.

Protein extracts from human brain tissue (N=5 for MS patients and N=6 for non-demented controls (NDCs)) were obtained after homogenization with TRIzol® Reagent (Invitrogen) and final protein pellets were resuspended in 1% SDS.

Total protein concentration of the astrocyte lysates was measured using NanoDrop 2000 (Thermo Scientific) at an absorbance of 280 nm. The Pierce BCA

Protein Assay Kit (Thermo Scientific) was used according to manufacturer's instructions for protein quantification of human brain lysates.

5.2.6 Flow cytometry

Primary human astrocytes (Sciencell) were analyzed for SPAG16 expression using flow cytometry. Incubations were done on ice. Extracellular antibody binding was measured by incubation of 1×10^5 cells with 100 µl anti-SPAG16 antibodies (in-house produced, 30 µg/ml) for 1 h. Next, the cells were incubated for 30 min with a FITC-conjugated goat anti-mouse IgG (1:100, Jackson, Uden, The Netherlands). Isotype control antibodies were used as a negative control (anti-hCG, in-house produced, 30 µg/ml) and 7-AAD (BD Biosciences, Erembodegem, Belgium) was used to gate on living cells. Intracellular antibody binding was measured similarly, using the BD Cytofix/Cytoperm™ Kit (BD Biosciences). The cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

5.2.7 Immunohistochemistry and immunocytochemistry

Analysis of SPAG16 expression in human MS brain lesions was performed as described earlier [205]. Subcellular localization of SPAG16 in HEK293 cells was analyzed by immunocytochemistry. Briefly, HEK293 cells were cultured on poly-L-lysine-treated (PLL; Sigma-Aldrich) glass coverslips. Next, cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.2% (v/v) PBS-Triton and 3 rinses in PBS. Samples were blocked with 3% goat serum in PBS for 30 min and incubated with anti-SPAG16 monoclonal antibodies (in-house produced, 30 µg/ml) in PBS for 2h at room temperature (RT). After 3 washes in PBS, coverslips were further incubated with goat anti-mouse-Alexa555 (Invitrogen; 1:400) in PBS during 1h. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). For negative controls, a set of culture slides was incubated under similar conditions without the primary antibodies. The coverslips were mounted with fluorescent mounting medium (Dako, Heverlee, Belgium) and evaluated with an Eclipse 80i microscope (Nikon, Brussels, Belgium), using standard objectives and NIS-Elements Basic Research Software (Nikon).

5.2.8 Immunoblotting

Protein extracts after subcellular protein fractionation, human brain tissue and primary human astrocyte lysates were analyzed for SPAG16 protein expression using Western blot. Briefly, samples (7,5-10 µg total protein) were diluted 1:1 and boiled in reducing SDS sample-buffer with 5% 2-mercaptoethanol (Sigma-Aldrich). Denatured samples were separated using electrophoresis on 12% SDS gels and proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Overijse, Belgium). The membranes were blocked for 2h in 5% Marvel-PBS-0,1% Tween-20 (5% M-PBS-T) at RT. Subsequently, the membranes were incubated with primary anti-SPAG16 monoclonal antibodies (30 µg/ml; in-house produced) in 5% M-PBS-T at RT for 2h. Next, the membranes were washed in PBS-T and incubated for 1h with HRP-labeled rabbit anti-mouse IgG (1:1500 in 5% M-PBS-T, Dako), followed by chemiluminescent detection (Thermo Fisher Scientific) using the ImageQuant LAS 4000 detector (GE Healthcare Life Sciences). A mouse isotype monoclonal antibody (IgG1) directed against an irrelevant protein was used as a negative control. Protein loading was monitored by stripping the membranes with mild stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween-20, pH 2.2) followed by 5 washes in PBS-T and PBS. Next, the membranes were blocked and reprobed with HRP-labeled mouse anti-GAPDH monoclonal antibodies (1:20,000 in 5% M-PBS-T; Thermo Fisher Scientific), followed by chemiluminescent detection.

5.2.9 Densitometric Analysis

SPAG16 protein bands were analyzed and quantified using the ImageQuant LAS 4000 software (GE Healthcare Life Sciences) and bands were normalized to their respective GAPDH loading control. Data are representative of the average fold change with respect to control for three replicate samples.

5.2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Software Inc.). A P-value <0.05 was considered statistically significant.

5.3 Results

5.3.1 SPAG16 is a cytoplasmic protein

Since we have previously shown elevated autoantibodies against SPAG16 in a subgroup of MS patients [160, 205], and since SPAG16 expression is only thoroughly investigated in the context of fertility (mostly in mice), it was verified whether SPAG16 isoforms were also expressed as membrane proteins or were present in other compartments (e.g. nuclear or cytoskeletal).

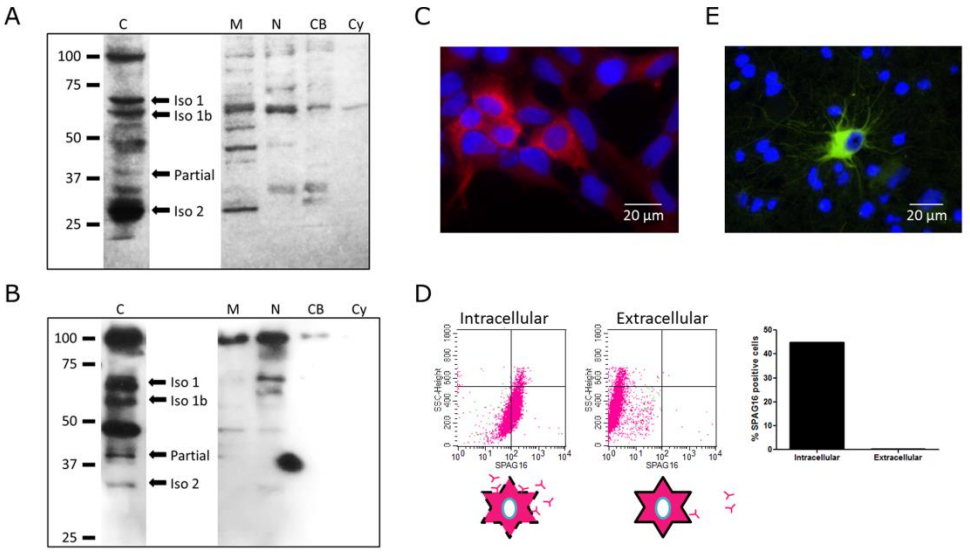

Fig. 5.2: Subcellular localization of SPAG16 and isoform expression. Subcellular protein fractionation was performed on (A) transfected HEK293 cells with SPAG16 isoform 2 and (B) U373 astrocytoma cells. Western blot results of the different fractions are shown. C: cytoplasmic, M: membrane, N: nuclear (soluble), CB: chromatin-bound, Cy: cytoskeletal. (C) Immunocytochemistry of HEK293 cells stained for SPAG16 (red), nuclei are stained blue (DAPI). (D) Intracellular (permeabilized cells) and extracellular flow cytometry analysis was performed on primary human astrocytes to determine SPAG16 localization. Side scatter (SSC) and SPAG16 fluorescence are shown in representative scatter plots. The bar graph shows the percentage of SPAG16 positive cells. Gating was performed according to an isotype control staining. (E) Immunohistochemistry of an MS lesion shows that SPAG16 (green) expression is upregulated in astrocytes, nuclei are stained blue (DAPI). Scale bar; 20 µm.

Performing initial Western blots on cellular lysates indicated multiple specific bands, however the ~20 kDa SPAG16-2 band was not detected, albeit a ~27 kDa band was present. Therefore, HEK293 cells were transfected with SPAG16-2 to verify its expression. As shown in Fig. 5.2A, indeed SPAG16-2 transfected cells, had an elevated expression of a ~27 kDa protein band in the cytoplasmic fraction (the band in the membrane fraction is due to contamination between fractions). Mass spectrometry analysis confirmed 3 peptides 'AIQMAQEQATD TEILER', 'TVGNVPDVYTQIMLLENENK' and 'VLEEALGMGLTAAGDAR' with a peptide probability of 100% corresponding to amino acids 79-95, 14-30, 146-165, respectively of SPAG16-2. SPAG16-1 (~71 kDa), SPAG16-1b (~65 kDa) and partial SPAG16 (~41 kDa) were also present in the cytoplasmic fraction of HEK293 cells and U373 astrocytoma cells (Fig. 5.2A-B). Other bands were also visible and isotype control staining (not shown) indicated that these bands are specific, including a 35 kDa band in transfected HEK293 cells (Fig. 5.2A) in the cytoplasm, nuclear and chromatin-bound fractions, probably corresponding to the SPAG16S nuclear speckle protein described by Zhang et al [230]. Whilst SPAG16-1b was present in all other fractions in HEK293 cells, including the membrane and cytoskeletal fraction, bands were faint and could be due to contamination between fractions. Next, immunocytochemistry of HEK293 cells was performed to confirm SPAG16 expression in the cytoplasm. Indeed, Fig. 5.2C shows SPAG16 staining in the cytoplasm of HEK293 cells. Finally, flow cytometry analysis of primary human astrocytes demonstrated intracellular SPAG16 expression (Fig. 5.2D) which was confirmed in MS lesions, where SPAG16 expression in astrocytes is also cytoplasmic (Fig. 5.2E).

5.3.2 SPAG16 isoform 1 and partial SPAG16 are upregulated in MS

We have previously shown that SPAG16 is upregulated in MS brain lesions, more specifically in reactive astrocytes [205]. To gain more insight into a potential role of SPAG16 in astrocytes, SPAG16 isoform expression was analyzed in age- and gender matched MS and NDC brain tissue (same donors as [205], Table 5.1) using Western blot. As shown in Fig. 5.3A, SPAG16-1, SPAG16-1b and partial SPAG16 were clearly present in MS1, MS2 and MS5. 2/5 MS patients (MS3 and MS4) lacked SPAG16-1 expression. Interestingly, MS3 and MS4 had a very small MS lesion in the used tissue for protein extraction or an inactive lesion, respectively.

In NDCs, SPAG16 protein expression (SPAG16-1 and partial) was less pronounced and SPAG16 expression was significantly lower compared to MS patients (Fig. 5.2B-C). SPAG16-2 expression was low in both MS lesions and NDC brain tissue. These results indicate that SPAG16-1 and partial SPAG16 expression are elevated in neuroinflammatory diseases such as MS.

Table 5.1: Characteristics of the MS patients and controls used for Western blot analysis

Diagnosis	**Age**[a]	**Gender**[b]	**Lesion type**
MS	54.2±5.1	60%	N.A.
MS 1	53	F	Active
MS 2	59	M	Chronic active
MS 3	43	M	Active (small lesion)
MS 4	45	F	Chronic Inactive
MS 5	71	F	Active and chronic active
NDC	65.0±2.4	67%	N.A.
NDC 1	64	M	N.A.
NDC 2	64	F	N.A.
NDC 3	70	M	N.A.
NDC 4	68	F	N.A.
NDC 5	69	F	N.A.
NDC 6	65	F	N.A.

a. Mean age in years ± SEM; b. % females, F: female, M:male; NDC: non-demented control

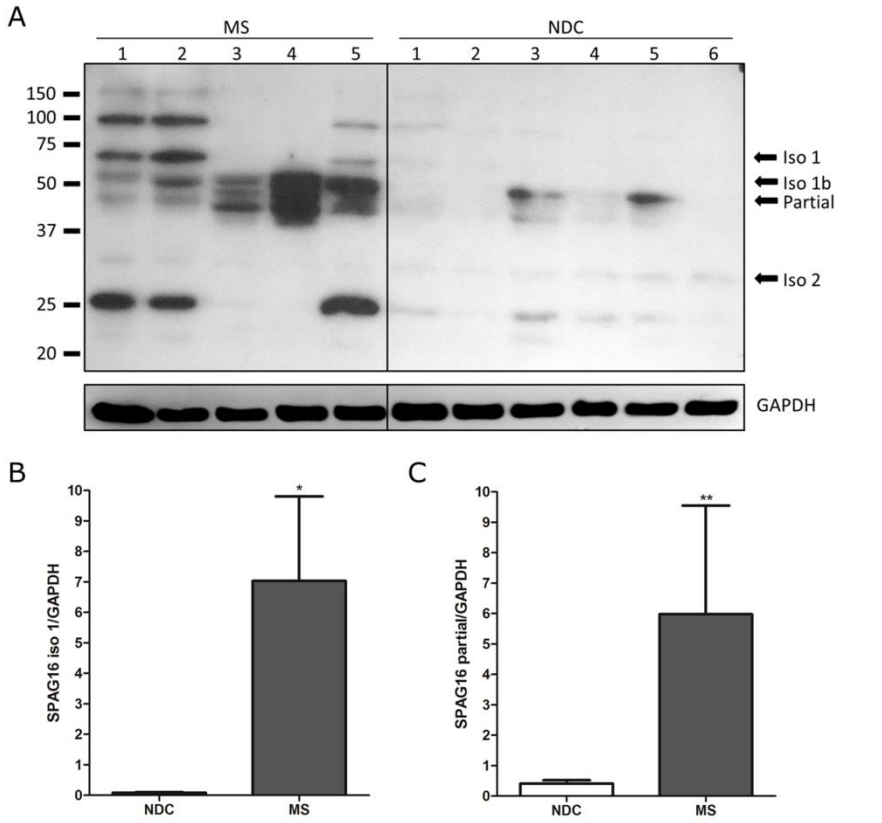

Fig. 5.3: SPAG16 isoform expression in MS and controls. Protein extracts (brain lysates) were prepared from 5 MS patients and 6 non-demented controls (NDCs), (A) followed by analysis of SPAG16 protein levels using Western blot. (B) SPAG16 isoform 1 bands (~71 kDa) and (C) partial SPAG16 (~41 kDa) were quantified and normalized against the loading control GAPDH. Results represent mean ± S.E.M. "*", $P<0.05$; "**", $P<0.01$

5.3.3 Different stimuli upregulate SPAG16 isoform 1 in primary human astrocytes

SPAG16 is upregulated in astrocytes in MS lesions [205], more specifically SPAG16-1 and partial SPAG16 (section 5.3.2), but little is known about the function of SPAG16 in astrocytes and associated mechanisms that upregulate SPAG16 in these cells. We examined if different pro-inflammatory cytokines and other stimuli generally used to activate astrocytes *in vitro* [213, 241, 242] could modulate the expression of SPAG16 in primary human astrocytes. Indeed we

demonstrated that SPAG16-1 is expressed in primary human astrocytes (Fig. 5.4A) and its expression is increased after 24h of treatment with 10 ng/ml CNTF (P=0.0302) and decreased after 48h of treatment with 10 ng/ml CNTF (P=0.0212). After 12h treatment with 10 ng/ml CNTF, SPAG16-1 levels were also increased, but this was not significant (P=0.0578) (Fig. 5.4B).

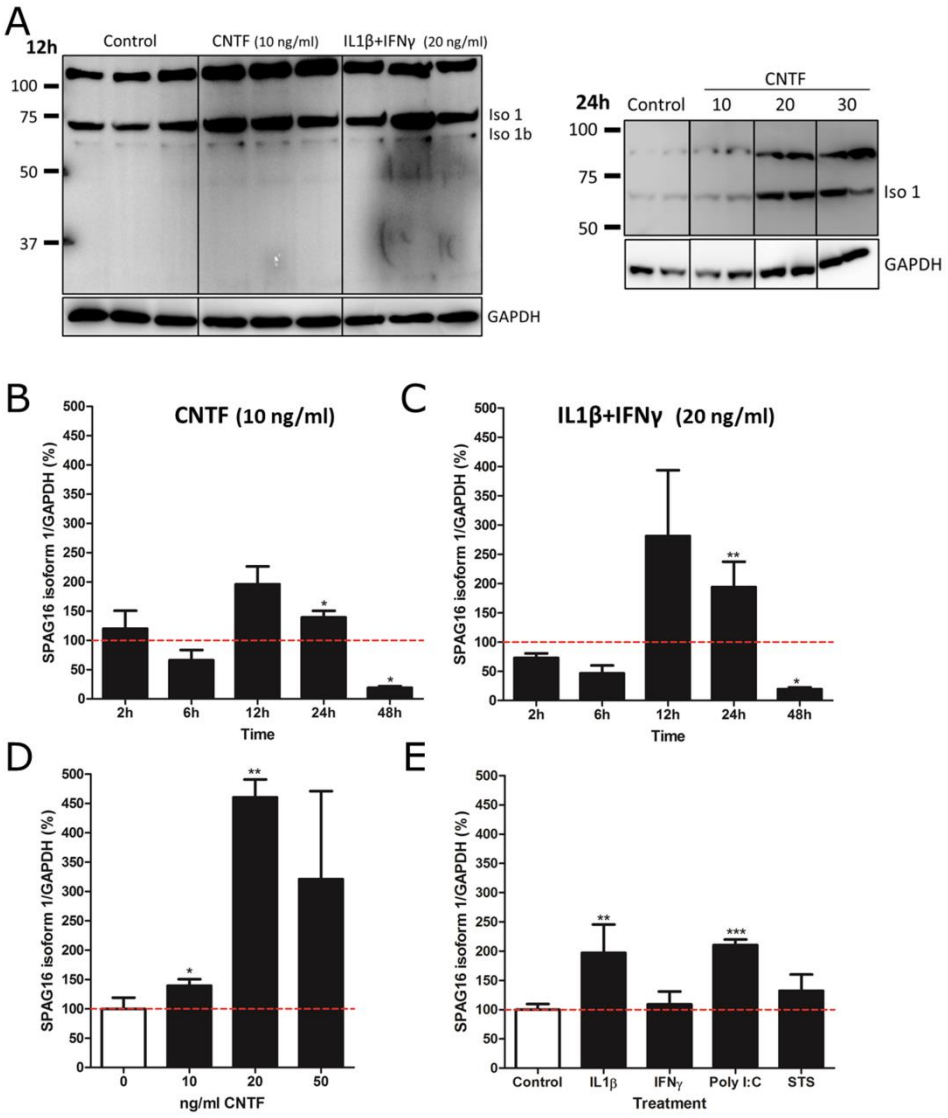

Fig. 5.4: SPAG16 protein expression in primary human astrocytes after *in vitro* exposure to different stimuli. Primary human astrocytes were treated with different

stimuli during 2-48 hours followed by analysis of SPAG16 protein levels using Western blot. SPAG16 isoform 1 bands were quantified and normalized against the loading control GAPDH and expression in control cells (culture medium) was set at 100% (red dotted line). Results represent mean ± S.E.M. of triplicate samples of one experiment. (A) Representative Western blot indicating different SPAG16 isoforms in primary human astrocytes (control, 10 ng/ml CNTF, 20 ng/ml IL1β and 20 ng/ml IFNγ). A ~71 kDa isoform; "iso 1" and the 571 AA isoform; "iso 1b" is visible. (B) Primary human astrocytes were treated with 10 ng/ml CNTF or (C) 20 ng/ml IL1β and 20 ng/ml IFNγ for different time periods followed by Western blot analysis. (D) Primary human astrocytes were treated for 24h with different concentrations of CNTF or (E) 10 ng/ml CNTF, 20 ng/ml IL1β, 20 ng/ml IFNγ, 20 ng/ml IL1β and 20 ng/ml IFNγ, 50 µg/ml Poly I:C or 100 nM staurosporine (STS). "*", P<0.05. "**", P<0.01. "***", P<0.001 versus control.

A similar response was seen after treatment with 20 ng/ml IL1β+20 ng/ml IFNγ (Fig. 5.4C). Furthermore, the upregulation of SPAG16-1 by CNTF seems to be dose-dependent (Fig. 5.4D). Finally, since 24h treatment with CNTF or IL1β+IFNγ, resulted in an upregulated SPAG16-1 expression, more stimuli were tested, including IL1β and IFNγ separately, Poly I:C and staurosporine (Fig. 5.4E). Whilst IL1β alone was able to mimic the previous upregulation of the combination with IFNγ; IFNγ alone or staurosporine had no significant effect on SPAG16-1 protein levels in primary human astrocytes. Further, Poly I:C treatment also resulted in upregulated SPAG16-1 levels. These results indicate, that in an inflammatory milieu like MS lesions, where pro-inflammatory cytokines and mediators activate astrocytes, SPAG16-1 expression can be upregulated as result of this pro-inflammatory environment. However, after 48h a significant downregulation of SPAG16-1 was observed, indicating a more regulating role of astrocyte activation on SPAG16 expression.

5.4 Discussion

SPAG16 is known for its essential role in sperm and cilia motility [185, 236]. However, little is known about the role of this protein in another context, for example in neuroinflammatory demyelinating diseases such as MS.

Here, we investigated the subcellular localization and isoform expression of SPAG16 in MS and primary human astrocytes. Furthermore, SPAG16 is specifically upregulated in activated astrocytes in MS lesions, which suggests possible stress-induced mechanisms of SPAG16 accumulation. To further investigate this possibility, the response of SPAG16 to different stress conditions was examined in primary human astrocytes.

To investigate the subcellular localization of SPAG16, protein fractionation was performed in HEK293 and U373 cell lines, followed by Western blot analysis. SPAG16-2 (~27 kDa), SPAG16-1 (~71 kDa), SPAG16-1b (~65 kDa) and partial SPAG16 (~41 kDa) were all present in the cytoplasmic fraction of HEK293 cells and U373 astrocytoma cells. This corresponds to results obtained in mice by Z. Zhang et al., where SPAG16L (SPAG16-1 in humans) was expressed in the cytoplasm and flagella of sperm cells [237]. Furthermore, Z. Zhang et al. found that similar SPAG16 isoforms were present in human sperm cells [180]. However, an additional 35 kDa SPAG16 isoform (named SPAG16S), representing the C-terminus of SPAG16-1, is expressed only in human and murine male germ cells, and is predominantly found in specific regions within the nucleus [180, 230]. Nevertheless, this 35 kDa isoform is not found in the human NCBI database but only in the murine NCBI database. The other immunoreactive bands (e.g. 50 and 100 kDa) may represent posttranslational processing of SPAG16, including proteolytic cleavage of SPAG16-1 and phosphorylation or other modifications [186]. Finally, we also found that SPAG16-2, which has a predicted molecular weight of ~20 kDa, is heavier and is found at ~27 kDa. Using online bio-informatics tools, SPAG16-2 has 7 predicted phosphorylation sites (including 2 protein kinase C (PKC) sites) and 1 glycosylation site. These and other post-translational modifications could explain the increased molecular weight of SPAG16-2 [238, 239].

Next, immunocytochemistry of HEK293 cells confirmed SPAG16 expression in the cytoplasm, and similar results are observed in the human protein atlas in 3 other cell lines [240].

To gain more insight into a potential role of SPAG16 in MS, SPAG16 isoform expression was analyzed in age- and gender-matched MS and NDC brain tissue. In NDCs, overall SPAG16 isoform expression was less pronounced. SPAG16-1 and partial SPAG16 expression was significantly higher in MS patients. Further, SPAG16-2 expression was low in both MS lesions and NDC brain tissue. These results indicate that SPAG16-1 and partial SPAG16 expression are mostly associated to neuroinflammatory diseases such as MS whilst SPAG16-2 expression is less pronounced.

Although only 5 MS patients were tested, it is evident that differences in SPAG16 isoform expression can be found among patients. This was also an observation by Z. Zhang et al. in sperm lysates from different donors [180]. Furthermore, to gain more insight in the role of SPAG16 in MS pathology, it would be interesting to evaluate SPAG16 isoform expression in more MS patients and in more detail in different lesion types such as early, late, active, chronic (in)active.

SPAG16 is upregulated in astrocytes in MS lesions [205], and results from the present study indicate that SPAG16-1 and partial SPAG16 expression is elevated in MS. However, little is known about the function of SPAG16 in MS lesion astrocytes and associated mechanisms that upregulate SPAG16 in these cells. Since MS lesions are sites with abundance of pro-inflammatory cytokines and cellular stress, we examined whether different pro-inflammatory cytokines and activators of astrocytes could stimulate the expression of SPAG16 in primary human astrocytes. Indeed, SPAG16-1 was upregulated in primary human astrocytes after 24h *in vitro* exposure to CNTF, IL1β and Poly I:C, commonly used to activate astrocytes *in vitro* [213, 241, 242]. In the case of CNTF, this upregulation seemed to be dose-dependent. Of note, each condition was performed in triplicate but most experiments were performed only once so additional repetition is needed. However, the different stimuli indicate a general mechanism of SPAG16-1 modulation in activated astrocytes and links SPAG16-1 expression to inflammation. This is also confirmed in murine primary astrocytes that upregulate SPAG16-1 after 24h and 48h exposure to Poly I:C (data not shown).

Interestingly, the isoform that is predominantly found in activated astrocytes is SPAG16-1. Since SPAG16-1 contains 7 WD-repeats, which are important for protein-protein interactions, it is interesting to find that specifically SPAG16-1

seems to be upregulated in activated astrocytes (*in vitro* and in MS lesions). By interacting with different protein partners, WD-repeat proteins like SPAG16-1 could play a role in different cellular functions, including cell proliferation (SPAG16 is also upregulated in cancers [182, 240]), extracellular matrix composition (in rheumatoid arthritis there is a link between SPAG16 and metalloproteinase 3 (MMP3) [204]), regulation of cytoskeletal assembly (structural stability) and cell motility [231, 232]. Nevertheless, the exact function of SPAG16-1 in astrocytes and its role in MS pathology remains to be elucidated.

In conclusion, our results demonstrate that SPAG16 is a cytoplasmic protein. Furthermore, compared to SPAG16-2, SPAG16-1 is the isoform which is predominantly expressed in human astrocytes. Finally, 24h *in vitro* activation of primary human astrocytes resulted in upregulation of SPAG16-1, indicating that the pro-inflammatory environment of MS lesions could be responsible for the previously observed upregulation of SPAG16 in astrocytes in MS lesions. Future studies investigating the effect of SPAG16 regulation in astrocytes are necessary to provide information about the actual function of SPAG16 beyond sperm cells and other ciliated cells. Furthermore, it is apparent that SPAG16 is more than just a sperm protein. As such, a name change is maybe recommended: SPAG16: **SP**ecial **a**nti**g**en 16.

6

Summary and general discussion

Multiple sclerosis (MS) is a chronic inflammatory and disabling disease of the CNS. Over the past 10 years, MS research has led to a better understanding of RRMS disease mechanisms and the development of different disease-modifying therapies reducing both severity and frequency of new relapses [243]. However, therapeutic options for progressive MS are rather disappointing and remain challenging, due to a lack of understanding of the pathogenic mechanisms driving progressive MS.

In general, there is a great need for novel diagnostic and prognostic biomarkers in MS. Diagnostic biomarkers would facilitate the diagnosis so that patients could start treatment earlier, which is greatly recommended. Furthermore, prognostic biomarkers are needed to predict which patients will convert from CIS to definite MS, predict secondary progression and further progression in PPMS patients. Moreover, there are no approved therapies for PPMS patients , so there is a great need for biomarkers that identify those patients that could benefit from immune therapy and the development of new therapeutic targets [244].

Antibody biomarkers are good candidates for diagnosis and prognosis of autoimmune diseases such as MS, because they reflect the presence, nature and intensity of certain immune responses [105]. In the search for novel antigenic disease markers for MS, we previously identified SPAG16 as a candidate autoantibody target, which had never been linked to MS [160]. In this thesis, the humoral immune response against SPAG16 in MS was characterized in depth. Furthermore, the expression – including potential regulating mechanisms – of SPAG16 in MS were investigated. In this chapter, the main findings are summarized and discussed, and suggestions for future research are proposed.

PART 1: SPAG16 as a target of the humoral autoimmune response in MS

Is SPAG16 targeted by the humoral immune response in MS?

When SPAG16 was first identified as a novel candidate autoantigen in MS, phage-ELISA was used to detect anti-SPAG16 antibodies in the CSF of MS patients and controls. The results demonstrated that anti-SPAG16 antibodies were present in the CSF of 19% of MS patients (sensitivity) with 93% specificity [160]. To validate these results, we investigated the anti-SPAG16 antibody reactivity in MS in more detail in **chapters 2 and 3**.

OCBs and elevated IgG are important hallmarks of MS CSF and are produced in the context of sustained antigenic stimulation in the intrathecal compartment [196]. Furthermore, detection of OCBs in CSF and an elevated IgG index are important diagnostic markers in MS [101]. However, the antigenic targets of these antibodies still remain unknown. Since anti-SPAG16 antibodies were first discovered in MS CSF, isoelectric focusing was performed to detect SPAG16-specific OCBs in the CSF of MS patients and confirm SPAG16 as an antigenic target. We found that ~22% of the MS patients had specific OCBs binding recombinant SPAG16 together with an elevated IgG index, indicating an intrathecal anti-SPAG16 antibody response in these patients. Next, with the biomarker potential of anti-SPAG16 antibodies and easier access to blood in mind, we investigated anti-SPAG16 autoantibodies in plasma in large cohorts of MS patients and controls.

In the two large MS patients' cohorts (N=153, N=374) that we tested, with MS patients from different locations (e.g. Belgium, The Netherlands, Spain), elevated levels of anti-SPAG16 antibodies were present in the plasma of 21%-22% of MS patients with 93%-95% specificity. Hereby we confirmed that 1 in 5 MS patients has an elevated plasma anti-SPAG16 antibody response. We also demonstrated that plasma anti-SPAG16 antibodies were present in ~15% of CIS patients, indicating that these antibodies can be detected early in the disease process but their presence was not correlated to MS conversion. Furthermore, different reactivities were found in RR, SP and PPMS, underlining the heterogeneity of the MS disease course in the different subgroups.

Since SPAG16 is a sperm protein, it is often assumed that anti-SPAG16 antibodies are associated with gender. However, in all our studies, we found no correlation between anti-SPAG16 antibodies and gender, although logistic regression analysis in PPMS patients indicated that female sex is a possible independent predictor of anti-SPAG16 seropositivity. Future studies investigating anti-SPAG16 antibodies should always investigate whether gender is a confounding factor.

In conclusion, in different cohorts we demonstrated that anti-SPAG16 antibodies can be consistently detected in ~21-22% of MS patients. In general we can conclude that SPAG16 is a target of the autoimmune humoral immune response in MS.

Can anti-SPAG16 antibodies be used for diagnosis in MS?

Many different antigenic targets and biomarkers have been described in MS [101, 208] and in this section we will compare anti-SPAG16 antibodies to a selection of these other biomarkers (Table 6.1).

Table 6.1: Anti-SPAG16 antibodies as a biomarker in MS: comparison[a]

	Category	Body Fluid	Biomarker Test	Sensitivity	Specificity
Anti-SPAG16 antibodies	Diagnostic? Prognostic?	CSF, plasma	ELISA	21%	95%
IgG OCBs	Diagnostic	CSF, serum	IEF/IB	>90%	95%[101]
Elevated IgG index	Diagnostic	CSF, serum	Formula	70%	96%[101]
Anti-KIR4.1 antibodies	Diagnostic	Serum	ELISA	47% 7.5% 57%	99%[159] 95%[245] 100%[246]

IEF: isoelectric focusing; IB: immunoblot. * Biomarkers in grey are in use in clinical practice [a] Only astrocyte targets in MS are shown

Anti-SPAG16 antibodies are present in a subgroup of MS patients (~21%) in all tested cohorts. Compared to IgG OCBs and an elevated IgG index, anti-SPAG16

antibodies are present in a smaller group of MS patients but these antibodies can be detected in the blood, whilst for the other biomarkers a lumbar puncture is necessary. Using them as a diagnostic marker would only be recommended in combination with other biomarkers to increase sensitivity. Compared to anti-KIR4.1 antibodies, against another astrocytic target, which initially was a very promising diagnostic marker, when Srivastava et al.[159] demonstrated that 47% of MS patients had antibodies (99% specificity), anti-SPAG16 antibodies are less abundant. However, more recent studies demonstrate that anti-KIR4.1 antibodies are potentially less reliable in MS[245] and are possibly a biomarker for children with acquired demyelinating disease[246]. These studies indicate that antibody biomarkers are good candidates, including anti-SPAG16 antibodies which can be measured and quantified using a stable test (ELISA), but there is a long road from biomarker discovery to clinical application.

Can anti-SPAG16 antibodies be used for prognosis in MS?

To investigate whether anti-SPAG16 antibodies are prognostic, we studied the anti-SPAG16 antibody reactivity in more detail in the different MS subtypes and compared clinical characteristics in **chapter 3.** In **chapter 4** we investigated the prognostic implications of anti-SPAG16 antibody seropositivity and MS conversion in optic neuritis patients.

In our previous *in vivo* EAE experiments, we demonstrated a disease exacerbation in mice injected with anti-SPAG16 antibodies, confirming that these antibodies are related to worse disease outcome (**chapter 2**) [205]. Next, we investigated the different disease characteristics of MS patients when they were stratified according to anti-SPAG16 serostatus (**chapter 3**) and found that anti-SPAG16 seropositive MS patients had a significantly higher EDSS. This indicates a potential role of anti-SPAG16 antibodies in MS pathology and their use as a prognostic biomarker. Although further long-term follow-up studies are needed, in the clinic this could mean that anti-SPAG16 seropositive MS patients would benefit from a more aggressive treatment. Further, when we then divided the MS patients according to MS subtype, we found that a significantly higher proportion of PPMS patients (34%) was positive for anti-SPAG16 antibodies compared to RRMS patients (19%). No significant differences were found compared to the SPMS patient group, whose antibody levels were intermediate

between RRMS and PPMS. We also demonstrated that PPMS patients, in contrast to RRMS and SPMS, were significantly younger when anti-SPAG16 antibodies were detected. Further, seropositive PPMS patients had a significantly shorter disease duration and comparable EDSS as seronegative PPMS patients, which was confirmed by a significantly elevated progression index (EDSS/disease duration) in seropositive PPMS patients. These findings, and our results of elevated anti-SPAG16 antibodies in PPMS, raise the possibility that disease progression in MS is related to antibody-mediated CNS damage and anti-SPAG16 antibodies could be used as a possible prognostic biomarker in PPMS. However, this needs to be confirmed by future follow-up studies of seropositive and seronegative PPMS patients. Additionally, it would be interesting to investigate whether MS patients with anti-SPAG16 antibodies present with a more "inflammatory" PPMS, with a generally worse disease outcome, and whether these antibodies could be used as a biomarker to determine the treatment approach. The results obtained in this thesis suggest that the humoral immune response, or at least anti-SPAG16 antibodies, is involved in a proportion of PPMS patients. It is then tempting to speculate that these PPMS patients could also be sensitive to the current immunomodulatory therapies. The very recent success that was obtained using the B cell depleting anti-CD20 monoclonal antibody ocrelizumab in a phase III trial in PPMS patients, confirms this hypothesis. PPMS patients who were treated with ocrelizumab had a 24% lower risk of confirmed disability progression after 12 weeks, and a 25% lower risk after 24 weeks, compared to patients who were treated with the placebo. Ocrelizumab treatment also resulted in a 29% reduction in walking time, lower T2-weighted lesion volumes and lower brain volume loss [50]. Further, it would be of interest to investigate whether anti-SPAG16 seropositive PPMS patients also present with CSF IgM OCB which has been identified by Villar et al. as a potential biomarker for a subset of PPMS patients with a more active inflammatory disease, who may benefit from immune-directed treatments. Villar et al. also revealed that stratification of PPMS patients according to the presence or absence of CSF IgM OCB identified a subset of PPMS patients who experienced a more aggressive prior clinical course, exhibited an increased number and distinct profile of CSF B cells, and were substantially more likely to demonstrate imaging evidence of active CNS inflammation [212]. These results and

the positive outcomes of the ocrelizumab trial further underline the importance of B cells, and possibly also autoantibodies, in the disease process of PPMS.
In **chapter 4** we investigated the prognostic potential of anti-SPAG16 seropositivity in optic neuritis patients. We found that 29% of ON patients were seropositive for anti-SPAG16 antibodies but we could not provide evidence for a link between anti-SPAG16 serostatus and conversion to MS. Whilst the follow-up period of these patients was approximately 3 years, future follow-up studies of >5 years are crucial to make final conclusions regarding the prognostic value of anti-SPAG16 antibodies in optic neuritis.

How could an autoimmune response arise against SPAG16?
An important question regarding the anti-SPAG16 antibody response in MS, is its origin. In **chapter 5,** we investigated the subcellular localization of SPAG16. We found that SPAG16 is a cytoplasmic protein, in other words it is an intracellular target. Antibody reactivity towards other intracellular targets like SPAG16 (e.g. neurofilament, tubulin) is not uncommon in MS[131, 199]. There are several hypotheses on the generation of an antibody response towards intracellular proteins. These include:

a) Breakdown of self-tolerance of the immune system in autoimmune diseases, including MS. This can be the result of an altered expression pattern (i.e. upregulated SPAG16 expression in reactive astrocytes in MS lesions, as described in **chapter 2**) which could lead to breaking of immune tolerance as exemplified in systemic sclerosis and cancer, where an altered expression pattern of other sperm-associated antigens elicited an autoantibody response[182, 201].

b) Cell damage and death of for example astrocytes and neurons (which express SPAG16) could lead to the exposure of intracellular antigens (epitope spreading). Astrocyte damage has been shown in MS and may be an important feature in lesion formation[247]. Furthermore, glial fibrillary acidic protein (GFAP), a marker for astrogliosis, is a potential biomarker for MS progression[248, 249] and notably astrocytes have recently been suggested as important players in MS progression as well [250]. It would therefore be very interesting to investigate anti-

SPAG16 antibodies in progressive MS in relation to astrocyte damage in those patients (measured by GFAP levels), especially since we have shown that PPMS patients with anti-SPAG16 antibodies have an elevated progression index and possibly a worse prognosis.

c) Molecular mimicry[200, 251]. Interestingly, when searching for possible epitopes of SPAG16 which show sequence similarity with viral or bacterial proteins, we observed that SPAG16 and BLRF2 (an Epstein Barr virus (EBV) tegument protein) show sequence similarity (63%, Fig. 6.1). Studies have suggested the involvement of viruses in the MS pathogenesis by either bystander activation of the immune system or via molecular mimicry with the strongest association with EBV [4, 25, 26]. However, experimental confirmation of binding of anti-SPAG16 antibodies to both peptides is necessary.

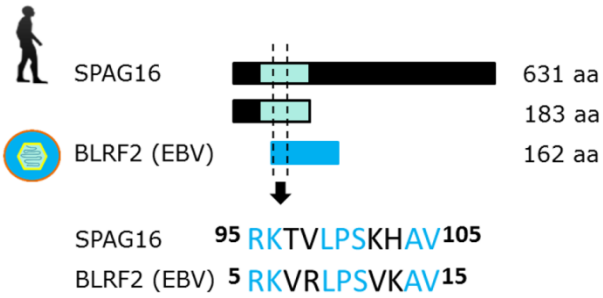

Fig. 6.1: Protein sequence similarity between SPAG16 and BLRF2. The EBV BLRF2 tegument protein (GenBank: CGH11869.1), involved in viral replication, shows 63% amino acid (aa) sequence similarity with SPAG16 isoform 1 (631 aa) and isoform 2 (183 aa); indicated in blue.

Intracellular antigens (e.g. intracellular pathogens or cytosolic targets) can also be processed via autophagy (degradation of unnecessary or dysfunctional cellular components via lysosomes) which is a novel pathway for MHC class II presentation. Autophagy is tightly linked with autoimmune diseases, including MS. Classically, extracellular antigens were thought to be the sole source of peptides for MHC class II presentation[252-254]. Although astrocytes are often described as CNS-resident antigen-presenting cells, their capacity to present antigen (e.g. SPAG16) and activate T cells remains controversial[255].

How do anti-SPAG16 antibodies play a role in MS pathology?

Anti-SPAG16 antibodies are found in a subgroup of MS patients and our results indicate that they play a potential harmful role in MS. In **chapter 2** we studied the pathologic relevance of circulating anti-SPAG16 antibodies by investigating the effect of passive antibody transfer of anti-SPAG16 mAbs in MOG_{35-55} peptide-induced EAE. *In vivo* experiments demonstrated that anti-SPAG16 mAbs significantly exacerbated EAE, compared to the isotype control group. We did not investigate whether anti-SPAG16 antibodies could induce EAE in mice and we did not demonstrate that they could be pathogenic, since we hypothesize (see Fig. 6.2) that anti-SPAG16 antibodies are formed after damage to astrocytes or neurons (resulting in the release of intracellular SPAG16). We therefore did not assume that anti-SPAG16 antibodies were pathogenic but that they are involved in MS pathology. Furthermore, potential mechanisms by which anti-SPAG16 mAbs mediated EAE exacerbation were investigated and we found increased infiltration of macrophages and activation of the complement cascade with formation of the membrane attack complex (C5b-9). In MS patients, fixation of complement by anti-SPAG16 antibodies is also a possible mechanism by which these antibodies could be harmful, since we observed that most patients had IgG1, either alone or in combination with IgG3, which could activate the complement system. Notably, the complement system has long been suspected to play an important detrimental role in MS pathology[256]. Further, Ingram et al. demonstrated that MS plaques were consistently positive for complement proteins and interestingly, cellular staining for complement components was largely restricted to reactive astrocytes[257]. It would be interesting to study whether astrocyte damage and neuronal damage is associated with anti-SPAG16 antibodies, providing further evidence of SPAG16 and anti-SPAG16 antibody involvement in MS pathology.

PART 2: The role of SPAG16 protein in MS

Is SPAG16 expressed differently in MS patients?

Several SPAG16 isoforms exist, but isoform 1 (SPAG16-1; 71 kDa) and isoform 2 (SPAG16-2; 20.4-kDa) are mostly documented. In sperm cells, SPAG16-1 is part of the axoneme and plays a role in sperm motility and thus fertility. In other cells with motile cilia (e.g. lung), SPAG1-1 also plays a role in motility. The function of SPAG16-2 and other isoforms is unknown [179]. In **chapter 2 and 5** we investigated the SPAG16 expression in more detail in the context of MS. Immunohistochemistry analysis revealed that SPAG16 is expressed in neurons in MS patients and controls. In MS brain lesions, SPAG16 was upregulated in reactive astrocytes. Later, we confirmed that SPAG16-1 and partial SPAG16 were mostly upregulated in MS.

Other biomarker targets in MS have been demonstrated to be upregulated in astrocytes such as αB-crystallin and YKL-40 (human analog of the murine breast regression protein chitinase 3-like 1). αB-crystallin is a stress-induced heat shock protein that is increased within or surrounding MS lesions[202] but only low antibody titers towards αB-crystallin could be detected in MS patients and healthy donors[157]. Another target is YKL-40, which is elevated in the CSF of patients with different neuroinflammatory conditions, such as MS and traumatic brain injury but also in cancers and is a biomarker for conversion from CIS to MS. Furthermore, YKL-40 is predominantly expressed in reactive astrocytes near inflammatory lesions[258, 259]. Also, YKL-40 transcription is induced *in vitro* in primary astrocytes by inflammatory mediators (e.g. IL-1β) released by macrophages which was accompanied by morphological changes and altered astrocytic motility[260]. In the case of SPAG16, we have focused on a) antibody reactivity and b) SPAG16 expression in the brain. However, future studies should investigate whether SPAG16 is present or elevated in the CSF (like YKL-40) and plasma of MS patients, e.g. as a result of astrocyte damage, providing further evidence of SPAG16 involvement in MS pathology.

How is SPAG16 upregulated in astrocytes?

Whilst reactive astrogliosis is a prominent feature of MS lesions, the astrocyte is often forgotten as an important player in MS lesion pathology. Traditionally,

astrocytes are mostly associated with their secondary scarring role and not with a role in lesion formation or repair [261]. However, astrocytes have become star players since the interesting findings in the demyelinating condition neuromyelitis optica (NMO) which identified astrocytes as the primary target of damage, resulting from a humoral immune response against the water channel aquaporin-4 [158]. Since we hypothesized that the upregulation of SPAG16 in reactive astrocytes in MS lesions is due to 'local stress' and inflammatory mediators present in the lesion, the effect of *in vitro* stimulation of primary human astrocytes on SPAG16 isoform expression was analyzed in **chapter 5**. Our results demonstrated that SPAG16-1 was upregulated in primary human astrocytes after *in vitro* exposure to CNTF, IL1β and Poly I:C, commonly used to activate astrocytes *in vitro* [213, 241, 242]. The different stimuli indicate a general mechanism of SPAG16-1 upregulation in activated astrocytes (astrogliosis). Future studies should investigate the pathways responsible for SPAG16 upregulation in astrocytes, but also in other cells and other neuroinflammatory diseases (e.g. NMO), to investigate whether upregulation of SPAG16 is a general cellular stress response or specifically linked to MS pathology. Astrocyte damage has been shown early in MS, concomitant with the appearance of perivascular inflammation, and may be an important feature in lesion formation[247, 261]. Depending on the lesion stage and lesion location, astrocytes play multiple roles, participating actively in both lesion development and repair [261]. Furthermore, signs of 'stress' in astrocytes in active lesions are also apparent by cytoplasmic accumulation of oxidized lipids, proteins and sometimes presence of oxidized DNA within their nuclei [262, 263]. Acute apoptotic cell death of astrocytes is rare, suggesting that astrocytes can cope much more efficiently with an oxidative insult in comparison to neurons and oligodendrocytes. Indeed, astrocytes express more anti-oxidant defense molecules for a longer time compared to oligodendrocytes or neurons[264]. Therefore, it would also be interesting to investigate SPAG16 expression in more detail in astrocytes (since these cells cope so well in stress conditions) in the different MS lesion types including early MS lesions.

What are potential functions of SPAG16?

In **chapter 5**, we have shown that SPAG16-1 expression is upregulated after activating primary human astrocytes *in vitro*. However, the exact role of an elevated SPAG16-1 expression in astrocytes is still unknown and an important research question. Based on the role of SPAG16 in sperm cells and motile cilia, its expression in cancer and the role of other SPAG proteins, several potential functions of SPAG16-1 in astrocytes can be proposed:

A. Since SPAG16-1 is part of the axoneme of motile cilia [185], in other cells, SPAG16-1 could also be involved in structural stability and motility of the cell.

B. SPAG16-1 expression has been shown to be increased in cancers, including malignant adenocarcinomas [181, 182]. Therefore, SPAG16-1 could be important for the survival of these cancer cells and be involved in proliferation but also metastasis. Furthermore, in rheumatoid arthritis, there is a link between SPAG16 and matrix metalloproteinases (MMP-3). Since MMPs are important in neuroinflammatory conditions such as MS [71], and for tumor metastasis, and astrocytes have been shown to be involved in modulation of the extracellular matrix [71], SPAG16-1 could somehow be linked to MMP expression.

C. So far, the group of SPAG proteins has grown to reach 15 members, including SPAG16. SPAG16 is not the only 'SPAG' protein that has alternative functions and an expression beyond sperm cells. For example, SPAG9 functions as a scaffold protein that structurally organizes mitogen-activated protein (MAP) kinases and mediates c-Jun-terminal signaling, important for responding to stress stimuli, such as cytokines, ultraviolet irradiation and heat shock. Further, SPAG9 is possibly involved in microtubule-based membrane transport and has been identified as an oncoprotein in human astrocytoma by promoting cell proliferation and invasion [265]. Furthermore, SPAG9 overexpression has been demonstrated in various other cancers including renal, cervical, breast and colon carcinomas [265]. Table 6.2 provides an overview of the other SPAG proteins and possible functions, indicating the wide variety of functions of SPAG proteins and their common involvement in ciliary motility and cancer.

Table 6.2: Other SPAG proteins and their functions

SPAG protein	Potential functions	References
SPAG1	Ciliary motility, cancer cell motility	266, 267
SPAG2	Sperm motility	268
SPAG4	Sperm axoneme stability, cancer migration and invasion	269, 270
SPAG5	Ciliary structural stability, related to poor prognosis in cervical cancer	271, 272
SPAG6	Ciliary motility	184, 273
SPAG7	Unknown	
SPAG8	Cell cycle regulation	274
SPAG9	Signal transduction, cell proliferation and invasion (e.g. astrocytoma, lung cancer, prostate cancer)	265, 275-277
SPAG10	Promotes phagocytosis of apoptotic cells, cancer development	278, 279
SPAG11	Sperm maturation, epididymal innate immunity	280
SPAG12	Unknown	
SPAG13	Role in colorectal cancer, cytoskeletal-associated protein (structural integrity)	281 282
SPAG15	Hyaluronidase involved in oocyte penetration (fertilization), tumor invasion and metastasis	283
SPAG17	Primary ciliary function, regulation of skeletal growth and mineralization	284

Final conclusion and future perspectives

The results obtained in this thesis provide essential insights into the humoral immune response against a previously unknown target in the CNS, SPAG16. We show that in different MS cohorts, anti-SPAG16 antibodies are found in ~21% of MS patients. Furthermore, anti-SPAG16 antibodies are associated with a worse prognosis in overall MS, indicated by an elevated EDSS, and in PPMS, indicated by an elevated progression index. The prognostic value of anti-SPAG16 antibodies should be investigated further in long-term follow-up studies of seropositive and seronegative patients. Also, although we found no evidence for a link between anti-SPAG16 serostatus and conversion to MS in optic neuritis, future follow-up studies are crucial to make final conclusions regarding the prognostic value of anti-SPAG16 antibodies in these patients. Our *in vivo* passive transfer experiments in EAE confirmed that anti-SPAG16 antibodies are detrimental since EAE disease was exacerbated.

In this thesis we have also provided the first evidence of a role for SPAG16 in reactive astrocytes in MS lesions, although our current understanding about the exact function of SPAG16 in these cells remains unknown. Figure 6.2 provides a schematic representation of the current working hypothesis regarding SPAG16 and anti-SPAG16 antibodies in MS. Briefly, astrocytes become activated and SPAG16 is upregulated due to the presence of proinflammatory cytokines and other inflammatory mediators in active MS lesions. This leads to damage to the astrocytes which results in the release of intracellular SPAG16. Neurons, which also express SPAG16, could also be damaged and release SPAG16. Next, antibodies against SPAG16 can be formed and finally anti-SPAG16 antibodies bind their target and mediate further damage, possibly via complement activation, as indicated by our *in vivo* EAE studies.

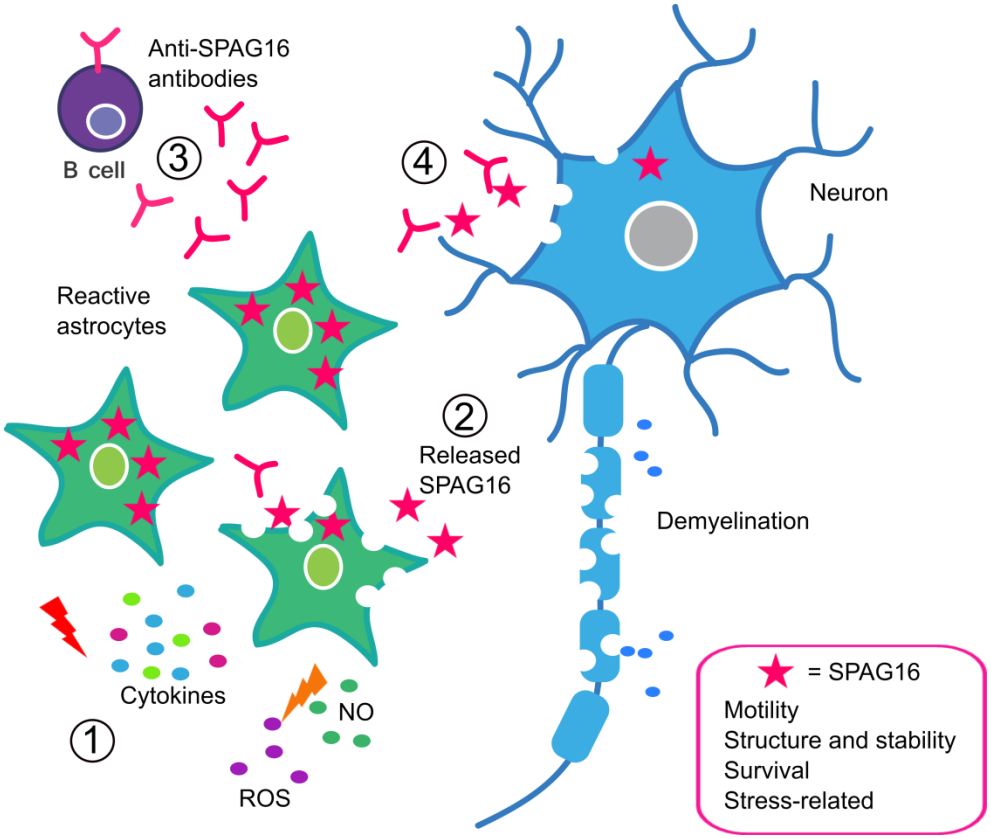

Fig. 6.2: Schematic representation of SPAG16 and anti-SPAG16 antibodies in MS. **1)** Proinflammatory cytokines and other inflammatory mediators, such as nitric oxide (NO) and reactive oxygen species (ROS) present in active MS lesions, activate astrocytes resulting in the upregulation of SPAG16 (★). **2)** Damage to astrocytes releases SPAG16 and **3)** antibodies against SPAG16 can be formed. **4)** Finally, anti-SPAG16 antibodies bind their target and mediate damage. Possible functions of SPAG16 are shown.

Whilst it has become clear in this thesis that SPAG16 is novel antibody target in MS and that SPAG16 has alternative functions besides its role in sperm motility, future research is necessary to unravel more of SPAG16's role in MS pathology. We have shown that activating primary human astrocytes *in vitro* upregulated SPAG16-1 expression. The next step would be to investigate the effect of an elevated SPAG16-1 expression in astrocytes. One strategy would be to transfect astrocytes with the different SPAG16 isoforms, including SPAG16-1 and SPAG16-2, and investigate the effect on astrocyte morphology, viability, physiology and translational alterations. The functional gene pathways associated with SPAG16-

1 upregulation in astrocytes could be studied using next-generation RNA sequencing. Alternatively, the role of SPAG16-1 could be investigated using the SPAG16L knockout model in mice after EAE induction.

In conclusion, SPAG16 is a novel antigenic target of the humoral immune response in MS but much is still unclear regarding the role of SPAG16 in astrocytes and in MS. However, it is apparent that SPAG16 is more than just a sperm protein and future research regarding this interesting antigenic target is warranted in MS.

7

Nederlandse samenvatting

Multiple sclerose (MS) is een chronische aandoening van het centrale zenuwstelsel (CZS). In de afgelopen 10 jaar, heeft MS-onderzoek geleid tot een beter begrip van RRMS ziektemechanismen en de ontwikkeling van verschillende ziekte modificerende therapieën [243]. Echter, zijn de therapeutische opties voor progressieve MS nogal teleurstellend en blijft het zoeken naar nieuwe therapieën een uitdaging vanwege een gebrek aan begrip van de pathogene mechanismen van progressieve MS.

In het algemeen is er een grote behoefte aan nieuwe diagnostische en prognostische biomarkers in MS. Diagnostische biomarkers dragen bij tot een betere diagnose, zodat patiënten eerder met een behandeling kunnen starten, wat sterk wordt aanbevolen. Bovendien zijn prognostische biomarkers nodig om te voorspellen (i) welke patiënten zullen converteren van CIS naar MS, (ii) welke patiënten secundair progressieve MS zullen ontwikkelen en (iii) welke PPMS-patiënten een snellere achteruitgang zullen hebben. Bovendien zijn er geen goedgekeurde therapieën voor PPMS-patiënten, zodat er een grote behoefte is aan biomarkers die patiënten kunnen identificeren die baat kunnen hebben bij de huidige immunotherapie en de ontwikkeling van nieuwe therapeutische targets [244].

Antilichaam biomarkers zijn goede kandidaten voor diagnose en prognose van auto-immuunziekten zoals MS, omdat ze de aanwezigheid, aard en intensiteit van bepaalde immuunreacties weerspiegelen [105]. Bij de zoektocht naar nieuwe antigene ziektemarkers voor MS, hebben we eerder SPAG16 geïdentificeerd als kandidaat autoantilichaam target, dat nooit eerder was gekoppeld aan MS [160].

In dit proefschrift, werd de humorale immuunrespons tegen SPAG16 in MS in meer detail bestudeerd. Verder werd de expressie - met inbegrip van potentiële regulerende mechanismen - van SPAG16 in MS onderzocht. In dit hoofdstuk worden de belangrijkste bevindingen samengevat en besproken, en suggesties voor toekomstig onderzoek worden voorgesteld.

DEEL 1: SPAG16 als een doelwit van de humorale auto-immuun respons in MS

Is SPAG16 een doelwit van de humorale immuunrespons bij MS?

In een eerste fase van het onderzoek naar SPAG16 als nieuw kandidaat autoantigen in MS, werden anti-SPAG16 antilichamen onderzocht in cerebrospinaal vocht (CSV) van MS-patiënten en controles met een faag-ELISA. De resultaten toonden aan dat anti-SPAG16 antilichamen aanwezig waren in het CSV van 19% van de MS-patiënten (gevoeligheid) met 93% specificiteit [160]. Om deze resultaten verder te valideren, onderzochten we de anti-SPAG16 antilichaamreactiviteit in MS in meer detail in **hoofdstukken 2 en 3**.

Oligoclonale banden en een verhoogde IgG zijn belangrijke kenmerken van MS CSV en worden geproduceerd in de context van een aanhoudende antigene stimulatie in de intrathecale ruimte [196]. Voorts zijn de detectie van OCB in CSV en een verhoogde IgG index belangrijke diagnostische markers bij MS [101], maar zijn de antigene doelwitten nog niet bekend. In **hoofdstuk 2** hebben we aangetoond dat SPAG16 een doelwit is van de humorale immuunrespons. Enerzijds hebben we aangetoond dat ~ 22% van de MS-patiënten SPAG16-specifieke OCB hebben in hun CSV. Dit wijst op een intrathecale anti-SPAG16 antilichaam respons bij deze patiënten. Vervolgens onderzochten we anti-SPAG16 autoantilichamen in plasma in grote cohorten van MS-patiënten en controles. Hierbij toonden we aan dat verhoogde niveaus van anti-SPAG16 antilichamen aanwezig waren in het plasma van 21%-22% van de MS-patiënten met 93%-95% specificiteit. We hebben ook aangetoond dat plasma anti-SPAG16-antilichamen aanwezig waren in ~15% van de CIS-patiënten, wat aangeeft dat deze antilichamen vroeg in het ziekteproces kunnen worden gedetecteerd, maar hun aanwezigheid is niet gecorreleerd met MS conversie. Bovendien werden verschillende reactiviteiten gevonden in RR, SP en PPMS (**hoofdstuk 3**), wat de heterogeniteit van het ziekteverloop van MS in de verschillende subgroepen onderstreept.

Concluderend, we hebben in verschillende cohorten aangetoond dat anti-SPAG16 antilichamen consequent werden gedetecteerd in ~21-22 % van de MS-patiënten. In het algemeen kunnen we concluderen dat SPAG16 een doelwit is van de humorale autoimmuunrespons bij MS.

Kunnen anti-SPAG16 antilichamen worden gebruikt voor de diagnose van MS?

Er zijn reeds veel verschillende antigene targets en biomarkers beschreven in MS [101, 208], maar deze worden zelden gebruikt in de kliniek voor de diagnose van MS wegens te lage gevoeligheid en specificiteit. Hoewel anti-SPAG16 antilichamen aanwezig zijn in een subgroep van patiënten met MS (~ 21%) in alle geteste cohorten, toch is het gebruik ervan als diagnostische merker alleen aanbevolen in combinatie met andere biomarkers om de gevoeligheid te verhogen.

Kunnen anti-SPAG16 antilichamen worden gebruikt voor de prognose van MS?

Om te onderzoeken of anti-SPAG16 antilichamen ook een prognostische waarde hebben, hebben we deze antilichamen in meer detail bestudeerd in de verschillende MS subtypes en klinische kenmerken vergeleken in **hoofdstuk 3**. In **hoofdstuk 4** hebben we de prognostische implicaties van anti-SPAG16 antilichaam seropositiviteit en MS conversie onderzocht in neuritis optica patiënten.

Eerdere *in vivo* experimentele autoimmune encephalomyelitis (EAE; diermodel voor MS) experimenten toonden aan dat injectie met anti-SPAG16 antilichamen resulteerde in een verergerde ziekte, hetgeen bevestigt dat deze antilichamen zijn gerelateerd aan een slechtere ziekte uitkomst (**hoofdstuk 2**) [205]. Vervolgens onderzochten we de verschillende ziektekenmerken van MS-patiënten wanneer ze werden gestratificeerd volgens hun anti-SPAG16 serostatus (**hoofdstuk 3**) en vonden dat anti-SPAG16 seropositieve MS-patiënten een significant hogere ziektescore (EDSS) hadden. Dit wijst op een mogelijke rol van anti-SPAG16 antilichamen in MS pathologie en hun gebruik als een prognostische biomarker. Hoewel verdere langere termijn follow-up studies nodig zijn, in de kliniek zou dit kunnen betekenen dat de anti-SPAG16 seropositieve MS-patiënten baat zouden hebben bij een agressievere behandeling. Wanneer we vervolgens per MS subtype keken, vonden we dat een significant hoger percentage PPMS-patiënten (34%) positief was voor anti-SPAG16 antilichamen vergeleken met RRMS-patiënten (19%). Verder hadden PPMS seropositieve patiënten een significant kortere ziekteduur en vergelijkbare

EDSS als seronegatieve PPMS-patiënten, hetgeen werd bevestigd door een significant verhoogde progressie index in seropositieve PPMS-patiënten. Deze bevindingen, en de resultaten van verhoogde anti-SPAG16 antilichamen in PPMS, wijzen op het mogelijke gebruik van deze antilichamen als een prognostische biomarker in PPMS.

In **hoofdstuk 4** onderzochten we de prognostische mogelijkheden van anti -SPAG16 seropositiviteit bij neuritis optica patiënten. We vonden dat 29% van deze patiënten seropositief waren voor anti-SPAG16 antilichamen maar we konden geen bewijs aantonen voor een verband tussen anti-SPAG16 serostatus en conversie naar MS. Terwijl de follow-up periode van deze patiënten ongeveer 3 jaar was, zijn toekomstige follow-up studies van >5 jaar cruciaal om definitieve conclusies te trekken met betrekking tot de prognostische waarde van anti-SPAG16 antilichamen in neuritis optica.

Hoe kan een auto-immuunreactie ontstaan tegen SPAG16 ?

Een belangrijke vraag met betrekking tot de anti-SPAG16 antilichaamrespons in MS, is zijn oorsprong. In **hoofdstuk 5** onderzochten we de subcellulaire lokalisatie van SPAG16. We vonden dat SPAG16 een cytoplasmatisch eiwit was, met andere woorden een intracellulair doelwit. Antilichaamreactiviteit tegen andere intracellulaire doelwitten zoals SPAG16 is niet ongewoon in MS [131, 199]. Er zijn een aantal hypothesen voor het ontstaan van een antilichaamrespons tegen intracellulaire eiwitten. Deze omvatten:

a) Algemene ontregeling van het immuunsysteem bij auto-immuunziekten zoals MS. Dit kan het gevolg zijn van een veranderd expressiepatroon (dwz opgereguleerd SPAG16 expressie in reactieve astrocyten in MS-laesies, zoals beschreven in **hoofdstuk 2**) wat kan leiden tot het doorbreken van de immuuntolerantie.

b) Celschade en celdood van bijvoorbeeld astrocyten en neuronen (die SPAG16 tot expressie brengen) zou kunnen leiden tot het vrijzetten van intracellulaire antigenen zoals SPAG16 (epitoop verspreiding).

c) Moleculaire mimicry (kruisreactiviteit) [200, 251]. Bij het zoeken naar mogelijke epitopen van SPAG16 welke sequentiegelijkenis vertonen met de virale of bacteriële eiwitten, vonden we 63% overeenkomst tussen SPAG16 en BLRF2 (een Epstein Barr virus (EBV) eiwit). Studies suggereren de betrokkenheid van virussen in de pathogenese van MS met de sterkste associatie met EBV [4, 25, 26].

Hoe kunnen anti-SPAG16 antilichamen een rol spelen in MS pathologie?
Anti-SPAG16 antilichamen werden gevonden in een subgroep van MS-patiënten en onze resultaten geven aan dat zij een potentieel schadelijke rol spelen in MS. In **hoofdstuk 2** hebben we de pathologische relevantie van circulerende anti-SPAG16 antilichamen verder onderzocht door het effect van passieve transfer van anti-SPAG16 antilichamen in EAE te bestuderen. Deze *in vivo* experimenten toonden aan dat anti-SPAG16 antilichamen de EAE ziektesymptomen aanzienlijk verergerden, in vergelijking met de controle groep. Potentiële mechanismen voor deze anti-SPAG16 antilichaam gemedieerde EAE-exacerbatie werden vervolgens onderzocht en we vonden een verhoogde infiltratie van macrofagen en activatie van de complement cascade. Het zou interessant zijn om te onderzoeken of de schade aan astrocyten en neuronen geassocieerd is met anti-SPAG16 antilichamen. Dit zou verder bewijs leveren voor de betrokkenheid van SPAG16 en anti-SPAG16 antilichaam in de pathologie van MS.

DEEL 2: De rol van het SPAG16 eiwit in MS

Is SPAG16 expressie anders in MS-patiënten?

Er bestaan verschillende SPAG16 isovormen, maar isovorm 1 (SPAG16-1, 71 kDa) en isovorm 2 (SPAG16-2; 20,4 kDa) zijn het beste gedocumenteerd. In zaadcellen, is SPAG16-1 een onderdeel van het axoneem en speelt het een rol in sperma motiliteit en dus vruchtbaarheid. In andere beweeglijke cellen met cilia (bijvoorbeeld longen), is SPAG1-1 ook betrokken in motiliteit. De functie van SPAG16-2 en andere isovormen is onbekend [179]. In **hoofdstuk 2 en 5** onderzochten we de SPAG16 expressie in meer detail in de context van MS. Immunohistochemische analyse toonde aan dat SPAG16 tot expressie komt in neuronen in MS-patiënten en controles. In MS-leasies, werd SPAG16 expressie opgereguleerd in reactieve astrocyten. Later, bevestigden we dat SPAG16-1 meestal wordt opgereguleerd in MS.

Hoe wordt SPAG16 opgereguleerd in astrocyten?

Volgens onze hypothese is de opregulatie van SPAG16 in reactieve astrocyten in MS-laesies het gevolg van 'locale stress' en ontstekingsmediatoren in de laesie. Om deze hypothese te toetsen, werd het effect van *in vitro* stimulatie van primaire humane astrocyten op SPAG16 expressie geanalyseerd in **hoofdstuk 5**. Onze resultaten toonden aan dat SPAG16-1 werd opgereguleerd in primaire humane astrocyten na *in vitro* blootstelling aan CNTF, IL1β en Poly I:C, die vaak gebruikt worden om astrocyten *in vitro* te activeren [213, 241, 242]. In toekomstige studies dienen de pathways die resulteren in een opregulatie van SPAG16 in astrocyten, maar ook in andere cellen en andere neuro-inflammatoire ziekten, verder onderzocht te worden.

Wat zijn mogelijke functies van SPAG16?

In **hoofdstuk 5** hebben we aangetoond dat SPAG16-1 expressie opgereguleerd wordt na *in vitro* activatie van primaire humane astrocyten. Echter, de precieze rol van een verhoogde SPAG16-1 expressie in astrocyten is nog onbekend en dit blijft een belangrijke onderzoeksvraag. Op basis van de rol van SPAG16 in spermacellen en beweeglijke cilia, de expressie in kanker en de rol van andere

SPAG proteïnen, worden verschillende mogelijke functies van SPAG16-1 in astrocyten voorgesteld:

A. Aangezien SPAG16-1 behoort tot het axoneem van beweeglijke cilia [185], in andere cellen zou SPAG16-1 ook betrokken kunnen zijn bij structurele stabiliteit en motiliteit van de cel.

B. Een verhoogde SPAG16-1 expressie is aangetoond in kankers, waaronder kwaadaardige adenocarcinomen [181, 182]. Daarom kan SPAG16-1 belangrijk zijn voor de overleving van deze kankercellen en betrokken zijn bij proliferatie, maar ook metastase. Bovendien, bij reumatoïde artritis, is er een verband tussen SPAG16 en matrix metalloproteinasen (MMPs). Omdat MMPs belangrijk zijn in neuro-inflammatoire aandoeningen zoals MS en tumormetastasen, en astrocyten zijn ook betrokken bij de modulatie van de extracellulaire matrix kan SPAG16-1 ook gekoppeld zijn aan MMP expressie [71].

C. Tot nu toe, bevat de groep van SPAG eiwitten 15 leden, waaronder SPAG16. SPAG16 is niet het enige 'SPAG' eiwit dat alternatieve functies en een expressie in andere cellen naast zaadcellen heeft. Bijvoorbeeld, SPAG9 fungeert als een helpereiwit dat structureel mitogeen geactiveerde proteïne (MAP) kinasen organiseert en medieert c-Jun-terminale signalering, belangrijk voor het reageren op stimuli, zoals cytokinen, ultraviolette straling en warmte shock. Verder is SPAG9 mogelijk betrokken bij microtubulus gestuurd membraantransport en is geïdentificeerd als een onco-eiwit in humane astrocytoma waar het celproliferatie en invasie bevordert [265]. Over het algemeen zijn de andere SPAG eiwitten betrokken bij ciliaire motiliteit en kankergroei.

Finale conclusie en toekomst perspectieven

De resultaten in dit proefschrift leveren essentiële inzichten in de humorale immuunrespons tegen een voorheen onbekend doelwit in het CZS, SPAG16. We hebben aangetoond in verschillende MS cohorten dat anti-SPAG16 antilichamen in ~21 % van de MS-patiënten aanwezig zijn. Bovendien lijken anti-SPAG16 antilichamen geassocieerd te zijn met een slechtere prognose in MS maar de prognostische waarde van deze antilichamen dient verder onderzocht te worden in de lange-termijn follow-up studies.
In dit proefschrift hebben we ook het eerste bewijs geleverd voor een rol voor SPAG16 in reactieve astrocyten in MS-laesies, hoewel onze huidige kennis over de precieze functie van SPAG16 in deze cellen nog niet bekend is. De huidige werkhypothese met betrekking tot SPAG16 en anti-SPAG16 antilichamen in MS wordt hieronder kort opgesomd:

1. Astrocyten worden geactiveerd en SPAG16 wordt opgereguleerd door de aanwezigheid van pro-inflammatoire cytokines en andere ontstekingsmediatoren in actieve MS-laesies.
2. Dit leidt tot schade aan de astrocyten (en neuronen) wat resulteert in het vrijkomen van intracellulair SPAG16.
3. Vervolgens kunnen antilichamen tegen SPAG16 gevormd worden.
4. Tenslotte kunnen anti-SPAG16 antilichamen hun doel binden en verdere schade veroorzaken, eventueel via complement activatie.

Hoewel het in dit proefschrift duidelijk is geworden dat SPAG16 een nieuw antilichaam target is in MS en dat SPAG16 alternatieve functies heeft naast zijn rol in de beweeglijkheid van de zaadcellen, is toekomstig onderzoek nodig om de exacte rol van SPAG16 in MS pathologie te ontrafelen. We hebben aangetoond dat het *in vitro* activeren van primaire humane astrocyten leidt tot een opregulatie van SPAG16-1 expressie. De volgende stap is om het effect van een verhoogde SPAG16-1 expressie in astrocyten verder te onderzoeken, eventueel via transfectie studies of met het SPAG16 knockout model in muizen.

Concluderend, SPAG16 is een nieuw doelwit van de humorale immuunrespons in MS maar er is nog veel onduidelijkheid over de exacte rol van SPAG16 in astrocyten en MS. Toch blijkt uit dit proefschrift dat SPAG16 meer is dan een zaadcel eiwit en toekomstig onderzoek rond dit interessante doelwit in MS is fundamenteel.

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Curriculum vitae

Laura de Bock werd geboren op 21 februari 1986 in Brugge. In 2004 behaalde zij haar diploma Algemeen Secundair Onderwijs (ASO) in de afstudeerrichting Wiskunde (6u)-Wetenschappen aan het St.-Jan Berchmanscollege in Zonhoven. In datzelfde jaar startte ze haar opleiding Biomedische Wetenschappen aan de Universiteit Hasselt waar ze in 2007 haar diploma Bachelor in de Biomedische Wetenschappen met onderscheiding behaalde. Aansluitend behaalde ze in 2009 haar diploma Master in de Biomedische Wetenschappen, in de afstudeerrichting Klinische moleculaire wetenschappen, met grootste onderscheiding. Haar masterthesis getiteld "The effects of prenatal exposure to flavanoids" voerde zij uit aan de Universiteit Maastricht in de onderzoeksgroep "Health Risk Analysis and Toxicology" van Dr. S. Khosrovani. Tijdens haar masteropleiding behaalde ze tevens de certificaten voor Proefdierkunde I (FELASA B) en Stralingsbescherming (Uhasselt en SCK-CEN). In september 2009 startte zij haar doctoraat aan het Biomedisch Onderzoeksinstituut (BIOMED) in de Immunologie/Biochemie groep onder het promotorschap van Prof. Dr. Veerle Somers en het co-promotorschap van Prof. Dr. Piet Stinissen. In 2010 schreef en behaalde zij een FWO-beurs voor haar doctoraat, gericht op het verder bestuderen van een nieuw autoantigen in multiple sclerose: SPAG16. Verder behaalde ze in 2009 extra onderzoeksfinanciering (€40.000) van de Belgische Charcot Stichting en in 2014 van de Rotary International (€5.000). Daarnaast was ze lid van het onderwijsteam in de opleidingen Biomedische Wetenschappen en Geneeskunde, begeleidde stagestudenten in de bachelor en master opleiding, en volgde zelf de cursussen Proefdierkunde II (FELASA C), Project Management, Bioveiligheid, Good scientific conduct and lab book taking, Career Management, Patent Databanken, Masterclass MS pathologie, Parametrische en niet-parametrische statistische methoden voor de levenswetenschappen, en eiwit purificatie. Ze was ook betrokken bij de organisatie van het "International Life Sciences Master Student Research Conference" in 2012 te Diepenbeek en nam zelf deel aan verscheidene nationale en internationale congressen waar ze haar onderzoeksresultaten presenteerde aan andere wetenschappers. Verder werkte ze tijdens haar doctoraat ook samen met enkele andere internationale

onderzoeksgroepen (met gezamenlijke publicaties) waaronder de groep van Prof. Dr. van der Helm-van Mil A. (Leiden University Medical Center) en Dr. Villar L. (Hospital Ramón y Cajal, Madrid).

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Eleanor Roosevelt