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UNIVERSITÉ DE GENÈVE
FACULTÉ DE MÉDECINE

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BIOMARKERS OF DISEASE PROGRESSION IN MULTIPLE SCLEROSIS:

TOWARDS MEASURING THE FOREST BEHIND THE TREE

Thesis submitted to the Faculty of Medicine of
the University of Geneva

for the degree of Privat-Docent
by

Claire Bridel

Geneva 2022

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Foreword

Neuroimmunology is the discipline that specializes in the care of people with multiple sclerosis (PwMS) and related diseases. It is one of the most dynamic fields of clinical neurology, with means to treat PwMS increasing steadily since the early 1990's. In MS, neurological disability accumulation is driven by two clinically distinct processes: relapses and progression. Relapses are defined as episodes of acute or subacute focal neurological deficits followed by varying degrees of recover. They are the most conspicuous initial clinical manifestations of MS in most patients, but their frequency decreases with time, becoming sparse after 10 to 15 years of disease evolution. Progression is defined as slow neurological disability accumulation occurring independently of relapses and MRI signs of acute focal inflammatory disease activity. It is an insidious process that is difficult to identify clinically, especially early in the disease course, as it may be masked by relapses. Its rate increases with time, becoming the major process driving disability accumulation later in the disease course in a majority of PwMS. All currently available disease modifying therapies (DMTs) are modulators of the peripheral immune system, targeting relapses and acute focal inflammatory disease activity with varying degrees of efficacy. Treatments specifically targeting progression are lacking, although this process contributes overwhelmingly to long-term physical and cognitive disability. Evaluating new drugs to reduce progression rate requires robust tools to quantify this process over the relatively short time frame of clinical trials. Such tools are lacking, but there are promising candidates. The absence of drugs to significantly slow progression and its devastating effect on the life of PwMS is a reality I witness daily in clinical practice. In the last years, I have thus focused my research on identifying and evaluating candidate body fluid biomarkers of progression, as well as on developing tools to measure these biomarkers. It is a challenging endeavor because the physiopathological underpinnings of progression are poorly understood and likely manifold. Several biomarkers will probably be needed to capture this complexity. In this thesis, I start by giving an overview of clinical, immunological, and pathological aspects of MS. I then give an overview of the state of knowledge of one of the most promising body fluid candidate biomarker of progression, as a background to the published manuscripts I selected for the body of this thesis. Finally, I discuss the challenges ahead, provide an overview of a research project I am currently working on thanks to the support of the *Fondation privée des Hôpitaux Universitaires de Genève* and *the Fondation Schmidheiny*, and present a new project I would like to start shortly in collaboration with the laboratory of Professor Jean-Charles Sanchez.

Introduction to multiple sclerosis

MS is a clinically, radiologically, and pathologically heterogeneous chronic inflammatory and degenerative disease of the central nervous system (CNS) of unknown etiology. In Switzerland, its prevalence is of approximately 1 in 1000, with a clear female predominance and a peak incidence in the late twenties and early thirties.[1] The risk of developing MS is higher among relatives of PwMS than in the general population, indicating a genetic susceptibility to MS. Genome wide association studies (GWAS) identified more than 200 gene variants linked to MS, most of which are regulators of the innate or adaptive immune system.[2] Human leucocyte antigen gene cluster (HLA) haplotype DRB1*1501 is to date the strongest identified genetic risk factor of MS.[2] Monozygotic twins have a disease concordance rate of about 25%, suggesting the genetic risk of MS is modulated by environmental factors.[2] The further from the equator, the higher the prevalence of MS, indicating geographical latitude is an important modulator of MS risk.[3] Epidemiological studies demonstrated that individuals migrating from an area of low to high prevalence of MS in childhood, or conversely, acquire the endemic risk of MS.[4] Ultraviolet (UV) radiation and/or vitamin D, both of which are lower in regions of high MS prevalence, have been suggested to mediate this latitude effect. UV radiation is suggested to protect against MS both through vitamin D dependent and independent effects on the immune system.[5] Vitamin D has broad effects on the immune system, including suppression of B and T cell proliferation, skewing of T cells from inflammatory responses towards T regulatory responses, and promoting monocyte and dendritic cell tolerogenic phenotypes.[6] Studies showed that increased vitamin D levels before the age of 20 were associated with a lower risk of MS, and that increasing serum levels of its metabolite 25-hydroxyvitamine D were associated with low disease activity in PwMS or subgroups thereof.[7,8] However, recent randomized controlled trials failed to reveal a significant effect of vitamin D supplement on disease activity or time to confirmed progression.[9,10] Other environmental factors associated with MS include cigarette smoking, which has been associated both with an increased susceptibility to MS and a poorer disease course,[6,11] and childhood obesity.[12,13] Viral infections have long been considered in the pathogenesis of MS, in particular Epstein-Barr virus (EBV), a highly B-cell tropic virus. Recently, a large epidemiological study highlighted the key role of EBV infection in MS susceptibility.[14] The mechanisms linking EBV infection to MS remain, however, to be elucidated.[15]

Clinical and radiological aspects of multiple sclerosis

There is no specific diagnostic biomarker for MS. Diagnosis relies on clinical, radiological, and biological evidence supporting MS, together with reasonable exclusion of alternative diagnoses. MRI plays a central role in the diagnosis of MS. White matter plaques, the hallmark of MS pathology, can be visualized on

standard clinical MRI scans.[16,17] Although they can occur virtually anywhere in the CNS, there is a predilection for five regions of the CNS, consisting of the optic nerves, periventricular and subcortical white matter, posterior fossa, and spinal cord.[17] The presence of plaques in 2 out of the latter 4 characteristic regions, together with their morphology and signal abnormalities on conventional MRI sequences (T2-weighted, T2-FLAIR, pre- and post-contrast T1-weighted scans), provide high specificity for the diagnosis of MS.[17] McDonald et al published the first consensus diagnostic criteria for MS in 2001.[18] These criteria have been revised periodically over the years, but the foundations have remained the same, and consist of demonstrating disease dissemination in space and time. In other words, they aim at attesting the multifocal and recurrent nature of the disease. Clinically isolated syndrome (CIS) is diagnosed when a patient experiences a clinical episode suggestive of MS, but fails to fulfill dissemination in time.[19] Most patients with CIS will evolve towards a diagnosis of MS, and the risk can be stratified according to biological, radiological and demographical predictors.[20]

For a majority of PwMS (80-90%), the initial phase of the disease is characterized by acute or subacute episodes of neurological deficits followed by varying degrees of recovery, called relapses (relapsing remitting MS, RRMS). Relapses correspond pathologically to acute white matter plaques, as will be discussed later, and radiologically to Gadolinium enhancing lesions. Ten to fifteen years after diagnosis, most people with RRMS transition to secondary progressive MS (SPMS), characterized by unrelenting disability accrual. A minority of PwMS (10-20%) are diagnosed with primary progressive MS (PPMS), which lacks the initial relapsing-remitting phase of the disease, and follows a clinical course similar to that of SPMS.[21] Current diagnostic nomenclature separates PwMS into active or inactive, according to the presence or not of clinical and/or radiological signs of acute focal inflammatory disease activity, regardless of the underlying MS subtype (RRMS, SPMS, or PPMS).[22]

Progression is defined as the irreversible accumulation of neurological disability occurring in the absence of acute focal inflammation, which includes relapses, new or enlarging T2 lesions, or Gadolinium enhancing lesions. Initially thought to be a hallmark of PPMS and SPMS, recent evidence indicate progression can occur from disease onset in people with RRMS. Indeed, with the advent of highly efficient drugs which silence acute focal inflammatory disease activity, disability accrual has become apparent in a proportion of these patients.[23] Incomplete recovery from relapses contributes to disability accumulation during the relapsing phase of the disease, but progression is the major contributor to long-term physical and cognitive disability.[24] In clinical practice, progression is considered if a patient shows an increase in disability, usually measured with the expanded disease status score (EDSS), which is confirmed over time and not attributable to a relapse.[25,26] The most widely used definition of progression considers the patient's baseline EDSS. If it is < 6, ≥ 1 point of sustained EDSS impairment is needed for progression to be diagnosed, whereas ≥ 0.5 points are required if baseline EDSS is > 6.0.[27] Brain and spinal cord atrophy,

which is observed at all stages of MS including CIS, is strongly associated with disability accumulation.[28,29] However, the use of CNS atrophy as biomarker of progression in daily clinical practice is prevented by multiple confounding factors that can alter its measurement, such as technical limitations, natural physiological fluctuations in brain volume, MS-related edema and gliosis, and superimposed brain volume loss due to ageing.[30,31]

Immunology of multiple sclerosis

The prevailing physiopathological hypothesis of MS proposes an autoimmune origin triggered by environmental factors that act on a genetically susceptible host. Specifically, myelin autoreactive T lymphocytes triggered in the periphery are thought to infiltrate the CNS and cause, together with activated innate immune cells (microglia and macrophages), an acute inflammatory reaction leading to demyelination and neuroaxonal loss. This hypothesis was initially formulated based on studies of a mouse model of MS, experimental autoimmune encephalomyelitis (EAE). EAE is generated by active immunization with whole myelin proteins or major histocompatibility class II (MHC II)-restricted myelin epitopes, in combination with adjuvants, and leads to an inflammatory disease of the CNS that is clinically, immunologically and histopathologically similar to MS.[32] Adoptive transfer of activated $CD4^+$ T cells that recognize CNS antigens from a mouse with EAE to a syngeneic healthy mouse directly induces EAE in the recipient mouse, indicating autoreactive $CD4^+$ T cells are sufficient to trigger the inflammatory demyelinating disease.[33] Further evidence for a central role of $CD4^+$ T cells in the physiopathology of MS came from GWAS. Single nucleotide polymorphisms in genes coding for T cell survival/growth cytokines interleukin (IL)-2 and IL-7 are associated with MS risk.[34,35] The strongest association identified wo far is with genes that encode the MHCII molecules, which are necessary for the activation of $CD4^+$ T cells by antigen-presenting cells.[36]

Autoreactive lymphocytes in experimental autoimmune encephalomyelitis and multiple sclerosis

Not all CNS autoreactive CD4+ T cells are able to induce EAE in wild-type mice. Interferon (IFN)γ-producing CD4+ T helper (Th) 1 cells were initially thought to be the main autoreactive T cell subset implicated in EAE and MS because IFNγ and macrophage infiltrates are a central pathological feature of both EAE and MS lesions, suggesting a Th1-driven inflammatory response.[37] However, it was later established that Th17 cells, another effector subset of $CD4^+$ T cells, can also trigger EAE.[38,39] Th17 cells are present in active white matter MS lesions, among peripheral blood mononuclear cells (PBMCs) and in the cerebrospinal fluid (CSF) of PwMS.[40,41] The majority of EAE models are driven by CD4+ T cells and MHCII, and MS research has thus mostly focused on these cells. The role of $CD8^+$ T cells in MS is much less well characterized, despite being the most abundant T cell subset in the perivascular and parenchymal infiltrates of MS lesions, as will be discussed in the next section. In MS lesions, $CD8^+$ T cells were reported

to interact with microglia, oligodendrocytes, and transected axons in MS lesions, and granzyme-B expressing $CD8^+$ T cells have been observed near demyelinated axons with cytotoxic granules polarized towards axons.[42,43] These data suggest a direct role for $CD8^+$ T cells in the damage to myelin and axons. In addition, MHCI alleles have been associated with increased susceptibility (HLA-A3A*0301) or resistance (HLA-A*0201) to MS, suggesting an implication of cytotoxic pathways in MS.[43] Although autoreactive T cells are considered the major effector cells driving CNS injury in MS, B cells emerged recently as key players in the physiopathology of MS because treatment with B cell depleting molecules is very effective in reducing acute focal inflammatory disease activity. The implication of B cells in MS is thought to be related mostly to their function as antigen presenting cells and cytokine producers rather than to their antibody-secreting function.[44] Under physiological conditions, central tolerance, that is the elimination of autoreactive B and T cell clones in the bone marrow and thymus, respectively, reduces the number of circulating autoreactive cells. The surviving autoreactive T and B cells are held in check by peripheral tolerance checkpoints.[45] T cell peripheral tolerance mechanisms include anergy when T cells are stimulated in the absence of costimulatory molecules, non-responsiveness to low antigen concentrations, and deletion of autoreactive T cells by activation-induced cell death.[46] Further control mechanisms include several types of regulatory T cells (Tregs). Autoreactive myelin-responsive $CD4^+$ T and CD8+ T cells have been detected among PBMCs isolated from PwMS, but also from age- and sex-matched healthy individuals.[47–51] Accumulating evidence suggests Treg frequency and suppressive function are altered in PwMS, their dysregulation facilitating myelin specific autoreactive T cells entry into the CNS.[52–56] B cell peripheral tolerance mechanisms include chronic antigen stimulation that leads to B cell receptor desensitization and anergy, and stimulation in the absence of cognate antigen-specific T cell help.[57,58–60] Defective peripheral checkpoints have been reported in PwMS, with an increased frequency of naive autoreactive B cells observed in the peripheral blood of patients compared to healthy controls.[61] Regulatory B cells (Bregs) can modulate humoral and cellular immunity by releasing anti-inflammatory cytokines IL-10, transforming growth factor-beta (TGFβ), granzyme B and IL-35.[62,63] Studies have reported decreased IL-10 expression by Bregs derived from PwMS compared to controls, and defective suppression of Th1 effector functions. [64–66]

Naïve lymphocytes are unable to penetrate the intact blood brain barrier. How autoreactive T cells are activated in the periphery to upregulate adhesion molecules and chemotactic receptors necessary to cross the blood brain barrier is unknown. Two main hypotheses prevail. The first suggests that disease-initiating lymphocytes reactive to microbial antigens cross react with structurally similar CNS epitopes (molecular mimicry). Following a systemic infection, these lymphocytes are activated, and cross the blood brain barrier. A second hypothesis supposes that antigens from the CNS are drained in deep cervical lymph-nodes, and are presented to T cells by autoreactive B cells.[44] More recently, a critical role for the

gut microbiota has been suggested, with the gut-associated lymphoid tissues as site for autoreactive T cell activation, which can then cross the blood brain barrier and a.[67] in the activation of autoreactive T cells in PwMS has been suggested,

Macrophages and microglia in multiple sclerosis

Macrophages are cells of the innate immune system involved in the phagocytosis of potentially harmful agents from their environment, including pathogens, dead cells, cell debris, or toxic molecules. Macrophages can either be tissue-resident non-migratory cells, or derive from blood monocytes that differentiate into macrophages upon their migration into tissues.[68] The most abundant and best characterized tissue-resident macrophage population of the CNS is microglia, located throughout the CNS parenchyma. Other tissue-resident macrophages include CNS border-associated macrophages that are located at the interface between the CNS and the blood brain barrier, namely perivascular, meningeal, and choroid plexus macrophages.[69,70] Tissue-resident macrophages derive mostly from myeloid precursors in the yolk sac which establish in the CNS prenatally, and exhibit a high potential for self-renewal and proliferation that is independent of blood myeloid precursors.[71] In contrast, circulating monocytes derive mostly from hematopoietic stem cells and are continually replaced by circulating myeloid cells after birth.[71] Most pathological studies have used markers common to microglia, border-associated macrophages, and monocyte derived macrophages, including ionized calcium-binding adapter molecule 1 (Iba1) and Cluster of Differentiation 68 (CD68). These cell types cannot be discriminated using these markers, and the term microglia/macrophages is used.[72] Many lines of evidence indicate microglia/macrophages play a dual role in the pathogenesis of MS.[73] Upon activation by cytokines secreted by activated Th1 and Th17 cells, intracellular components from necrotic or apoptotic cells, and components of the complement cascade, microglia/macrophages contribute to lesion formation and axonal damage via the secretion of pro-inflammatory mediators, reactive oxygen and nitrogen species, and glutamate, and modulate the adaptive immune response via their role as antigen presenting cells.[74,75] However, microglia/macrophages also support repair mechanisms, in particular by removing myelin debris which is necessary for axonal sprouting and remyelination.[74] Beyond their role in the immune response, microglia/macrophages play an active role in synaptic remodeling both during development and following injury, and the loss of critical homeostatic functions of microglia/macrophages is thought to contribute to increased damage and reduced repair in MS.[76]

Neuropathology of multiple sclerosis

White matter plaques

Multifocal areas of well demarcated white matter demyelination, referred to as plaques, are the pathological hallmark of MS regardless of its clinical course or stage. Large white matter plaques can be readily observed macroscopically on brain and spinal cord pathological specimens of PwMS. Acute white matter plaques are the pathological substrate of relapses, and the most frequent plaque stage in RRMS. Acute plaques are characterized pathologically by sharply demarcated areas of demyelination with relative axonal sparing, and intense activated microglia/macrophage infiltration throughout the lesion. Most macrophages are lipid-laden, indicating they have internalized myelin debris.[72] At the center of the lesion, a post-capillary venule displays a perivascular cuff of lymphocytes consisting mostly of $CD8^+$ T cells and to a lesser extent $CD4^+$ T cells, B cells, and plasma cells. Extensive blood brain barrier damage is present and $CD8^+$ and $CD4^+$ T cells infiltrate the parenchyma through the permeable barrier, while B cells and plasma cells remain mostly in the perivascular space and rarely spread into the parenchyma. Acute plaques are pathologically heterogeneous, and further categorized into four patterns (I to IV), based on the presence or not of complement and immunoglobulin deposition and apoptotic nuclei, as well as the myelin-associated protein that is predominantly lost.[77] This heterogeneity is observed both between patients and also in a single patient at different stages of the disease.[77] Inflammation and blood brain barrier disruption are auto limited, and active white matter plaques spontaneously mature into chronic active and inactive plaques, which predominate in patients with progressive MS. Chronic active plaques are characterized pathologically by sharply demarcated demyelination with numerous myelin-laden macrophages and microglia concentrated in the periphery of the lesion, forming a cellular rim around a hypocellular center. Smoldering plaques are characterized by a slowly expanding rim of activated microglia with only few myelin-contain macrophages.[78] Inactive plaques are completely demyelinated, hypocellular lesions, characterized by axonal and oligodendrocyte loss and astrogliosis, with minimal microglia/macrophage and lymphocyte infiltration and a largely restored blood brain. Shadow plaques are remyelinated plaques characterized by thinly myelinated axons with short internodal distances. They are more frequent in people younger than 55 or within 10 years of diagnosis compared to progressive MS where they are sparse, suggesting a time-dependent decrease in remyelination potential.[79] The mechanisms leading to plaque formation and those governing plaque maturation from acute inflammation and demyelination to remyelination and inactive astrogliosis are poorly understood. Virtually all lesion stages can be observed at any disease stage of MS. However the proportions differ, with active plaques most prevalent in early MS, and smoldering and inactive plaques prevailing in progressive MS.[80]

Grey matter plaques

In the last two decades, pathology and imaging research has demonstrated that focal demyelination is not limited to white matter, occurring in cortical and deep grey matter of the brain too.[81] Grey matter plaques are less conspicuous, requiring specific immunostaining approaches to be revealed, which may contribute to explain why they were not emphasized in the first 19th century pathological descriptions of the disease. Grey matter plaques, which are associated with neuronal and axonal loss, differ from white matter plaques in that the inflammatory infiltrate is less prominent, and demyelination less extensive. Cortical grey matter plaques are classified according to their location within the cortex: cortico-subcortical lesions (type I or leukocortical), intracortical (type II) lesions, and subpial (type III) lesions.[82] Type I and II are identified in all disease stages of MS, whereas type III is essentially seen in progressive MS, often adjacent to meningeal inflammation, as will be discussed below.

Diffuse white and grey matter injury

In addition to focal white and grey matter lesions, diffuse CNS changes are also present. Normal appearing white matter (NAWM) refers to the white matter between plaques which appears intact on clinical MRI scans. However, pathological examination of NAWM reveals some degree of microglial activation, lymphocytic infiltrate, demyelination, axonal loss, and astrogliosis, albeit to a much lesser extent than what is observed in focal white matter lesions.[83] The extent and severity of NAWM injury does not correlate with white matter lesion load, but rather with disease duration. It is more pronounced in late RRMS and SPMS as well as in PPMS, compared to early RRMS. Normal appearing grey matter (NAGM) refers to the grey matter between cortical demyelinating plaques, which shows neuronal loss and atrophy starting early in the disease course.

Meningeal inflammation

Widespread inflammation within the meninges of the brain and spinal cord is found in all MS disease courses. The formation of ectopic follicle-like structures (tertiary lymphoid follicles), consisting of T cells, B cells, and microglia/macrophages, is essentially observed in SPMS, and is most prominent in patients with high levels of brain inflammation, extensive and active subpial grey matter demyelination, and who have a rapidly progressive clinical disease course.[84–86] Tertiary lymphoid follicles are associated with a gradient of neuronal, astrocyte, and oligodendrocyte loss and microglial activation in subpial grey matter lesions that is greatest in the most external cortical layers, and it is hypothesized that inflammatory and/or cytotoxic mediators diffuse into the cortex induce damage directly or via microglial activation.[85–89]

Progressive multiple sclerosis

CNS inflammation is present both in RRMS and progressive MS, but its pattern differs. In progressive MS, active lesions are scarce, smoldering and inactive lesions predominate, and the blood brain barrier is largely repaired. Diffuse inflammation of the white and grey matter as well as the meninges is driven by tissue-resident populations of immune cells, including lymphocytes and activated microglia/macrophages, and is thought to drive widespread myelin, axonal, and neuronal damage.[92,93,90,91]. Although present from disease onset, brain and spinal cord atrophy accelerates in progressive MS and correlates best with disability accumulation, suggesting it is the pathological substrate of progression. [94] Demyelination and the resulting loss of trophic support from oligodendrocytes contributes to axonal degeneration, but evidence indicates axonal and neuronal injury can also occur independently of demyelination.[96–99] How and if inflammation and neurodegeneration are linked in MS is still a debated, part of the scientific community suggesting that the compartmentalized inflammation described above leads neuronal death, another part suggesting inflammation and neurodegeneration are independent processes. (correale)

Molecular pathways of neurodegeneration

The molecular mechanisms leading to neuronal death in PwMS remain to be elucidated, but several lines of evidence suggest a role for chronic oxidative and nitrosative stress and mitochondrial dysfunction. Immune cells, in particular activated macrophages and microglia, secrete reactive oxygen species (ROS) and reactive nitric species (RNS), which are toxic to neurons and glial cells.[100] Neurons with cytoplasmic accumulation of oxidized phospholipids and DNA strand breaks are abundant in cortical MS lesions, suggesting oxidative stress is present.[101] Application of oxygen and nitrogen donors to the spinal cord of healthy mice is sufficient to induce EAE-like axonal injury in the absence of demyelination, and scavengers that reduce levels of ROS and RNS attenuate focal axonal degeneration without altering the number of immune cells.[102] Anti-oxidative pathways, which mitigate the long term deleterious effects of oxidative stress on cells, include nuclear factor erythroid 2-related factor 2 (NRF2) pathway. NRF2 is a transcription factor that induces the expression of antioxidant enzymes including heme oxygenase 1 (HMOX1), which scavenges free radicals and removes damaged proteins. HMOX1 levels are increased in MS lesions and in the CNS of EAE mice, indicating the presence of compensatory mechanisms to chronic oxidative stress.[103,104] Mitochondria are particularly vulnerable to oxidative stress.[105] Lacking protective histones, mitochondrial DNA can rapidly accumulate mutations when oxidative stress increases, eventually compromising oxidative phosphorylation, ATP synthesis, and cell survival.[105] Chronic neuronal hypoxia is thought to result from impaired oxidative phosphorylation which cannot meet the high neuroaxonal ATP demands. Supporting this hypothesis, a hypoxic gene expression signature was identified in the CNS of

EAE, in particular with upregulation of hypoxia inducible factor 1 alpha (HIF-1α). Moreover, nuclear translocation of HIF-1α, which indicates its activation, was observed in the CNS of PwMS.[106–108]

Treating progression

Since the 1990's, more than 15 immunomodulatory or immunosuppressive molecules have been approved to treat MS. All DMTs modulate the peripheral immune system or the access of lymphocytes to the CNS, reducing clinical and MRI signs of acute focal inflammatory disease activity with variable levels of efficiency. A number of these DMTs were tested in progressive MS, most of them proving ineffective in reducing progression rate.[109] Two molecules have shown a modest effect on 12-week confirmed disability progression in PPMS and SPMS (ocrelizumab and siponimod, respectively).[110,111] In the EXPAND randomized controlled trial which investigated siponimod versus placebo in SPMS, a 21% reduction in 3-month confirmed disability progression was observed in the treatment arm. However, those who benefitted most from siponimod were patients who had clinical and/or radiological signs of acute focal disease activity over the prior 2 years (active SPMS).[70] Similarly, in the ORATORIO randomized controlled trial comparing ocrelizumab to placebo in people with PPMS, post-hoc analysis revealed that the subgroup that benefitted most was that with radiological inflammatory disease activity (active PPMS). Therefore, it is likely the effect of these drugs on disability accrual is predominantly due to the suppression of superimposed inflammatory disease activity rather than to a direct effect on progression. All clinical trials evaluating the effect of neuroprotective molecules or molecules stimulating remyelination on progression have failed so far, and DMTs specifically targeting progression are lacking.[112] Progression contributes significantly to long-term disability in PwMS, highlighting the importance of developing such treatments. Treatments targeting oxidative stress, mitochondrial failure, oligodendrocyte regulation, and non-T cell mediated inflammation are currently under evaluation in phase 2 and 3 trials. [112–114] To evaluate the potential of a drug to reduce progression rate, robust means to measure progression over the relatively short time frame of clinical trials. The development of biomarkers of progression, in particular body fluid biomarkers, is thus a thriving field of research.

Body fluid biomarkers of progression

Biomarkers of progression are needed to identify patients for inclusion into clinical trials evaluating molecules with neuroprotective potential as well as to be used as endpoints in these trials. Neurofilament light (NfL) is currently the body fluid biomarker with highest potential for clinical implementation and use in clinical trials. Neurofilaments are major components of the axonal cytoskeleton, of which 3 subunits exist (light, medium, and heavy) and associate to form intermediate filaments. They are exclusively expressed by neurons of both the central and peripheral nervous systems. Upon CNS damage, NfL is

released into the CSF, where it can be measured using conventional immunoassays. With the development and commercialization of high sensitivity immunoassays 5 years ago, NfL can now be measured in blood, allowing repetitive assessments.[115] NfL is increased in virtually all CNS conditions, and is thus an unspecific marker of neuroaxonal damage, as demonstrated in **manuscript 2**. However, there is abundant data indicating that in PwMS, serum NfL closely associates with acute focal inflammatory disease activity, which drives acute neuroaxonal damage. Serum NfL is associated with T2 lesion volume and Gadolinium enhancing lesion count in people with RRMS[116] and decreases after DMT initiation.[117] In individual patients, serum NfL has been demonstrated to mirror closely clinical and radiological signs of acute focal inflammatory disease activity.[118] Taken together, these data suggest high potential as a biomarker to monitor acute inflammation and assess treatment response. The potential of serum NfL to monitor progression or to predict progression remains disputed. Serum NfL was reported to be a predictor of disability at 10 years [119]and to associate with brain atrophy at 5 and 10 years[120]. Another study however found little prognostic power of baseline serum NfL for long-term disability progression.[121] In **manuscript 3**, we investigated the potential of serum NfL to monitor disease progression in people with RRMS treated with natalizumab and very little residual acute focal inflammatory disease activity. We found serum NfL could not discriminate between progressors and non-progressors. Additional research is needed to evaluate the potential of serum NfL to serve as a biomarker of progression in MS. The potential of neurofilament heavy (NfH) as a biomarker in MS has been less investigated than that of NfL. One of the reasons is that it is less abundant than NfL, and robust assessment of its concentration in body fluids is more challenging. In **manuscript 4**, we compare 3 highly sensitive immunoassays to measure NfH in serum, 2 of which we developed, and determine the most sensitive assay. Future studies will in cohorts of PwMS will determine its potential as a biomarker of progression. Given the complexity of MS physiopathology and its heterogeneity, it is unlikely a unique biomarker will be sufficient to capture the mechanisms driving progression. Additional biomarkers thus need to be discovered, and **manuscript 1** reports an attempt to do so in CSF.

Manuscript 1

Claire Bridel, Marleen J A Koel-Simmelink, Laura Peferoen, Claudio Derada Troletti, Sisi Durieux, Rianne Gorter, Erik Nutma, Priya Gami, Ellen Iacobaeus, Lou Brundin, Jens Kuhle, Hugo Vrenken, Joep Killestein, Sander R Piersma, Tran V Pham, Helga E De Vries, Sandra Amor, Connie R Jimenez, Charlotte E Teunissen. *Brain endothelial cell expression of SPARCL-1 is specific to chronic multiple sclerosis lesions and is regulated by inflammatory mediators in vitro*. Neuropathol Appl Neurobiol 2018 Jun; 44(4):404-416.

This study aimed at identifying novel CSF biomarkers that discriminate people with RRMS from people with SPMS. To this end, we performed a proteomics study applying nano liquid chromatography tandem mass spectrometry to CSF of a discovery cohort of people with RRMS or SPMS. We identified candidate biomarker Secreted Protein, Acidic and Rich in Cysteines-like 1 (SPARCL-1), a cell matrix modulating protein, that was upregulated in SPMS. We next sought to validate these findings in 3 independent cohorts. Unfortunately, we were only able to replicate the findings in one validation cohort, which may reflect the large heterogeneity of MS. However, analysis of SPARCL-1 expression in MS brain revealed that in chronic lesions but not active lesions or NAWM, endothelial cells express SPARCL-1. Further, we found that SPARCL-1 expression was regulated by MS-relevant inflammatory mediators in cultured human brain endothelial cells, suggesting a role in blood brain barrier permeability regulation.

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Brain endothelial cell expression of SPARCL-1 is specific to chronic multiple sclerosis lesions and is regulated by inflammatory mediators *in vitro*

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Brain endothelial cell expression of SPARCL-1 is specific to chronic multiple sclerosis lesions and is regulated by inflammatory mediators *in vitro*

Aims: Cell matrix modulating protein SPARCL-1 is highly expressed by astrocytes during CNS development and following acute CNS damage. Applying NanoLC-MS/MS to CSF of RRMS and SPMS patients, we identified SPARCL-1 as differentially expressed between these two stages of MS, suggesting a potential as CSF biomarker to differentiate RRMS from SPMS and a role in MS pathogenesis. **Methods**: This study examines the potential of SPARCL-1 as CSF biomarker discriminating RRMS from SPMS in three independent cohorts (n = 249), analyses its expression pattern in MS lesions (n = 26), and studies its regulation in cultured human brain microvasculature endothelial cells (BEC) after exposure to MS-relevant inflammatory mediators. **Results**: SPARCL-1 expression in CSF was significantly higher in SPMS compared to RRMS in a Dutch cohort of 76 patients. This finding was not replicated in 2 additional cohorts of MS patients from Sweden (n = 81) and Switzerland (n = 92). In chronic MS lesions, but not active lesions or NAWM, a vessel expression pattern of SPARCL-1 was observed in addition to the expression by astrocytes. EC were found to express SPARCL-1 in chronic MS lesions, and SPARCL-1 expression was regulated by MS-relevant inflammatory mediators in cultured human BEC. **Conclusions**: Conflicting results of SPARCL-1′s differential expression in CSF of three independent cohorts of RRMS and SPMS patients precludes its use as biomarker for disease progression. The expression of SPARCL-1 by

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Correction added on 27 October 2017, after first online publication: The name of the author Ellen Iacobaeus has been correctly detailed on this version.

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BEC in chronic MS lesions together with its regulation by inflammatory mediators *in vitro* suggest a role for SPARCL-1 in MS neuropathology, possibly at the brain vascular level.

Keywords: biomarker, blood brain barrier, cerebrospinal fluid, chronic lesions, Multiple sclerosis, SPARCL-1

Introduction

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of the central nervous system (CNS) of unknown aetiology. Initially, most MS patients present with a relapsing-remitting clinical course of disease (relapsing-remitting MS, RRMS) [1], characterized neuropathologically by a high proportion of active lesions [2,3]. A variety of disease-modifying treatments are now available to successfully prevent inflammatory relapses [4]. Despite these therapies, a relevant proportion of RRMS patients transition to secondary progressive multiple sclerosis (SPMS) within 10–15 years of diagnosis [2,3], during which irreversible disability accumulates [5] and chronic neuropathological lesion stages predominate [2,3]. The mechanisms driving neurodegeneration are poorly understood [6] and options to reduce disability progression in SPMS are scarce [7]. Objective means to assess progression are critically needed to evaluate and select those treatments that successfully modify the rate of disability accumulation. As a first step towards this goal, we performed a proteomic study on CSF of MS patients (RRMS, $n = 5$ and SPMS, $n = 5$) and other inflammatory neurological diseases (OIND, $n = 5$), aiming at identifying cerebrospinal fluid (CSF) protein biomarkers allowing discrimination of RRMS and SPMS. Secreted Protein Acidic and Rich in Cysteine-like 1 (SPARCL-1, a.k.a. hevin and SC1) emerged as a significantly differentially expressed protein between RRMS and SPMS. SPARCL-1 is an ubiquitous and highly conserved secreted protein with anti-adhesive properties [8], involved in tissue remodelling and extracellular matrix organisation [9]. SPARCL-1 is highly expressed by astrocytes in the developing CNS [10] and during the synaptogenic period of early rat postnatal CNS development [11]. It induces excitatory synaptogenesis in cultured rat retinal ganglion cells [12,13] through bridging of neuronal membrane receptors NRX1a and NL1 [14]. SPARCL-1 expression decreases in the healthy adult CNS [12,15,16], but is transiently overexpressed by astrocytes in mouse and rat models of acute brain injury such as status epilepticus [17,18] and stroke [19]. After demyelination, activated adult murine oligodendrocyte progenitor cells revert to a more immature gene expression profile, including higher SPARCL-1 mRNA expression levels [20], indicating its expression during neurogenesis and after CNS injury is not limited to astrocytes and may be relevant to MS. Little is known about the expression pattern of SPARCL-1 in CNS and CSF of MS patients. One study reported elevated levels of SPARCL-1 in CSF of RRMS patients compared to healthy controls [21]. In experimental autoimmune encephalomyelitis, spinal cord mRNA levels of SPARCL-1 were reported to correlate with paralysis severity [22]. Our initial finding that SPARCL-1 is differentially expressed in CSF of RRMS and SPMS patients, together with evidence from the literature of SPARCL-1′s upregulation following CNS injury prompted us to [1] further investigate the potential of SPARCL-1 as a CSF biomarker to discriminate SPMS from RRMS, and [2] analyse the expression pattern of SPARCL-1 in the CNS of MS patients and its relation to progression.

Methods

Patient selection

This study was performed according to the ethical principles of the Declaration of Helsinki. The use of CSF and tissue samples for the analyses described in this manuscript received approval by the local ethics committee. Patient characteristics of the cohorts are presented in Table 1. In Amsterdam, CSF samples from patients and controls were selected from the NUBIN biobank of the MS centre, VU medical centre, Amsterdam. The NUBIN biobank initiated in 2000 includes CSF samples of more than 250 patients with MS, other neurological diseases (OND) and OIND, recruited on a voluntary basis. Detailed clinical and MRI measures at time of CSF collection are available for all included cases. The discovery cohort consisted of 5 CSF samples from RRMS female patients with low disease activity (as determined by an experienced clinician based on clinical information, i.e. relapse rate and Expanded

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Table 1. Demographical characteristics of the patients included in the discovery cohort and the three validation cohorts

Cohort	*n*	*% Female*	*Mean age (SD) in years*	*Mean EDSS (SD)*	*Mean disease duration (SD) in years*	*Immunomodulatory treatment*
Discovery	15					
RRMS	5	100	45.4 (7.0)	4.3 (1.3)	14.9 (6.2)	No
SPMS	5	100	47.1 (9.8)	6.4 (1.3)	23.4 (4.3)	No
ONIDC	5	100	46.6 (5.8)	NA	NA	NA
Validation Dutch	76					
RRMS	47	60	40.4 (8.9)	3.1 (1.2)	7.9 (6.4)	No
SPMS	29	40	49.3 (7.5)	5.7 (1.3)	19.5 (8.8)	No
Validation Swiss	92					
RRMS	65	75	38.7 (11.9)	3.1 (1.5)	7.4 (6.3)	yes (21/65)*
SPMS	27	59	52.8 (8.9)	6.0 (1.3)	19. 1(11.4)	yes (22/27)†
Validation Swedish	81					
RRMS	42	67	34.3 (10.1)	2.0 (1.2)	3.9 (7.0)	No
SPMS	39	54	51.0 (8.9)	5.0 (1.6)	20.4 (8.3)	yes (5/39)‡

RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; EDSS, expanded disability status scale; SD, standard deviation; NA, not applicable.
*Treatment RRMS in Swiss cohort: interferon beta, glatiramer acetate, teriflunomide and natalizumab;
†Treatment SPMS Swiss cohort: azathioprine, interferon beta, methotrexate and fumarate;
‡Treatment SPMS Swedish cohort: natalizumab and glatiramer acetate.

Disability Status Scale (EDSS) progression and active T2 and gadolinium enhancing MRI lesions), five CSF samples from SPMS female patients with rapid disease progression (based on confirmed EDSS progression), and five CSF samples from female control patients with OIND. Validation was performed in a larger cohort that included the discovery cohort and comprised 47 RRMS CSF samples and 29 SPMS CSF samples of both sexes. In Stockholm, CSF samples were selected from the biobank of the Neurology clinic at Karolinska University Hospital that is part of Stockholm's Medical Biobank (SMB) with ID number 914. All CSF samples obtained from this biobank were collected during routine neurological diagnostic work up or during clinical follow-up. MRI-scans and detailed clinical measures were recorded for all included patients. The cohort included 42 CSF samples from RRMS and 39 CSF samples from progressive MS (SPMS and PRMS). In Basel, all CSF samples were collected as part of routine diagnostic work up within the biobank of the Department of Neurology, University Hospital Basel. The cohort included 65 CSF samples from RRMS and 27 CSF samples from SPMS.

MRI acquisition and analysis of the Dutch cohort

Magnetic resonance imaging (MRI) acquisition was performed at 1.0 Tesla (Siemens Magnetom Impact, Erlangen, Germany) and consisted of axial pre- and post-contrast T1-weighted (repetition time [TR] ¼ 700 ms, echo time [TE] ¼ 15 ms, 5.0 mm slice thickness, 0.5 mm inter-slice gap) images and T2-weighted ([TR] ¼ 2700 ms, [TE] ¼ 90 ms, 5.0 mm slice thickness with 0.5 mm inter-slice gap) images. MRI examination was performed within 3 weeks of CSF collection. T1 hypointense or black hole lesion load, T2 lesion load and presence of gadolinium enhanced lesions were quantified using home-developed semi-automated seed-growing software based on a local thresholding technique. To assess brain atrophy, we measured normalized brain volumes (NBV) using Structural Image Evaluation, using Normalization of Atrophy Cross-sectionally (SIENAX) [23].

CSF sample collection and storage

In Amsterdam, Stockholm and Basel, CSF was collected and stored at −80°C in polypropylene tubes, after centrifugation within 1 h after withdrawal, according to international biobanking consensus guidelines that were optimised for CSF proteomics [24].

Proteomics analysis

CSF sample preparation and gel electrophoresis CSF samples were recoded and analysed in a blinded fashion. To minimize inter-run bias, each gel contained

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two patients from each clinical group. All the samples were processed with one spin cartridge. The depletion of top-14 high abundant proteins was achieved as previously reported [25]. Briefly, 1 ml aliquots of CSF from each patient were applied directly to the spin filter (Agilent Human 14 or Genway), following instructions from the manufacturer. Depleted CSF was further concentrated using 3 kDa filters prior (Millipore, Darmstadt, Germany) to loading the whole depleted CSF fraction on 1-D gradient gels from Invitrogen (Carlsbad, CA, USA; NuPAGE 4–12% Bis-Tris gel, 1.5mmx10 wells). The gels were then stained with Coomassie brilliant blue G-250 (Pierce, Rockford, USA).

In-Gel Digestion Before nano liquid chromatography tandem mass spectrometry (NanoLC-MS/MS) analysis, separated proteins were in-gel digested as previously described [25]. Briefly, gels were washed and dehydrated once in 50 mM ammonium bicarbonate (ABC) and twice in 50 mM ABC/50% acetonitrile (ACN). Cysteine bonds were reduced by incubation with 10 mM DTT/50 mM ABC at 56°C for 1 h and alkylated with 50 mM iodoacetamide/50 mM ABC at room temperature (RT) in the dark for 45 min. After washing sequentially with ABC and ABC/50% ACN, whole gel lanes were cut into 10 equal bands. Each gel band was sliced up into approximately 1-mm cubes and collected in tubes, washed in ABC/ACN and dried in a vacuum centrifuge. Gel cubes were incubated overnight at 23°C with 6.25 ng/ml trypsin and covered with ABC to allow digestion. Peptides were extracted once in 1% formic acid and twice in 5% formic acid/50% ACN. The volume was reduced to 50 µL in a vacuum centrifuge prior to nanoLC-MS/MS analysis.

Nano-LC separation Peptides were separated by an Ultimate 3000 nanoLC system (Dionex LC-Packings, Amsterdam, The Netherlands) equipped with a 20 cm × 75 µm ID fused silica column custom packed with 3 µm 120 Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 6 µl/min. (1.6% acetonitrile in 0.05% formic acid) on a 1 cm × 100 µm ID precolumn packed with 5 µm ReproSil Pur C18 aqua. Peptides were separated in a 60 min gradient (8–32% acetonitrile in 0.05% formic acid) at 300 nl/min. followed by washing (72% acetonitrile in 0.05% formic acid) and equilibration (4% acetonitrile in 0.05% formic acid). The inject-to-inject time was 90 min.

Mass spectrometry Intact peptide MS spectra and MS/MS spectra were acquired on a LTQ-FT hybrid mass spectrometer (Thermo Fisher, Bremen, Germany) as described in detail in [1,2]. Intact masses were measured at 50,000 resolution in the ICR cell. In parallel, following an FT prescan, the top five peptide signals (charge-states 2+ and higher) were submitted to MS/MS in the linear ion trap (3 amu isolation width, 30 ms activation, 35% normalized activation energy, Q value of 0.25 and a threshold of 5000 counts). Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s.

Protein identification and quantification MS/MS spectra were searched against the human IPI database 3.62 (83 947 entries) using Sequest (version 27, rev 12) with a maximum allowed deviation of 10 ppm for the precursor mass and 1 amu for fragment masses. Methionine oxidation and cysteine carboxamidomethylation were allowed as variable modifications, two missed cleavages were allowed. Scaffold 3.00.04 (Proteomesoftware, Portland, OR) was used to organize the gel-slice data and to validate peptide and protein identifications. Identifications with a Peptide Prophet probability>95% were retained. Subsequently, protein identifications with a ProteinProphet probability of >99% with two peptides or more in at least one of the samples were retained. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped. For quantitative protein analysis across samples, spectral counts (number of identified MS/MS spectra for each protein) were normalized on the sum of the spectral counts per biological sample. Differential analysis of samples was performed using the BetaBinominal test as described previously [26]. Protein identification and quantification details can be found in [26, 27].

Candidate biomarker selection

Candidate biomarkers for validation were prioritized based on the following criteria: (i) fold change >1.2 and P-value < 0.05; (ii) mean spectral count of one of the clinical groups >2; (iii) number of identified peptide sequences (coverage). Next, we searched for availability of ELISA assays, and selected an ELISAs with a detailed

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validation report (i.e. recovery, linearity results and coefficients of variation presented of individual samples in the datasheet).

Elisa

SPARCL-1 DuoSet ELISA Development kit (R&D systems, Abingdon, United Kingdom) was used to measure the concentration of SPARCL-1 in CSF. The wells were in-house coated with goat anti-human SPARCL-1 and recombinant human SPARCL1 protein was used as standard, according to the instructions of the manufacturer. We tested the analytical performance of this assay for CSF. This was done with two different pools of CSF, prepared from leftovers of routine diagnostics. The optimal dilution of CSF for SPARCL-1 measurement was found to be 1/500. Initial validation of linearity was performed by evaluating serial dilutions of the CSF pools from 1/100 to 1/1200. A spike-in experiment with recombinant SPARCL-1 yielded a mean recovery of 70.6%. Inter- and intra-assay coefficients of variation were 12% and 5.6% respectively, measured in two pools over two plates.

Autopsy material

Post mortem brain material was obtained from the Netherlands Brain Bank, Amsterdam, the Netherlands, with the approval of the VU University Medical Ethical Committee (Amsterdam, The Netherlands). Patients and controls, or their next of kin, had given informed consent for the use of their brain tissue and clinical details for research purposes. Clinical characteristics and post mortem intervals are provided in Table 2.

Table 2. Demographical characteristics of the autopsy cases included in the study

Case number	*Age at death*	*Gender*	*Diagnosis*	*PM delay*	*Lesion type*
1	74	M	SPMS	10.15	active
2	60	F	SPMS	10.40	active
3	54	M	SPMS	8.15	Active, chronic active
4	50	F	SPMS	7.35	active
5	53	M	SPMS	10.00	active
6	66	F	SPMS	9.35	active
7	61	M	SPMS	9.15	Chronic active
8	64	F	SPMS	10.10	Chronic active and inactive
9	66	M	SPMS	7.30	Chronic active
10	48	M	SPMS	8.00	Chronic active
11	54	M	SPMS	10.50	Chronic active
12	56	F	SPMS	10.30	Chronic active
13	35	F	SPMS	10.20	Chronic active
14	54	M	SPMS	8.15	Chronic active
15	60	F	SPMS	10.40	Chronic active
16	67	F	SPMS	9.15	Inactive
17	45	M	SPMS	7.45	Inactive
18	77	F	SPMS	10.00	Inactive
19	64	F	SPMS	10.10	Inactive
20	75	M	SPMS	7.45	Inactive
21	59	F	SPMS	4.45	NAWM
22	57	F	SPMS	28.10	NAWM
23	73	M	SPMS	8.00	NAWM
24	76	F	SPMS	7.55	NAWM
25	75	M	SPMS	10.10	NAWM
26	56	M	SPMS	9.50	NAWM
27	58	F	AD	5.15	NA
28	88	F	AD	12.15	NA
29	49	M	Neurologically healthy individual	6.15	NA

MS, multiple sclerosis; NAWM, normal appearing white matter; NA, not applicable.

Immunohistochemistry

5 μm-thick paraffin sections were deparaffinized in xylene and rehydrated through descending alcohol concentrations. Endogenous peroxidase activity was blocked by incubating the slides 30 min in phosphate buffered saline (PBS) containing 0.3% H2O2. For HLA-DR, SPARCL-1 and AT8 (Tau) stainings, the sections were heated in 0.01 M citrate buffer (pH 6.0). After allowing the heated sections to regain room temperature (RT), sections were rinsed in PBS. Sections were incubated with primary antibodies directed against PLP (AbD Serotec, Oxford, UK), HLA-DR (clone LN3, eBioscience, Huissen, The Netherlands), SPARCL-1 (R&D systems, Abingdon, United Kingdom) and AT8 (ThermoFisher, Waltham, USA) for 1 h at RT. After incubation, sections were thoroughly rinsed in PBS and incubated with ready-to-use goat-anti-mouse

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EnVision+™-HRP (Dako, Glostrup, Denmark) for 30 min at RT for PLP, HLA-DR and AT8 and mouse anti-goat IgG-HRP (Dako) for SPARCL-1. Sections were rinsed in PBS, incubated in 3,3′-diaminobenzidine (DAB; Dako) to visualize staining and counterstained with haematoxylin. White matter MS lesions were classified according to the degree of myelin damage and the activity of the microglia/macrophage [3]. Briefly, active lesions were characterised by a focal area of myelin loss filled with myelin-laden 'foamy' macrophages; chronic active lesions were identified by a rim of activated microglia/macrophages surrounding a hypocellular centre, and inactive white matter lesions as a demyelinated area with few or no HLA-DR + cells.

Confocal imaging

5 μm-thick cryosections were fixed in acetone for 10 min. Sections were incubated with the same anti-SPARCL-1 antibody as for immunohistochemistry (R&D systems), and anti-CD31 (DAKO) to identify endothelial cells. For SPARCL-1, an Alexa 488-labelled donkey anti-goat (Molecular Probes, Thermo-Fisher Scientific, Waltham, USA) was used as secondary antibody, and for CD31 an Alexa 555-labelled goat anti-mouse (Molecular Probes). Incubations with secondary antibodies were performed consecutively: between the Alexa 488 donkey anti-goat and the Alexa 555 goat anti-mouse, a 30 min blocking step at RT with 1% goat serum was performed. Incubation with Hoechst was performed for 1 min. Slides were then mounted and stored at 4C in the dark. Images were acquired using a standard confocal scanning microscope (TCS SP2, Leica Microsystems, Heidelberg GmbH, Mannheim, Germany). Images were scanned in three channels, 488 for SPARCL-1, 555 for CD31 and 360 for the nucleus.

Cell culture and treatments

The human brain endothelial cell line hCMEC/D3 was kindly provided by Dr. P.-O. Couraud (Institut Cochin, Université Paris Descartes, Paris, France). hCMEC/D3 cells were grown in endothelial growth basal medium 2 supplemented with human epidermal growth factor, hydrocortisone, GA-1000, vascular endothelial growth factor, human fibroblastic growth factor B, R3-IGF-1, ascorbic acid, and 2.5% FCS (Lonza, Basel, Switzerland). The immortalized human brain endothelial cell line hCMEC/D3 was cultured as described previously [28]. To investigate the regulation of SPARCL-1 gene expression, hCMEC/D3 cells were stimulated for 48 or 72 h with different combination of cytokines including TNFα (Peprotech, Rocky Hill, USA) (10 ng/ml), IFNγ (PeproTech) (10 ng/ml), TGF-β1 (R&D Systems) (10 ng/ml) and IL-1β (PeproTech) (10 ng/ml).

Real-time quantitative PCR

Gene expression analysis was performed on confluent monolayers of hCMEC/D3 cells in 24 well culture plates (Corning, Lowell, USA). RNA isolation was performed using TRIzol (Invitrogen) according to manufacturer's protocol. Complementary DNA (cDNA) was synthesized with the Reverse Transcription System kit (Promega, Madison, USA) following manufacturer's guidelines and RT-PCR was performed as described previously [29]. Primer sequences used are as follows, describing the forward and reverse sequence, respectively: SPARCL-1: GTTCCTTCACAGATTCTAACCA,TTTACTGCTCCTGTTCAACTG;GAPDH:CCATGTTCGTCATGGGTGTG,GGTGCTAAGCAGTTGGTGGTG.

Statistical analysis

Statistics were performed in SPSS version 3 (Table 1, 2 and 3) and GraphPad Prism version 6 (Figures 1 and 4). Differences in mean values between clinical groups obtained in the ELISA experiments were analysed with ANCOVA, correcting for age and gender. Pearson's coefficients were calculated to evaluate correlation. Differences in mean values between the expression levels obtained in the cell culture experiments were analysed by 1-way ANOVA.

Results

Nano LC-MS/MS on CSF samples identifies SPARCL-1 as differentially expressed in RRMS and SPMS

To facilitate in-depth coverage of the CSF proteome, samples were subjected to high-abundant protein depletion, followed by SDS-PAGE fractionation, in-gel tryptic digestion and nanoLC-MS/MS analysis. In total, 925 proteins were identified in CSF. The beta binomial test for comparison of protein spectral counts [26] between RRMS and SPMS yielded six differentially regulated

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Table 3. Correlations between SPARCL-1 CSF concentrations and clinical characteristics.

	EDSS *Pearson's correlation coefficient* (*P* value)	*Age* *Pearson's correlation coefficient* (*P* value)	*Disease duration* *Pearson's correlation coefficient* (*P* value)
SPARCL-1(ng/ml)			
Dutch cohort, $n = 76$	0.185 (0.119)	**0.383 (0.001)**	**0.292 (0.01)**
Swedish cohort, $n = 81$	0.100 (0.409)	0.087 (0.449)	0.069 (0.563)
Swiss cohort, $n = 92$	0.027 (0.801)	**0.320 (0.002)**	0.101 (0.386)

EDSS, expanded disability status scale; SD, standard deviation; NA; not applicable. Significant correlations are bold.

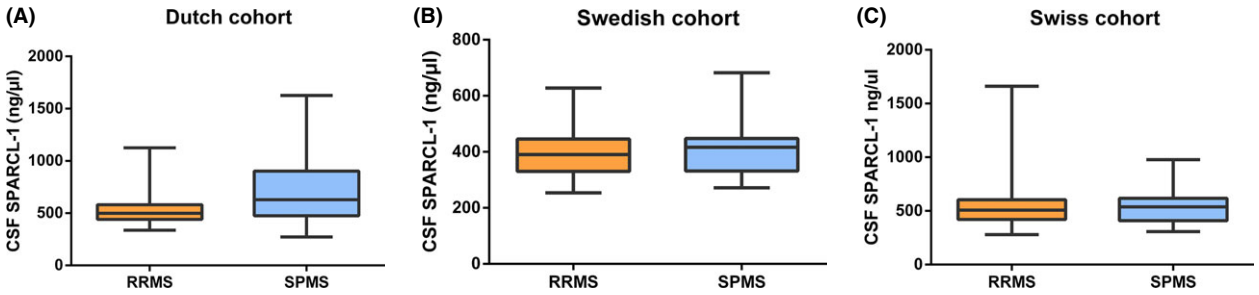

Figure 1. SPARCL-1 expression in CSF of RRMS and SPMS patients. RRMS: relapsing remitting multiple sclerosis. SPMS; secondary progressive multiple sclerosis. CSF SPARCL-1: SPARCL-1 expression in cerebrospinal fluid. **A** Dutch cohort **B** Swedish cohort **C** Swiss cohort. Star indicates statistically significant difference in mean expression levels ($P = 0.001$) as assessed by ANCOVA with correction for age and gender. [Colour figure can be viewed at wileyonlinelibrary.com]

proteins (Table S1), amongst which SPARCL-1 (fold change 2.49, *P*-value = 0.014) (Table S1). SPARCL-1 was also differentially expressed between SPMS and OIND (fold change 2.34, $P = 0.023$), together with 14 other proteins, but not between RRMS and OIND (data not shown).

SPARCL-1 expression in CSF of RRMS and SPMS patients

We proceeded to the measurement of SPARCL-1 in a partly independent and larger validation cohort of RRMS and SPMS patients by ELISA (Table 1). The results in Figure 1**A** show that in the Dutch cohort, SPARCL-1 levels were significantly higher in the SPMS group compared to the RRMS group (fold change 1.44, $P < 0.001$), after correction for age and gender. SPARCL-1 levels were not differentially regulated in two independent cohorts of RRMS and SPMS patients from Sweden (Figure 1**B**) and Switzerland (Figure 1**C**) after correction for age and gender. The three cohorts were comparable in terms of mean age, gender ratio, mean EDSS and mean disease duration (Table 1). In the Swiss cohort, a majority of SPMS patients were treated with immunomodulatory drugs, whereas none were treated in the Dutch cohort (Table 1). There was no correlation between SPARCL-1 CSF levels and EDSS in all three cohorts (Table 3). In the Dutch cohort, for which we have extensive MRI measurements at time of CSF collection, we found no significant correlation between SPARCL-1 levels and total number of T2 lesions, total T2 lesion volume, total black hole volume, brain normalized volume, total Gd+ lesions and total T2 lesions/total Gd+ ratio (data not shown).

Vessel expression pattern of SPARCL-1 is specific to chronic MS lesion stages

To investigate the pattern of SPARCL-1 expression in the CNS of MS patients in relation to MS progression, we performed an immunohistochemical analysis of five active lesions, eight chronic active lesions, five inactive lesions and five normal appearing white matter (NAWM) regions from independent autopsy material (Table 2). White matter from one healthy control and two Alzheimer's disease (AD) patients was also examined. We found that SPARCL-1 is expressed by astrocytes in all MS lesion stages and NAWM (Figure 2), as

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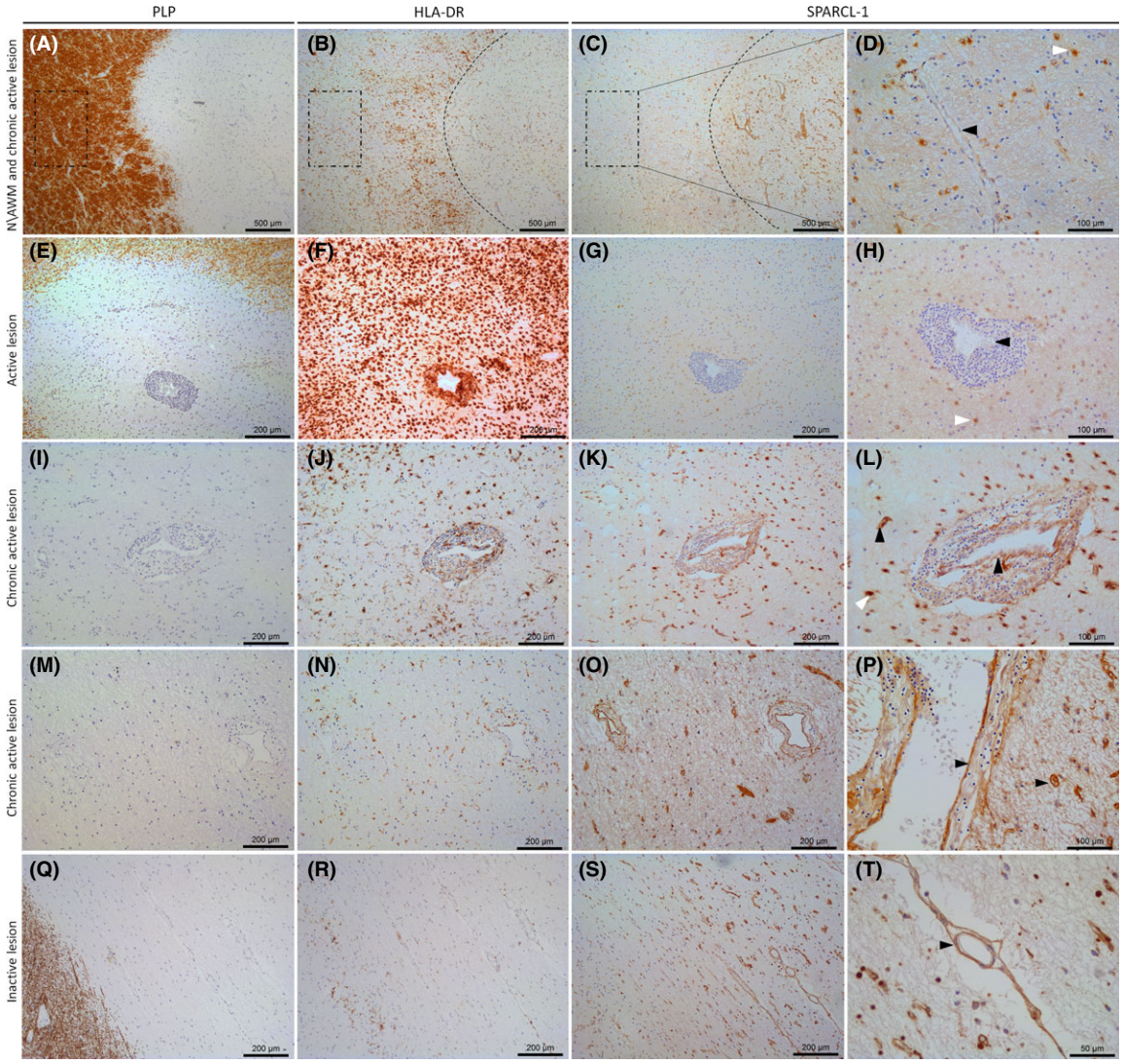

Figure 2. Immunostainings of NAWM, active, chronic active and inactive lesions in MS. (**A–C**): NAWM (left half of the pictures) and chronic active lesion (right half of the pictures). Rectangle delineates the region displayed in higher magnification in **D.** In **B** and **C**, NAWM and the chronic active lesion are demarcated by a dotted line. (**A**) PLP staining showing myelin in NAWM and absence of myelin in chronic active lesion. (**B**) HLA-DR staining shows typical activated foamy macrophages rim around chronic active lesion. (**C**) SPARCL-1 staining shows vascular pattern of SPARCL-1 expression in chronic active lesion but not NAWM. **(D)** Higher magnification of SPARCL-1 staining corresponding to NAWM region delimited by rectangle in pictures **A** to **C**. White arrowhead: astrocyte expressing SPARCL-1, black arrowhead: vessel without SPARCL-1 expression. (**E–H**): Active lesion displaying in its centre a vessel with typical perivascular lymphocyte cuff. (**E**) PLP staining showing absence of PLP detection in active lesion. (**F**) HLA-DR staining shows activated foamy macrophages throughout the lesion. **G** and **H**: SPARCL-1 staining shows SPARCL-1 expression by astrocytes (**H**, white arrowhead), but no vascular pattern of SPARCL-1 expression (**H**, black arrowhead). (**I–L**): Chronic active lesion displaying in its centre a vessel with some degree of inflammation. (**I**): PLP staining showing absence of PLP detection in chronic active lesion. (**J**): HLA-DR staining shows typical activated foamy macrophages rim around chronic active lesion. **K** and **L**: SPARCL-1 staining shows SPARCL-1 expression by astrocytes (**L**, white arrowhead), and vascular pattern of SPARCL-1 expression (**L**, black arrowheads) in both small and large vessels. (**M–P**): Chronic active lesion displaying in its centre a vessel with little perivascular inflammation. (**M**): PLP staining showing absence of PLP detection in chronic active lesion. (**N**): HLA-DR staining shows activated foamy macrophages in chronic active lesion. **O** and **P**: SPARCL-1 staining shows vascular pattern of SPARCL-1 expression (**P**, black arrowheads) in both small and large vessel. (**Q–T**): Inactive lesion displaying in its centre a vessel with no perivascular inflammation. (**Q**): PLP staining showing absence of PLP detection in inactive lesion. (**R**): HLA-DR staining shows few foamy macrophages around inactive lesion. **S** and **T**: SPARCL-1 staining shows vascular pattern of SPARCL-1 expression (**T**, black arrowheads) in both small and large vessel. Original magnifications: A, B, C 80x, E, F, G, I, J, K, M, N, O, Q, R, S 200× and D, H, L, P 400x, T 800x. [Colour figure can be viewed at wileyonlinelibrary.com]

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well as to a lesser degree in the white matter (WM) of the healthy control (Figure 3**E,F**) and AD patients (Figure 3**B,C**). The expression of SPARCL-1 by astrocytes is stronger in chronic active (Figure 2**K,L,O,P**), and inactive lesions (Figure 2**S, T**), compared to active lesions (Figure 2**G,H**) and NAWM (Figure 2**C, D**). In addition, we identified a specific vessel expression pattern of SPARCL-1 in chronic active (Figure 2**K,L,O,P**) and inactive (Figure 2**S, T**) lesions. This expression is highly specific to chronic lesion stages in MS, as we did not observe this vessel pattern of expression in any active lesion (Figure 2**G,H**) or NAWM (Figure 2**C, D**). Moreover, the areas of SPARCL-1 vessel expression pattern are sharply demarcated from the regions where this pattern is not observed, and correspond exactly to the transition from a chronic lesion to NAWM (Figure 2**A–C**). We did not observe this vessel pattern of expression in white matter of a healthy control or AD patients (Figure 3). Confocal imaging of a vessel in a chronic active lesion showed that SPARCL-1 is detected in the cytosol of BEC (Figure 4), consistent with the secreted nature of the protein.

Endothelial expression of SPARCL-1 is regulated by inflammatory mediators in vitro

Next, we investigated the expression regulation of SPARCL-1 in human brain endothelial cell line hCMEC/D3[28]. We hypothesized that SPARCL-1 expression is regulated by inflammatory mediators associated with the pathogenesis of MS and its progression [30]. hCMEC/D3 cultures grown to confluence were incubated for 48 or 72 h with TNFα, IFNγ or a combination thereof, and TGFβ, IL1β or a combination thereof. SPARCL-1 expression studied by real time quantitative PCR showed an upregulation after 48 and 72 h incubation with a combination of TGFβ and IL1β, but not in the presence of one of the two only (Figure 5). After 48 h incubation, TNFα, IFNγ and a combination thereof induced an upregulation of SPARCL-1, which was not visible anymore after 72 h (Figure 5). Together, these data suggest that SPARCL-1 expression is regulated in a time-dependent manner and transiently by inflammatory mediators, with different effect according to the incubation time.

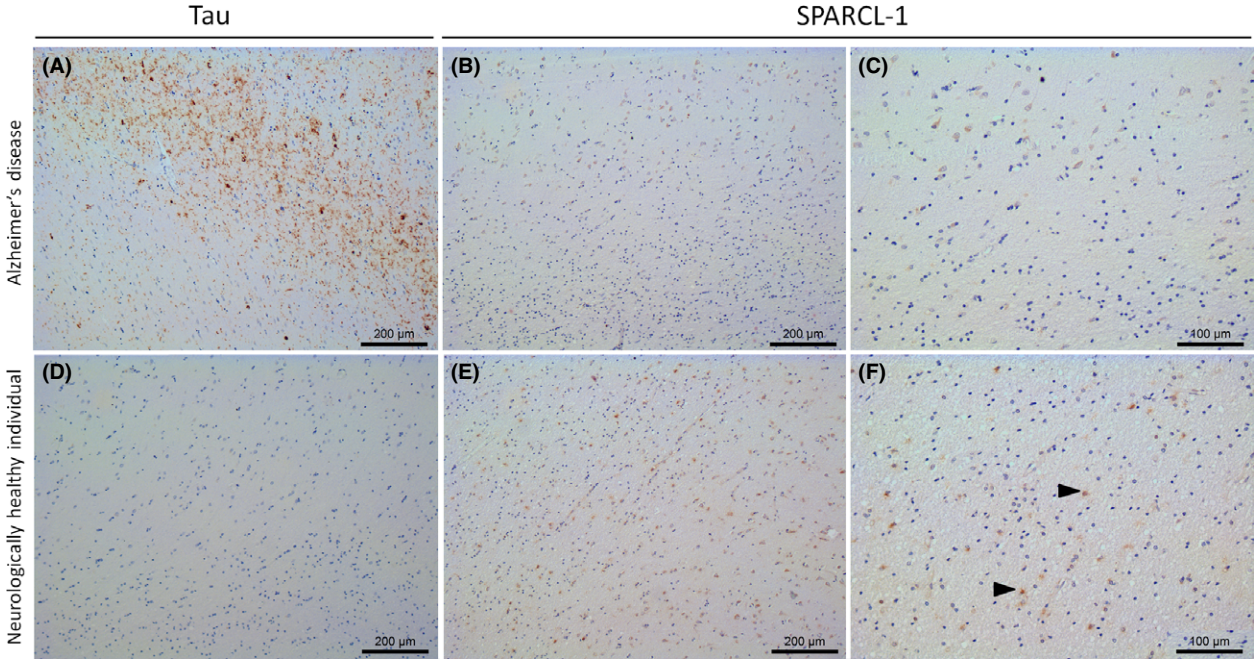

Figure 3. Immunostainings of AD and neurologically healthy individual. (**A–C**): Temporal grey and white matter of Alzheimer's disease patient. (**D–F**): Temporal grey and white matter of neurologically healthy individual. **A** and **D**: Tau staining. Tau deposits visible in temporal grey matter of AD individual (**A**); no Tau staining detected in temporal grey matter of neurologically healthy individual (**D**). **B**, **C**, **E** and **F**: SPARCL-1 staining. In AD (**B**, **C**) and neurologically healthy individual (**E**, **F**), no vessel pattern of SPARCL-1 expression detectable. In neurologically healthy individual (**E**, **F**), SPARCL-1 expression mostly visible in white matter astrocytes (**F**), black arrowheads). Original magnifications: A, B, D, E 200×, C, F 400×. [Colour figure can be viewed at wileyonlinelibrary.com]

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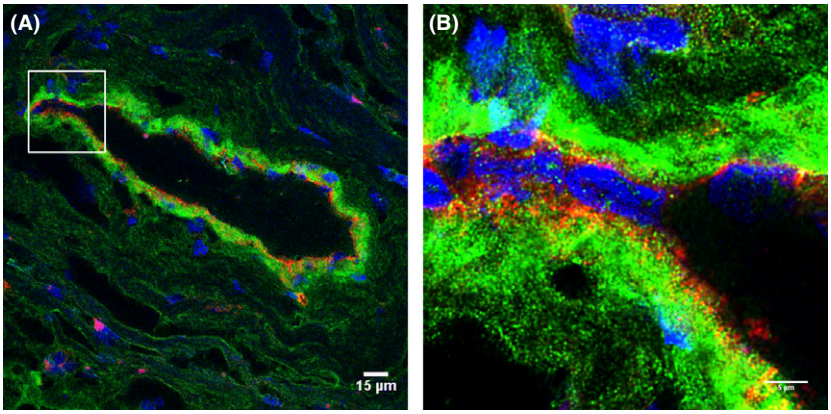

Figure 4. SPARCL-1 is expressed by endothelial cells in vessel of chronic active lesion. Confocal image of vessel (**A**) and endothelial cells (**B**) in a representative vessel of a chronic active lesion. CD31 (red) is used as an endothelial cell marker. SPARCL-1 (green) is detectable in the cytosol of endothelial cells, consistent with the secreted nature of the protein. Nucleus: blue. [Colour figure can be viewed at wileyonlinelibrary.com]

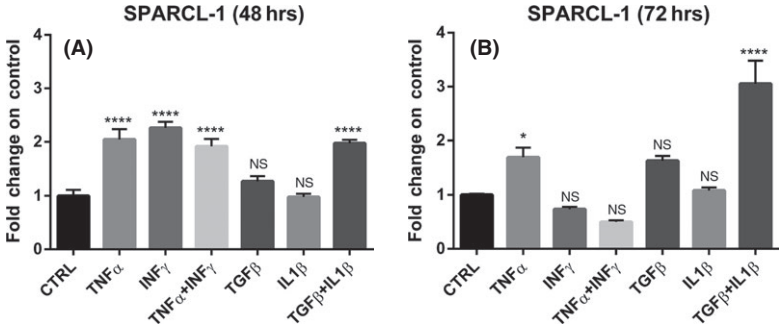

Figure 5. Inflammatory mediators regulate SPARCL-1 expression *in vitro.* Relative mRNA **e**xpression of SPARCL-1 to GAPDH in brain endothelial cell line hCMEC/D3, normalized to control (ctrl) expression level, after 48 h treatment (**A**) and 72 h treatment (**B**) with inflammatory mediators TNFα, IFNγ, TGFβ and IL1β or combinations thereof. Ctrl: vehicle only. * is *P*-value < 0.05; ** is *P*-value < 0.01; *** is *P*-value < 0.001; **** is *P*-value < 0.0001. The data represented are the mean of 3 independent experiments ± SEM. [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

In this study, we identified SPARCL-1 as being differentially expressed in the CSF of RRMS and SPMS in a Dutch cohort of 76 patients. Significantly higher levels of SPARCL-1 were measured in CSF of SPMS compared to RRMS. In *post mortem* white matter (WM) of MS patients, we found that SPARCL-1 is expressed by astrocytes in all lesion stages, but its expression is stronger in chronic active and inactive lesions than in active lesions and NAWM, mirroring the higher activation state of astrocytes. In addition, we observed a stage-specific expression pattern of SPARCL-1 by brain vessels. Indeed, brain vessel expression of SPARCL-1 was highly specific and limited to chronic active and inactive lesion stages, as it was not observed in active lesions, NAWM and white matter of healthy controls and AD patients. Confocal microscopy identified BEC as the source of SPARCL-1 expression in vessels of chronic lesion stages.

SPMS patients have a higher proportion of chronic lesion stages than RRMS patients [2], and we hypothesized that the higher CSF levels of SPARCL-1 detected in CSF of SPMS patients reflected the higher proportion of chronic lesions in these patients. We did not observe a correlation between SPARCL-1 CSF levels and EDSS. However, the EDSS being strongly lesion-location dependant, a lack of correlation does not necessarily preclude SPARCL-1′s potential to reflect chronic lesion load. SPARCL-1 CSF levels also failed to correlate with total T2 lesion volume or count, black hole lesion

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volume, brain normalized volume, total Gd+ count and total T2 count/ total Gd+ count ratio.

In independent cohorts from Switzerland and Sweden, we did not observe the differential CSF expression of SPARCL-1 as identified in the Dutch cohort. Demographic characteristics including mean age, gender ratio, mean EDSS and mean disease duration were comparable in all 3 cohorts. Inclusion criteria did differ (inclusion on a voluntary basis in Amsterdam vs. systematic inclusion in Sweden and Switzerland), leading to a collection bias. In the Swiss cohort, a majority of SPMS patients were treated with immunomodulatory drugs at time of CSF collection, while none were treated in the Dutch cohort and only a minority (12.8%) in the Swedish cohort, which could affect CNS and CSF levels of SPARCL-1. However, excluding the treated patients from the analysis in the Swedish cohort did not modify the conclusions. Many factors may contribute to explain our divergent results. Access to high quality CSF samples of SPMS patients with extended clinical information is difficult, but ideally, a large multicentre study would be needed to clarify our findings.

Expression of SPARCL-1 by astrocytes, and to a lesser extent neurons, is well-characterized in healthy CNS, during development and following acute injury [12,31,32], but its expression by BEC has not been reported before. Here, we show that SPARCL-1 expression by BEC is restricted to chronic active and inactive MS lesions, suggesting a stage-specific role of SPARCL-1 in MS. In an *in vitro* model of BEC, we found that Th1-related molecules TNFα and IFNγ, whose levels are elevated in CNS lesions of MS patients [33,34], induce an increase in SPARCL-1 expression after 48 h exposure, and that this upregulation recedes upon sustained exposure for 72 h. Sustained co-exposure to TGFβ and IL-1β, both of which are highly expressed in MS lesions [35,36], also upregulate SPARCL-1 expression by endothelial cells. Together, these data show that endothelial cell expression of SPARCL-1 is modulated by the cytokine milieu. Further studies are needed to relate these *in vitro* findings to the cytokine milieu of MS lesions.

Founding family member Secreted protein acidic and rich in Cysteine (SPARC), a matricellular protein bearing high structural homology to SPARCL-1, has recently been reported to increase transendothelial permeability of the blood brain barrier (BBB) *in vitro* [37]. SPARCL-1 and SPARC exert antagonistic effects on cell adhesion [38] and excitatory neuron synaptogenesis [22], but no function for SPARCL-1 in BBB regulation has been ascribed so far. In light of our findings, SPARCL-1 expression may also exert an effect on BBB permeability, and further experiments are needed to test this hypothesis.

In conclusion, the lack of consistency of our findings in CSF of MS patients precludes the use of SPARCL-1 as CSF biomarker of SPMS at this stage. The tight restriction of BEC SPARCL-1 expression to chronic MS lesions suggests a role for SPARCL-1 in BBB repair mechanisms, which peak during chronic MS lesion stages [39,40]. The modulation of BEC SPARCL-1 expression by inflammatory mediators offers new perspectives on SPARCL-1′s function in chronic inflammatory brain diseases, in particular at the BBB level.

Acknowledgements

This research has been given ethical approval by local ethical committees. Ethical committee protocol number 00.080 (Amsterdam).

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Author contributions

CB designed the study, planned the experiments, did the immunohistochemistry, confocal microscopy and ELISA experiments, analysed all the data, performed the statistics and wrote the manuscript. MJAKS prepared the CSF samples for mass spectrometry. LP helped with the analysis of the immunohistochemistry and critically reviewed the manuscript. CDT performed and analysed the cell culture and qPCR experiments and critically reviewed the manuscript. SD, RG, EN, and PG provided technical help. EI and LB provided the CSF samples from Sweden and critically reviewed the manuscript. JK provided the CSF samples from Switzerland and critically reviewed the manuscript. HV performed the MRI analysis and critically reviewed the manuscript. JK selected the patients and critically reviewed the manuscript. SRP did the mass spectrometry experiments and wrote the proteomics material and methods section of the manuscript. TVP performed the

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analysis of the mass spectrometry data. HEdV, CJ and SA helped design the study, analyse the data and critically reviewed the manuscript. CT designed the study, planned the experiments, analysed the data and wrote the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Differentially regulated proteins in CSF of RRMS and SPMS patients.

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Manuscript 2

Claire Bridel, Wessel N van Wieringen, Henrik Zetterberg, Betty M Tijms, Charlotte E Teunissen, and the NFL Group. *Diagnostic Value of Cerebrospinal Fluid Neurofilament Light Protein in Neurology: A Systematic Review and Meta-analysis*. JAMA Neurol 2019 Sep 1; 76(9):1035-1048.

This study aimed at comparing the levels of CSF NfL across neurological conditions including MS, to evaluate its potential in discriminating clinically similar conditions. To do so we performed a large meta-analysis based on individual-level data. We found that CSF NfL was elevated compared with healthy controls in most neurological conditions. Highest levels were observed in cognitively impaired HIV-positive individuals, amyotrophic lateral sclerosis, frontotemporal dementia, and Huntington disease. CSF NfL overlapped in most clinically similar conditions, in particular MS subtypes and other inflammatory diseases of the CNS.

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JAMA Neurology | Original Investigation

Diagnostic Value of Cerebrospinal Fluid Neurofilament Light Protein in Neurology
A Systematic Review and Meta-analysis

Claire Bridel, MD, PhD; Wessel N. van Wieringen, PhD; Henrik Zetterberg, MD, PhD; Betty M. Tijms, PhD; Charlotte E. Teunissen, PhD; and the NFL Group

IMPORTANCE Neurofilament light protein (NfL) is elevated in cerebrospinal fluid (CSF) of a number of neurological conditions compared with healthy controls (HC) and is a candidate biomarker for neuroaxonal damage. The influence of age and sex is largely unknown, and levels across neurological disorders have not been compared systematically to date.

OBJECTIVES To assess the associations of age, sex, and diagnosis with NfL in CSF (cNfL) and to evaluate its potential in discriminating clinically similar conditions.

DATA SOURCES PubMed was searched for studies published between January 1, 2006, and January 1, 2016, reporting cNfL levels (using the search terms *neurofilament light* and *cerebrospinal fluid*) in neurological or psychiatric conditions and/or in HC.

STUDY SELECTION Studies reporting NfL levels measured in lumbar CSF using a commercially available immunoassay, as well as age and sex.

DATA EXTRACTION AND SYNTHESIS Individual-level data were requested from study authors. Generalized linear mixed-effects models were used to estimate the fixed effects of age, sex, and diagnosis on log-transformed NfL levels, with cohort of origin modeled as a random intercept.

MAIN OUTCOME AND MEASURE The cNfL levels adjusted for age and sex across diagnoses.

RESULTS Data were collected for 10 059 individuals (mean [SD] age, 59.7 [18.8] years; 54.1% female). Thirty-five diagnoses were identified, including inflammatory diseases of the central nervous system (n = 2795), dementias and predementia stages (n = 4284), parkinsonian disorders (n = 984), and HC (n = 1332). The cNfL was elevated compared with HC in a majority of neurological conditions studied. Highest levels were observed in cognitively impaired HIV-positive individuals (iHIV), amyotrophic lateral sclerosis, frontotemporal dementia (FTD), and Huntington disease. In 33.3% of diagnoses, including HC, multiple sclerosis, Alzheimer disease (AD), and Parkinson disease (PD), cNfL was higher in men than women. The cNfL increased with age in HC and a majority of neurological conditions, although the association was strongest in HC. The cNfL overlapped in most clinically similar diagnoses except for FTD and iHIV, which segregated from other dementias, and PD, which segregated from atypical parkinsonian syndromes.

CONCLUSIONS AND RELEVANCE These data support the use of cNfL as a biomarker of neuroaxonal damage and indicate that age-specific and sex-specific (and in some cases disease-specific) reference values may be needed. The cNfL has potential to assist the differentiation of FTD from AD and PD from atypical parkinsonian syndromes.

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Supplemental content

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Identifying neuroaxonal damage and quantifying the intensity of this process is a critical step in patient care because it may support diagnosis and help estimate the prognosis of neurological conditions. In addition, it is essential for the evaluation of drug candidates with disease-modifying potential. Neurofilament light protein (NfL) is an abundant cytoskeletal protein exclusively expressed by central and peripheral neurons. Elevated levels of NfL in cerebrospinal fluid (CSF) were first reported in neurodegenerative conditions more than 20 years ago,[1] sparking interest in the potential of this neuron-specific protein as a biomarker. Since then, elevated levels of NfL in CSF (cNfL) have been described in a number of neurological and psychiatric conditions. The magnitude of the increase in inflammatory, degenerative, infectious, ischemic, and traumatic neurological conditions, as well as in psychiatric disorders, varies between conditions and studies. To date, cNfL levels have not been compared systematically between neurological disorders, and patient numbers in individual studies are often low. A positive association between cNfL and age has been reported in healthy controls (HC)[2] but was not systematically investigated in neurological conditions and may alter the performance of this biomarker across age categories. Together, these questions limit clinical implementation of cNfL. To compare cNfL levels between diagnoses, assess the association of age and sex with these variables, and evaluate the potential of cNfL level as a diagnostic biomarker, we performed a systematic review and meta-analysis on individual data collected from studies reporting cNfL levels in diseases and controls.

Key Points

Question How do levels of neurofilament light in cerebrospinal fluid (cNfL) compare between neurological conditions and with healthy controls?

Findings Among 10 059 individuals in this systematic review and meta-analysis, cNfL was elevated in most neurological conditions compared with healthy controls, and the magnitude of the increase varies extensively. Although cNfL overlaps between most clinically similar conditions, its distribution did not overlap in frontotemporal dementia and other dementias or in Parkinson disease and atypical parkinsonian syndromes.

Meaning The cNfL is a marker of neuronal damage and may be useful to differentiate some clinically similar conditions, such as frontotemporal dementia from Alzheimer disease and Parkinson disease from atypical parkinsonian syndromes.

Methods

Search Strategy

This systematic review and meta-analysis followed Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) reporting guidelines.[3] We searched PubMed for articles published in English between January 1, 2006, and January 1, 2016, reporting cNfL levels (using the search terms *neurofilament light* and *cerebrospinal fluid*) in neurological or psychiatric conditions and/or in HC. Titles and abstracts were reviewed, and relevant studies were selected. The quality of primary articles was assessed using relevant criteria from the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[4] and the QUADAS-2 guidelines.[5] All studies were approved by local ethics committees.

Inclusion Criteria

Studies were included if lumbar cNfL was reported for neurological patients and/or HC and/or individuals with subjective neurological or cognitive complaints and/or a psychiatric condition and/or a systemic disease that may affect the central nervous system (CNS). A reference method for the measurement of cNfL is lacking to date. To limit between-cohort heterogeneity due to the measurement tool, we included only those studies that used the same commercially available immunoassay (NF-light ELISA [enzyme-linked immunosorbent assay]; UmanDiagnostics) on the market since 2006. This assay was selected because it was used in a majority of publications (71 of 112) since 2006 and was reported to be sensitive and robust.[6]

Data Collection

We contacted the corresponding authors to request access to individual-level cNfL, age at CSF sampling, sex, and diagnosis. An individual's data were included only if all of those variables were available. For patients with multiple sclerosis (MS) and HIV-positive individuals, treatment status was also collected.[7,8] Information on study procedures was extracted from the publication or requested from the corresponding author.

Diagnostic Categories

Diagnosis was established by the original study authors according to published criteria when applicable (**Table 1**). Information about the clinical subtype of neurodegenerative conditions was not retained, and all clinical subtypes of a condition were pooled in a single diagnostic group. Stroke, cardiac arrest, HIV infection, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), Guillain-Barré syndrome (GBS), Cushing disease in remission, and optic neuritis (ON) were diagnosed according to clinical guidelines. Presymptomatic genetic frontotemporal dementia (pgFTD), Huntington disease (HD), and premanifest HD (pHD) were diagnosed by genetic testing. The HIV-infected individuals with cognitive impairment (iHIV) included individuals with mild neurocognitive impairment and individuals with HIV-associated dementia.

Individuals with subjective neurological complaint (SNC) or subjective cognitive decline (SCD) had complaints but no objectifiable neurological condition after extensive workup. Inflammatory neurological diseases (IND) were inflammatory diseases of the CNS, excluding MS, clinically isolated syndrome (CIS), and ON. Noninflammatory neurological diseases (NID) were any CNS disease that was not of inflammatory nature. Mixed dementia (MD) was dementia of assumed mixed pathology, and dementia not specified (DNS) was dementia of uninvestigated origin. Healthy controls were individuals who did not have neurological complaints or signs of a neurological condition.

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Diagnostic Groups

We clustered a subset of frequent neurological conditions into 3 groups of clinically similar disorders. These included the following: (1) untreated relapsing-remitting MS (uRRMS), individuals with relapsing-remitting MS treated with disease-modifying therapy (tRRMS), CIS, ON, primary progressive MS (PPMS), secondary progressive MS (SPMS), and IND; (2) Alzheimer disease (AD), FTD, combined FTD and amyotrophic lateral sclerosis (FTD/ALS), vascular dementia (VaD), dementia with Lewy bodies (DLB), idiopathic normal-pressure hydrocephalus (iNPH), mild cognitive impairment of suspected AD pathology (MCI), SCD, and iHIV; and (3) Parkinson disease (PD), PD dementia (PDD), DLB, multiple system atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal syndrome of suspected tau underlying pathology (CBS).

cNfL Measurement

The cNfL was measured at 17 different centers using the commercially available kit (NF-light ELISA assay). The cNfL values were reported in picograms per milliliter or nanograms per liter. A systematic error in the reported concentration of cNfL was identified at 8 centers due to a misinterpretation of the assay's protocol. The protocol indicated to perform a 1:1 dilution of CSF before performing the assay. However, because this dilution is included a priori in the value assignment of the standard curve, this initial dilution should not be corrected for at calculation of the concentration. Raw NfL values obtained from the 8 implicated centers were corrected for the systematic error (divided by 2).

Statistical Analysis

We performed an individual-level meta-analysis based on cNfL measurements provided by the corresponding authors. Linear mixed-effects models were used to estimate the fixed effects of age, sex, and diagnosis on log-transformed NfL levels, with cohort of origin modeled as a random intercept, using the R packages "lme4" and "lmerTest" (R Project for Statistical Computing). Age was centered according to the mean. First, we tested all 2-way and 3-way interaction terms between all fixed effects, which were retained in the model when statistically significant. No 2-way interaction of age and sex or 3-way interaction of age, sex, and diagnosis on cNfL was observed, and the best-fitting model included all fixed effects and interaction terms for diagnosis by age and diagnosis by sex. Next, we used the R package "emmeans" to obtain marginalized change folds and 95% CI cNfL and cNfL-age slope estimates for all diagnoses and to perform post hoc pairwise comparisons between diagnoses in the mean cNfL levels and in the strength of the associations between cNfL age, adjusting *P* values for multiple testing with the Tukey procedure. Finally, we calculated point estimates of fold-change increases for each diagnostic group compared with controls for specific ages. The consequences of study variability on the results was assessed using the intraclass correlation coefficient, which reflects the proportion of variance that can be attributed to between-study variation, for the total sample and per diagnostic group (analyses for the latter were performed on models the included the fixed effects of age and sex). Values higher than 0.60 were considered to be indicative of substantial heterogeneity. The results were considered statistically significant when they had an adjusted 2-sided *P* value below .05. All analyses were performed in R version 3.4.2.

Table 1. Diagnostic Criteria Used by the Original Study Authors

Diagnosis	Abbreviation	Diagnostic Criteria
Multiple sclerosis and clinically isolated syndrome	MS and CIS	McDonald criteria,[9] 2005 revisions,[10] and 2010 revisions[11]
Alzheimer disease and mild cognitive impairment	AD and MCI	Criteria by McKhann et al[12] and IWG-2 criteria[13]
Parkinson disease	PD	United Kingdom Parkinson Disease Society Brain Bank criteria[14] and National Institute of Neurological Disorders and Stroke criteria[15]
Parkinson disease dementia	PDD	Movement Disorder Task Force[16]
Progressive supranuclear palsy	PSP	Criteria by Litvan et al[17]
Multiple system atrophy	MSA	Criteria by Gilman et al[18]
Corticobasal syndrome	CBS	Criteria by Lee et al,[19] criteria by Litvan et al,[17] and criteria by Mathew et al[20]
Dementia with Lewy bodies	DLB	Criteria by McKeith et al[21]
Frontotemporal dementia (including all clinical subtypes)	FTD	Criteria by Neary et al[22] and The Lund and Manchester Groups[23]
Amyotrophic lateral sclerosis	ALS	Revised El Escorial criteria[24]
Combined frontotemporal dementia and amyotrophic lateral sclerosis	FTD/ALS	
Vascular dementia	VaD	Criteria by Erkinjuntti et al[25] and National Institute of Neurological Disorders and Stroke
Idiopathic normal-pressure hydrocephalus	iNPH	Criteria by Relkin et al[26]
Bipolar disorder	BD	*Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition)
HIV positive with cognitive impairment (including entire spectrum of cognitive impairment)	iHIV	Global Deficit Score[27]

Abbreviation: IWG-2, International Working Group 2.

Results

Data Set Characteristics, Population, and Demographics

The literature search resulted in 153 records. On the basis of title and abstract, 112 publications were selected for full-text review, and 44 data sets met our selection criteria and were included in the meta-analysis. In addition, 3 data sets unpublished at the time of data collection were provided by study authors, resulting in a total of 47 data sets (**Table 2** and eFigure 1 in the Supplement). Data were obtained for 10 059 individuals (mean [SD] age, 59.7 [18.8] years; 54.1% female), and 35 diagnoses were identified, including control groups (HC [n = 1332], SNC [n = 45], and SCD [n = 24] [eTable 1 in the Supplement]), inflammatory diseases of the CNS (CIS, ON, RRMS, SPMS, PPMS, and IND [n = 2795]) (eTable 1 in the

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Table 2. Data Sets Included in the Meta-analysis

Source	Contributed Diagnostic Categories (No. of Individuals)	Diagnostic Criteria	Healthy Controls Contributed, No.
Anckarsäter et al,[28] 2014	None	NA	34
Axelsson et al,[29] 2014	SPMS (n = 30), PPMS (n = 5)	McDonald criteria 2010 revisions[11]	14
Bäckström et al,[30] 2015	PD (n = 99), MSA (n = 11), PSP (n = 12)	PD: United Kingdom Parkinson Disease Society Brain Bank criteria[14]	30
		MSA: Criteria by Gilman et al[18]	
		PSP: Criteria by Litvan et al[17]	
Bjerke et al,[31] 2011	AD (n = 30), VaD (n = 26)	AD: Criteria by McKhann et al[12]	30
		VaD: Criteria by Erkinjuntti et al[25]	
Bjerke et al,[32] 2014 and Jonsson et al,[33] 2012	MCI (n = 31)	Criteria by McKhann et al[12]	15
Bruno et al,[34] 2012	None	NA	19
Burman et al,[35] 2014	RRMS (n = 43), SPMS (n = 20), National Institute of Neurological Disorders and Stroke (n = 7), SNC (n = 6)	McDonald criteria 2010 revisions[11]	2
Fialová et al,[36] 2013	CIS (n = 32), RRMS (n = 18)	McDonald criteria 2005 revisions[10]	24
Fialová et al,[37] 2017	AD (n = 25), DNS (n = 13), IND (n = 17)	AD: Criteria by McKhann et al[12]	25
Gunnarsson et al,[38] 2011	RRMS (n = 92)	McDonald criteria 2010 revisions[11]	0
Hall et al,[39] 2012 and Hall et al,[40] 2015	AD (n = 48), PD (n = 196), PDD (n = 56), PSP (n = 53), MSA (n = 67), CBS (n = 15), DLB (n = 69)	AD: Criteria by McKhann et al[12]	150
		PD: National Institute of Neurological Disorders and Stroke criteria[15]	
		PDD: Movement Disorder Task Force[16]	
		MSA: Criteria by Gilman et al[18]	
		PSP and CBS: Criteria by Litvan et al[17]	
		DLB: Criteria by McKeith et al[21]	
		CBS: Criteria by Mathew et al[20]	
Herbert et al,[41] 2015	PD (n = 64), MSA (n = 50)	PD: United Kingdom Parkinson Disease Society Brain Bank criteria[14]	70
		MSA: Criteria by Gilman et al[18]	
Hjalmarsson et al,[42] 2014	Stroke (n = 20)	Clinical	20
Jakobsson et al,[43] 2014 and Rolstad et al,[44] 2015	BD (n = 133)	*Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition)	38
Jeppsson et al,[45] 2013	iNPH (n = 27)	Criteria by Relkin et al[26]	20
Jessen Krut et al,[46] 2014	iHIV (n = 13)	Global Deficit Score[27]	152
Khademi et al,[2] 2013 Aeinehband et al,[47] 2015, and unpublished data	CIS (n = 203), RRMS (n = 682), IND (n = 387), National Institute of Neurological Disorders and Stroke (n = 370)	McDonald criteria[9]	30
Khalil et al,[48] 2013	CIS (n = 47), NID (n = 15)	McDonald criteria 2010 revisions[11]	0
Kuhle et al,[49] 2013	CIS (n = 62), RRMS (n = 38), SPMS (n = 25), PPMS (n = 23)	McDonald criteria 2005 revisions[10]	72
Kuhle et al,[50] 2013	RRMS (n = 30)	McDonald criteria 2005 revisions[10]	0
Kuhle et al,[51] 2015	RRMS (n = 36)	McDonald criteria 2005 revisions[10]	0
Magdalinou et al,[52] 2015 and unpublished data	AD (n = 26), CBS (n = 16), FTD (n = 16), MSA (n = 30), PD (n = 10), PSP (n = 29)	AD: Criteria by McKhann et al[12]	28
		CBS: Criteria by Mathew et al[20]	
		FTD: The Lund and Manchester Groups[23]	
		MSA: Criteria by Gilman et al[18]	
		PD: United Kingdom Parkinson Disease Society Brain Bank criteria[14]	
		PSP: Criteria by Litvan et al[17]	
Martínez et al,[53] 2015 and unpublished data	PPMS (n = 17), SPMS (n = 6), RRMS (n = 192), CIS (n = 109)	McDonald criteria[9]	0
Martínez et al unpublished data	CIS (n = 51), RRMS (n = 46)	McDonald criteria[9]	0
Martínez et al unpublished data	NID (n = 6), IND (n = 2), stroke (n = 4), GBS (n = 1), ON (n = 1)	Clinical	0
Meeter et al,[54] 2016	pgFTD (n = 42), FTD (n = 90)	Not specified	49

(continued)

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Table 2. Data Sets Included in the Meta-analysis (continued)

Source	Contributed Diagnostic Categories (No. of Individuals)	Diagnostic Criteria	Healthy Controls Contributed, No.
Menke et al,[55] 2015 and Lu et al,[56] 2015	ALS (n = 38)	Revised El Escorial criteria[24]	20
Modvig et al,[57] 2013 and Modvig et al,[58] 2016	ON (n = 56)	Clinical	27
Modvig et al,[59] 2015	ON (n = 85)	Clinical	0
Paterson et al,[60] 2015	AD (n = 94)	IWG-2 criteria[13]	30
Pérez-Santiago et al,[61] 2016	iHIV (n = 14), HIV (n = 14)	Global Deficit Score[27]	0
Pijnenburg et al,[62] 2015	FTD/ALS (n = 26), FTD (n = 4), AD (n = 25), SCD (n = 24)	ALS: Revised El Escorial criteria[24]	0
		AD: Criteria by McKhann et al[12]	
		Neuropathological confirmation (11 of 25 for AD, 15 of 23 for FTD)	
		Genetic confirmation (12 of 23 for FTD)	
Pyykkö et al,[63] 2014	iNPH (n = 29), MD (n = 3), AD (n = 8)	AD: Criteria by McKhann et al[12]	0
		iNPH: Clinical	
Ragnarsson et al,[64] 2013	Cushing disease (n = 12)	Clinical	6
Romme Christensen et al,[65] 2014	PPMS (n = 12), SPMS (n = 12)	McDonald criteria 2005 revisions[10]	0
Rosén et al,[66] 2014	Cardiac arrest (n = 21)	Clinical	20
Sandberg et al,[67] 2016	RRMS (n = 97), SPMS (n = 44), PPMS (n = 12)	McDonald criteria 2010 revisions[11]	0
Scherling et al,[68] 2014	FTD (n = 83), PSP (n = 23), CBS (n = 16), PD (n = 6), AD (n = 45)	FTD: Criteria by Neary et al[22]	54
		PSP: Criteria by Litvan et al[17]	
		AD: Criteria by McKhann et al[12]	
		CBS: Criteria by Lee et al[19]	
Skillbäck et al,[69] 2014	AD (n = 1417), PDD (n = 45), FTD (n = 146), LBD (n = 114), MD (n = 517), VaD (n = 465), DNS (n = 545)	AD: IWG-2 criteria[13]	107
		DNS: *International Statistical Classification of Diseases, 10th Revision*	
		PDD: Movement Disorder Task Force[16]	
		FTD: The Lund and Manchester Groups[23]	
		DLB: Criteria by McKeith et al[21]	
		VaD: National Institute of Neurological Disorders and Stroke	
Stilund et al,[70] 2015	RRMS (n = 44), PPMS (n = 15), CIS (n = 27), SNC (n = 39)	McDonald criteria 2010 revisions[11]	0
Tortelli et al,[71] 2015 and Tortelli et al,[72] 2012	CIDP (n = 25), ALS (n = 37), MCI (n = 3), AD (n = 15), MSA (n = 1), CBS (n = 2), NID (n = 5)	ALS: Revised El Escorial criteria[24]	0
		CIDP: Clinical	
		AD and MCI: Criteria by McKhann et al[12]	
		CBS: Criteria by Lee et al[19]	
		MSA: Criteria by Gilman et al[18]	
Tortorella et al,[73] 2015	CIS (n = 21)	McDonald criteria 2005 revisions[10]	0
Trentini et al,[74] 2014	PPMS (n = 21), SPMS (n = 10), National Institute of Neurological Disorders and Stroke (n = 15)	McDonald criteria[9]	0
Vågberg et al,[75] 2015	None	NA	53
Villar et al,[76] 2015	RRMS (n = 98), CIS (n = 29)	McDonald criteria 2010 revisions[11]	37
Wild et al,[77] 2015	HD (n = 30), pHD (n = 13)	Genetic testing	14
Zetterberg et al,[78] 2016	MCI (n = 193), AD (n = 95)	Criteria by McKhann et al[12]	111

Abbreviations: AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; BD, bipolar disorder; CBS, corticobasal syndrome; CIDP, chronic inflammatory demyelinating polyradiculopathy; CIS, clinically isolated syndrome; DLB, dementia with Lewy bodies; DNS, dementia not specified; FTD, frontotemporal dementia; FTD/ALS, combined frontotemporal dementia and amyotrophic lateral sclerosis; GBS, Guillain-Barré syndrome; HD, Huntington disease; iHIV, HIV positive with cognitive impairment; IND, inflammatory neurological disorders other than multiple sclerosis; iNPH, idiopathic normal-pressure hydrocephalus; IWG-2, International Working Group 2; MCI, mild cognitive impairment; MD, mixed dementia; MSA, multiple system atrophy; NA, not applicable; NID, noninflammatory neurological disorders; ON, optic neuritis; PD, Parkinson disease; PDD, Parkinson disease dementia; pgFTD, presymptomatic genetic frontotemporal dementia; pHD, premanifest Huntington disease; PPMS, primary progressive multiple sclerosis; PSP, progressive supranuclear palsy; SCD, subjective cognitive decline; SNC, subjective neurological complaint; SPMS, secondary progressive multiple sclerosis; RRMS, relapsing-remitting multiple sclerosis; VaD, vascular dementia.

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Supplement), dementias and predementia stages (MCI, AD, pgFTD, FTD, VaD, DLB, iNPH, DNS, MD, pHD, HD, iHIV, and FTD/ALS [n = 4339]) (eTable 1 in the Supplement), and parkinsonian syndromes (PD, PDD, MSA, PSP, CBS, and DLB [n = 984]) (eTable 1 in the Supplement). Three diagnostic categories were excluded from the statistical models because they had fewer than 5 observations per sex (Cushing disease, cardiac arrest, and HIV), resulting in 32 diagnostic categories and 10 012 individuals included in the analysis.

cNfL Distribution Across Diagnoses

We first examined the distribution of cNfL across diagnostic categories (**Figure 1**). The cNfL was increased compared with HC in most neurological conditions (Figure 1A). The fold changes compared with HC varied extensively between individual conditions, with the largest effect sizes observed in iHIV (21.36; 95% CI, 9.86-46.30), FTD/ALS (10.48; 95% CI, 4.85-22.67), ALS (7.58; 95% CI, 4.49-12.81), and HD (5.88; 95% CI, 2.43-14.27) (Figure 1B; eTable 2 in the Supplement).

Association of cNfL With Age and Sex

In HC, we observed a yearly increase of 3.30% (95% CI, 2.98%-3.62%) in cNfL levels (eTable 2 in the Supplement). A positive association between cNfL and age was also observed in individuals with subjective complaints, BD, and in most neurodegenerative conditions (eTable 2 in the Supplement). In MS, iHIV, and rapidly progressive neurodegenerative conditions (FTD, ALS, FTD/ALS, MSA, PSP, CBS, and HD), no such association was observed (eTable 2 in the Supplement). In HC, cNfL was higher in men (26.0%, 95% CI, 16.0%-37.0%) (eTable 3 in the Supplement). This was also the case in a minority of neurological conditions, including MS, AD, VaD, and PD (eTable 3 in the Supplement).

cNfL Levels Within 3 Groups of Clinically Similar Disorders

We next compared cNfL between neurological conditions within 3 groups of clinically similar disorders. In inflammatory conditions of the CNS, the mean cNfL levels were similar in ON, CIS, and MS subtypes (eTable 4A in the Supplement). The association between cNfL and age was positive in ON, CIS, and IND but was negative in uRRMS (**Figure 2**A; eFigure 2 and eTable 2 in the Supplement). The ratio of cNfL between ON and CIS, ON and IND, and CIS and IND remained stable across the age range of the study, while the ratio between uRRMS and CIS decreased with increasing age (eTable 5A in the Supplement). No association between cNfL and age was observed in tRRMS and PPMS (Figure 2A and eTable 4 in the Supplement). The ratio of cNfL between uRRMS and tRRMS and between uRRMS and PPMS remained stable across the age range of the study (eTable 5B in the Supplement). No association between cNfL and age was observed in SPMS (Figure 2A; and eTable 2 in the Supplement). Although cNfL levels tended to be higher in young uRRMS compared with age-corresponding SPMS, this did not reach statistical significance (eTable 5C in the Supplement). In dementias and related disorders, the mean cNfL levels were statistically significantly higher in FTD compared with other causes of dementia, such as AD (2.08; 95% CI, 1.72-2.56 [eTable 4B in the Supplement]), VaD (1.56; 95% CI, 1.25-1.96 [eTable 4B in the Supplement]), and DLB (2.50; 95% CI, 1.89-3.33 [eTable 4B in the Supplement]). An association of cNfL with age was positive in AD, VaD, and DLB but was absent in FTD (Figure 2B; eFigure 2B and eTable 4B in the Supplement). The ratio of cNfL between AD and FTD increased with age; in individuals 90 years and older, the distribution of cNfL in both conditions overlapped (eTable 5D in the Supplement). An association between cNfL and age was absent in FTD and FTD/ALS, while it was present in pgFTD (eFigure 2 and eTable 2 in the Supplement). A positive association with age was observed in AD, MCI, and SCD (Figure 2B; eTable 2 in the Supplement), and the ratio of cNfL between AD and MCI remained stable across the age range (eTable 5E in the Supplement). In parkinsonian syndromes, the mean cNfL levels did not differ between PD and PDD and between PDD and DLB, while they were higher in MSA, PSP, and CBS compared with PD (eTable 4C in the Supplement). In MSA, PSP, and CBS, no association with age was observed, while a positive association was found in PD, PDD, and DLB (Figure 2C and eTable 2 in the Supplement). The ratio of cNfL between MSA and PD, PSP and PD, and CBS and PD decreased with age but remained high across the age range of the study (Figure 2C and eTable 5G in the Supplement).

Assessment of Cohort Heterogeneity

In this meta-analysis, we pooled individual patient data originating from 42 different data sets. To estimate the proportion of the total variance of cNfL accounted for by the data set (cohort) of origin, we calculated the intraclass coefficient for cohort-related random intercepts. Across the total sample (n = 10 012), the intraclass coefficient was low at 0.15. Likewise, in a majority of diagnostic categories, the intraclass coefficient was low to moderate (<0.60). However, in 7 of the 32 diagnostic categories (MD, DNS, PDD, DLB, NID, iHIV, and stroke), the intraclass coefficients were high (>0.60), indicating that a large proportion of the variance in cNfL was due to the data set of origin (eTable 6 in the Supplement).

Discussion

In this meta-analysis that included 10 012 individuals, we found that cNfL was increased compared with HC in most neurological conditions studied. The largest effect sizes were observed in iHIV, FTD/ALS, ALS, and HD, while the effect sizes in inflammatory conditions of the CNS were low. Other neurological disorders showed much subtler increases that failed to reach statistical significance (PD and CIDP/GBS). However, the effect sizes in these conditions were positive, and larger sample sizes may allow for more robust estimates. In HC, we observed a positive association between cNfL and age. A positive association, albeit weaker, was also present in a majority of neurological conditions. An association with sex was absent in most diagnostic categories except for HC, PPMS, AD, VaD, and PD, where levels were higher in men. In clinically similar disorders, the distribution of cNfL relative to age mostly overlapped, suggesting limited use for differential diagnosis. Exceptions were FTD, which segregated from other common

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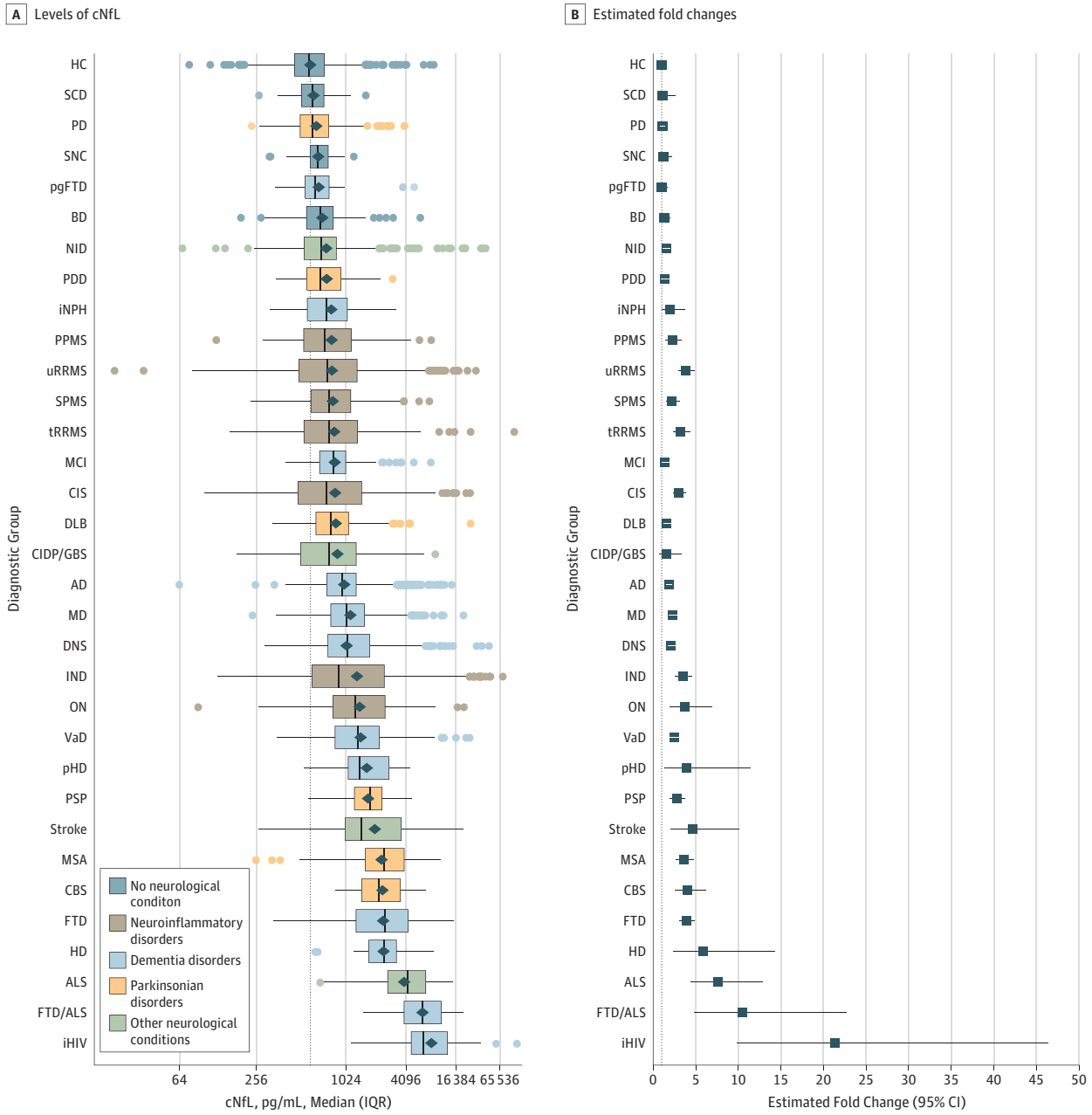
Figure 1. Neurofilament Light in Cerebrospinal Fluid (cNfL) Levels Across Diagnostic Categories



A, Levels of cNfL are shown corrected for age and sex. B, Estimated fold changes are compared with healthy controls (HC). AD indicates Alzheimer disease; ALS, amyotrophic lateral sclerosis; BD, bipolar disorder; CBS, corticobasal syndrome; CIDP/GBS, chronic inflammatory demyelinating polyradiculopathy and Guillain-Barré syndrome; CIS, clinically isolated syndrome; DLB, dementia with Lewy bodies; DNS, dementia not specified; FTD, frontotemporal dementia; FTD/ALS, combined frontotemporal dementia and amyotrophic lateral sclerosis; HD, Huntington disease; iHIV, HIV positive with cognitive impairment; IND, inflammatory neurological disorders other than multiple sclerosis; iNPH, idiopathic normal-pressure hydrocephalus; MCI, mild cognitive impairment; MD, mixed dementia; MSA, multiple system atrophy; NID, noninflammatory neurological disorders; ON, optic neuritis; PD, Parkinson disease; PDD, Parkinson disease dementia; pgFTD, presymptomatic genetic frontotemporal dementia; pHD, premanifest Huntington disease; PPMS, primary progressive multiple sclerosis; PSP, progressive supranuclear palsy; SCD, subjective cognitive decline; SNC, subjective neurological complaint; SPMS, secondary progressive multiple sclerosis; tRRMS, treated relapsing-remitting multiple sclerosis; uRRMS, untreated relapsing-remitting multiple sclerosis; and VaD, vascular dementia.

causes of dementia (including AD and VaD), and PD, which segregated from atypical parkinsonian syndromes. These data indicate that cNfL may contribute to the differentiation of these conditions, particularly in younger individuals.

cNfL and Age

In about two-thirds of the diagnoses, including HC, we observed a positive association between cNfL and age. In the control groups (HC, SNC, and SCD), as well as in pgFTD and BD,

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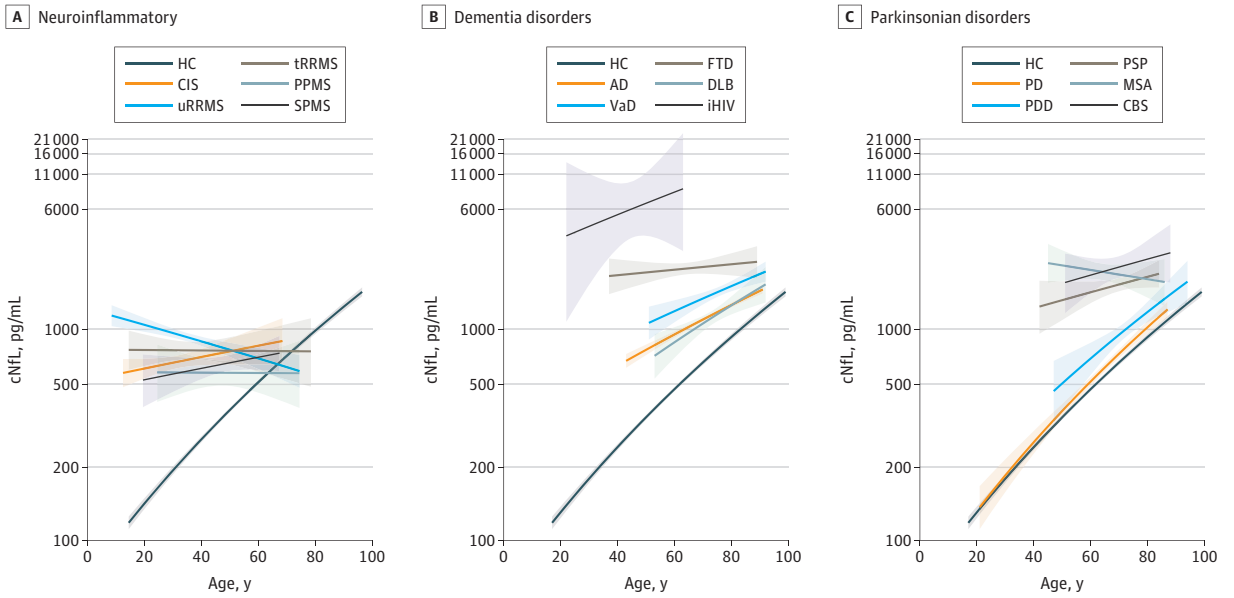
Figure 2. Neurofilament Light in Cerebrospinal Fluid (cNfL) in Neurological Conditions According to Age



A-C, Log cNfL values are shown according to age across diagnoses. Shading around regression lines represents standard errors. AD indicates Alzheimer disease; CBS, corticobasal syndrome; CIS, clinically isolated syndrome; DLB, dementia with Lewy bodies; FTD, frontotemporal dementia; HC, healthy controls; iHIV, HIV positive with cognitive impairment; MSA, multiple system atrophy; PD, Parkinson disease; PDD, Parkinson disease dementia; PPMS, primary progressive multiple sclerosis; PSP, progressive supranuclear palsy; SPMS, secondary progressive multiple sclerosis; tRRMS, treated relapsing-remitting multiple sclerosis; uRRMS, untreated relapsing-remitting multiple sclerosis; and VaD, vascular dementia.

the association of cNfL with age was strongest. This positive association in diagnostic categories without an overt neurological condition may reflect a decrease in CSF clearance with age, the presence of a preclinical age-related neurological condition, or age-related neuronal loss.[79] The association of cNfL with age in HC implies that age-specific reference values may be needed and that the diagnostic potential of cNfL may decrease with age. In neurological conditions with substantially elevated levels of cNfL, such as FTD, ALS, FTD/ALS, HD, and iHIV, as well as in atypical parkinsonian syndromes, no association with age was observed, suggesting that neuropathological processes may cause plateau levels or mask age associations. In MS, an association with age was absent or negative, which may reflect the observation that younger patients with MS have more active diseases.[2]

cNfL and Sex

In a minority of diagnoses, including HC, cNfL was higher in men than women. The clinical relevance of these findings is uncertain, but the results suggest that sex-specific reference values may be needed.

Other Determinants of cNfL Levels

Age, sex, and the random (cohort) association explained 46% of the variance of cNfL in the best-fitting model, indicating that many determinants of cNfL remain to be identified. Disease duration and severity could influence cNfL levels. However, these data were not available in the data sets that were included in this meta-analysis, and studies designed specifically to evaluate the association of these variables and others (eg, smoking, physical activity, and body size) are ongoing.

cNfL in Inflammatory Conditions of the CNS, Including MS

The cNfL was increased in all inflammatory conditions of the CNS examined in this meta-analysis, but the effect sizes were small. The distribution of cNfL in CIS, ON, and RRMS overlapped, which may be expected because CIS and a proportion of ON are initial manifestations of RRMS. Neurodegeneration has a central role in MS, contributing to disease progression and long-term disability.[80] Poor understanding of the processes driving neurodegeneration, together with the lack of biomarkers allowing dynamic measurement of its rate, hampers the development of specific treatments.[81] The cNfL has been reported to correlate with brain atrophy,[50,82] which is considered a marker of neurodegeneration.[83,84] We found that levels of cNfL did not differ statistically significantly between RRMS, PPMS, and SPMS, indicating that on a population level cNfL may not differentiate acute inflammation-induced neuronal damage in the context of relapses from progressive neurodegeneration if the consequences of recent relapses or novel lesion formation are not considered. In individual patients, cNfL has been reported to reflect acute neuronal and axonal damage in MS, with levels transiently increasing during relapse.[85-87] We found that cNfL levels in uRRMS and tRRMS did not differ statistically significantly. However, patients with the most active RRMS with potentially highest cNfL levels are also those who are most likely to be treated, and cNfL has been reported to decrease after treatment initiation in individual patients.[38,50,51]

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cNfL in Dementia and ALS

The higher levels of cNfL observed in FTD compared with other frequent causes of dementias, including AD, VaD and DLB, may be related to the anatomical location of neurodegeneration or the rate of neuronal death. This finding suggests that cNfL may support the differentiation of FTD from other dementias, in line with a recent study[88] not included in this meta-analysis, which reported that in combination with YKL40 and Aβ42 cNfL assists in the differentiation between FTD and AD with high accuracy. In iHIV, which included both mild cognitive impairment due to HIV and HIV-associated dementia, we observed highest levels of cNfL, setting it apart from neurodegenerative and vascular causes of dementia. This may reflect a high rate of neuroaxonal damage due to the presence of HIV and the inflammatory response to it in the CNS, or it may indicate additional peripheral nervous system damage contributing to the elevation of cNfL. In predementia stages, such as MCI and pgFTD, cNfL values were similar to levels in HC, suggesting that CNS damage must reach a certain extent before it is reflected by increased cNfL. However, the pgFTD cohort was small (n = 42); therefore, a small effect size could have been missed. The cNfL levels were highly elevated in ALS and FTD/ALS compared with HC. These results are in line with single-center studies not included in this meta-analysis that used different assays to measure NfL in CSF.[89] Together with the high levels of cNfL observed in stroke, these findings indicate that the rate of neuroaxonal damage may be an important determinant of the magnitude of NfL increase in CSF, possibly by overriding CSF clearance mechanisms.

cNfL in Degenerative Parkinsonian Syndromes

In degenerative parkinsonian syndromes, cNfL clustered into 2 groups. The first group consisted of PD, PDD, and DLB, in which cNfL levels were similar to those in HC, and the second group consisted of atypical parkinsonian syndromes MSA, PSP, and CBS, with elevated levels of cNfL compared with HC and the absence of association with age. This finding is in line with the results of another meta-analysis[90] that focused on parkinsonian disorders, examining data sets not included in the present meta-analysis, further underscoring the robustness of our findings. These data have important clinical implications because they suggest a potential for cNfL in supporting the differentiation of PD from atypical parkinsonian syndromes. Accurate and early differential diagnosis of these conditions is crucial because their prognosis and management differ substantially.

Serum NfL

A few years ago, an ultrasensitive assay was developed that allows measurement of NfL in serum (sNfL). This assay uses the same antibody pair as the immunoassay used in the studies included in this meta-analysis, and studies[91,92] have reported high correlations between serum and CSF levels. These findings indicate that sNfL may replace cNfL. In addition, it may likely be that the findings of the present meta-analysis, which collected data over 10 years, can be readily translated to sNfL.

Limitations of the Study

Our systematic review and meta-analysis has some limitations. In all studies included in the meta-analysis except one,[93] diagnosis was based on clinical criteria. This limitation is mostly a concern for dementias and parkinsonian syndromes, for which definitive diagnosis requires postmortem examination. However, the agreement between clinical and pathological diagnoses was reported to be high when diagnoses were established in specialized centers using consensus criteria.[94,95] For AD and MCI, 2 consensus criteria were applied (criteria by McKhann et al[12] and the International Working Group 2 [IWG-2] criteria[13]), for which a high concordance rate was reported.[96] For VaD, the 2 consensus diagnostic criteria used (criteria by Erkinjuntti et al[25] and the National Institute of Neurological Disorders and Stroke criteria) were also reported to have a high agreement.[97] For PD, 2 consensus criteria were applied, for which concordance evaluation is not available. For ALS, FTD, PSP, MSA, PDD, DLB, and iHIV, the same consensus criteria were applied in all studies. In MS, the McDonald criteria were revised over time, and this may have influenced classification of RRMS and CIS. A further limitation is the inability to capture dementia of multifactorial origin, which may have increased heterogeneity in the dementia diagnostic categories and blurred the difference in cNfL distributions between dementia subtypes. Further classification of neurodegenerative conditions into clinical phenotypes could not be performed because this information was absent in a majority of studies. Therefore, the specific value of cNfL in subphenotypes could have been missed in this meta-analysis. In addition, for some conditions, data and age ranges were limited, resulting in large standard errors and low statistical power, and conclusions for these conditions should be interpreted with caution. Finally, we included only those studies that used a specific immunoassay for cNfL in an attempt to reduce heterogeneity due to the analytical procedure. However, the range of conditions that were explored in the studies not included in the meta-analysis for the same reason did not differ from those included.

Conclusions

Our study was designed to compare cNfL levels across neurological conditions and controls, assess the association of age and sex with these variables, and evaluate the potential of cNfL to differentiate clinically similar conditions. Our meta-analysis found that cNfL was elevated in a majority of the neurological conditions included in this study. Although cNfL overlapped between most clinically similar conditions, its distribution did not overlap in FTD compared with other dementia subtypes or in PD compared with atypical parkinsonian syndromes, indicating clinical potential in differentiating these conditions.

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Manuscript 3

Claire Bridel, Cyra E Leurs, Zoë Y G J van Lierop, Zoé L E van Kempen, Iris Dekker, Harry A M Twaalfhoven, Bastiaan Moraal, Frederik Barkhof, Bernard M J Uitdehaag, Joep Killestein, Charlotte E Teunissen. *Serum Neurofilament Light Association With Progression in Natalizumab-Treated Patients With Relapsing-Remitting Multiple Sclerosis*. Neurology 2021 Nov 9; 97(19):e1898-e1905.

This study aimed at evaluating the potential of serum NfL to serve as a biomarker to predict or monitor progression in RRMS patients. To do so, we measured serum NfL longitudinally over a median time of 5.2 years in people with RRMS treated with natalizumab, with or without progression. This allowed us to evaluate the longitudinal dynamics of NfL mostly in the absence of clinical and radiological signs of acute focal inflammation, thereby allowing us to uncouple progression from inflammatory disease activity. We found that there was neither predictive value of baseline NfL levels nor a difference in longitudinal NfL dynamics between people with RRMS who did or did not progress during the follow-up time of the study.

RESEARCH ARTICLE

Serum Neurofilament Light Association With Progression in Natalizumab-Treated Patients With Relapsing-Remitting Multiple Sclerosis

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Abstract

Background and Objectives

To investigate the potential of serum neurofilament light (NfL) to reflect or predict progression mostly independent of acute inflammatory disease activity in patients with relapsing-remitting multiple sclerosis (RRMS) treated with natalizumab.

Methods

Patients were selected from a prospective observational cohort study initiated in 2006 at the VU University Medical Center Amsterdam, the Netherlands, including patients with RRMS treated with natalizumab. Selection criteria included an age of 18 years or older and a minimum follow-up of 3 years from natalizumab initiation. Clinical and MRI assessments were performed on a yearly basis, and serum NfL was measured at 5 time points during the follow-up, including on the day of natalizumab initiation (baseline), 3 months, 1 year, and 2 years after natalizumab initiation, and on last follow-up visit. Using general linear regression models, we compared the longitudinal dynamics of NfL between patients with and without confirmed Expanded Disability Status Scale (EDSS) progression between year 1 visit and last follow-up, and between individuals with and without EDSS$^+$ progression, a composite endpoint including the EDSS, 9-hole peg test, and timed 25-foot walk.

Results

Eighty-nine natalizumab-treated patients with RRMS were included. Median follow-up time was 5.2 years (interquartile range [IQR] 4.3–6.7, range 3.0–11.0) after natalizumab initiation, mean age at time of natalizumab initiation was 36.9 years (SD 8.5), and median disease duration was 7.4 years (IQR 3.8–12.1). Between year 1 and the last follow-up, 28/89 (31.5%) individuals showed confirmed EDSS progression. Data for the EDSS$^+$ endpoint was available for 73 out of the 89 patients and 35/73 (47.9%) showed confirmed EDSS$^+$ progression. We observed a significant reduction in NfL levels 3 months after natalizumab initiation, which reached its nadir of close to 50% of baseline levels 1 year after treatment initiation. We found no difference in the longitudinal dynamics of NfL in progressors vs nonprogressors. NfL levels at baseline and 1 year after natalizumab initiation did not predict progression at last follow-up.

Conclusion

In our cohort of natalizumab-treated patients with RRMS, NfL fails to capture or predict progression that occurs largely independently of clinical or radiologic signs of acute focal inflammatory disease activity. Additional biomarkers may thus be needed to monitor progression in these patients.

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Glossary

9HT = 9-hole peg test; **DMT** = disease-modifying therapy; **E+NP** = EDSS+ nonprogressors; **E+P** = EDSS+ progressors; **EDSS** = Expanded Disability Status Scale; **EDSS+** = a composite endpoint including the Expanded Disability Status Scale, the 9-hole peg test, and the timed 25-foot walk test; **ENP** = EDSS nonprogressors; **EP** = EDSS progressors; **GE** = gadolinium-enhancing; **IQR** = interquartile range; **MS** = multiple sclerosis; **NfL** = neurofilament light; **RRMS** = relapsing-remitting multiple sclerosis; **SPMS** = secondary progressive multiple sclerosis; **T25W** = timed 25-foot walk test.

Classification of Evidence

This study provides Class II evidence that serum NfL levels are not associated with disease progression in natalizumab-treated patients with RRMS.

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of the CNS. After 10–15 years of disease evolution, progressive irreversible disability accumulates in a majority of patients largely independently of acute focal inflammatory disease activity, which includes relapses and new T2 or gadolinium-enhancing (GE) MRI lesions. With the advent of highly effective disease-modifying therapies (DMT) to treat relapsing-remitting multiple sclerosis (RRMS), acute focal inflammatory disease activity can be silenced in a significant majority of these patients.[1] Once considered a characteristic of secondary progressive MS (SPMS), evidence now indicates that disability accrual can occur from disease onset, independently of acute focal inflammatory disease activity.[2,3] The uncoupling of these processes suggests the mechanisms underlying progression are, at least partly, independent of those causing relapse-related neuroaxonal damage. Treatments that significantly reduce the rate of disability progression are scarce but critically needed, as progression contributes significantly to long-term disability. In order to evaluate the potential of novel therapies to reduce progression rate, biomarkers to quantify or predict this process are needed. Neurofilament light (NfL) is a biomarker of neuroaxonal damage.[4] Its levels increase in serum of patients with RRMS during relapses and concomitantly to the appearance of new T2 or GE lesions, returning to baseline within a couple of months of the acute event, and decrease following DMT initiation.[5] These data suggest that NfL is a promising tool to monitor acute focal inflammatory disease activity in MS. In cross-sectional studies, NfL is associated with measures of disease severity such as the Expanded Disability Status Scale (EDSS), and in longitudinal studies, high baseline NfL predicts EDSS worsening in the following year, or up to 15 years later in patients with clinically isolated syndrome.[6-8] These data suggest NfL holds potential for prediction of short- and long-term neurologic disability.

We hypothesized that NfL levels increase over time in patients with disability progression and can be used to monitor and predict progression that occurs largely independently of acute focal inflammatory disease activity. We tested this hypothesis by comparing the longitudinal trajectories of NfL in natalizumab-treated patients with RRMS that either progressed or not over a period of at least 3 years. We then evaluated the potential of NfL at time of natalizumab initiation or 1 year after treatment initiation to predict progression during follow-up.

Methods

Cohort

Patients were selected from the natalizumab pharmacovigilance study, an ongoing prospective observational cohort study initiated in 2006 at the VU University Medical Center Amsterdam, the Netherlands.[9] Selection criteria for the present study were age 18 years or older and a minimum follow-up of 3 years from natalizumab initiation. Clinical assessments were performed at initiation of natalizumab (baseline) and repeated every 12 months, and included relapse history, EDSS assessment by trained personnel, timed 25-foot walk test (T25W), and 9-hole peg test (9-HPT) (Figure 1). The cohort was retrospectively divided into progressors and nonprogressors according to 2 outcomes: either the EDSS alone or the EDSS$^+$, a composite endpoint including the EDSS, the 9-HPT, and the T25FW.[10] EDSS progression was assessed by comparing EDSS at last follow-up with EDSS at year 1. Year 1 and not baseline was used as a reference EDSS in order to reduce the effect of focal inflammatory disease activity occurring prior to natalizumab initiation, which may potentially affect EDSS at baseline. EDSS progressors (EP) were defined as having a sustained EDSS increase at both the last follow-up and the penultimate EDSS assessment, compared to year 1 EDSS, fulfilling the criteria of confirmed EDSS progression. The increase was at least 1.5 (if reference EDSS score = 0), 1 (if reference EDSS score = 1–5.5), or 0.5 (if reference EDSS score ≥6.0) (Figure 1). EDSS nonprogressors (ENP) were defined as individuals not fulfilling the criteria of EP. EDSS$^+$ progressors (E$^+$P) were defined as having progression in 1 of the 3 components (EDSS, T25FW, or 9-HPT), with a worsening of ≥20% in the T25FW or the 9-HPT at last follow-up, confirmed at the penultimate T25FW and 9-HPT assessment, or in the EDSS as outlined above. EDSS$^+$ nonprogressors (E$^+$NP) were individuals not fulfilling the criteria for E$^+$P. All patients gave written informed consent for the

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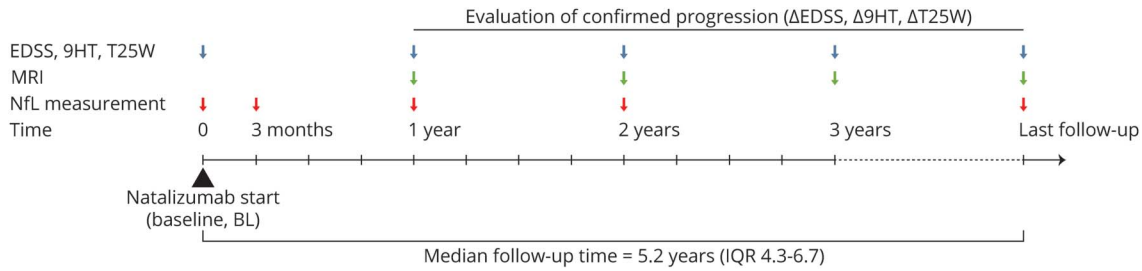
Figure 1 Study Setup



Blue arrows indicate when clinical assessment was performed with respect to natalizumab initiation (baseline). Green arrows indicate when MRI was performed with respect to natalizumab initiation (baseline). Red arrows indicate when neurofilament light (NfL) was measured with respect to natalizumab initiation (baseline). 9HT = 9-hole peg test; EDSS = Expanded Disability Status Scale; IQR = interquartile range; T25W = timed 25-foot walk test.

collection and use of medical data and biological fluids for research purposes. This study was in accordance with the ethical principles of the Declaration of Helsinki and received local ethics committee consent.

Serum NfL Measurement

Blood was collected at baseline, after 3 months, 1 year, 2 years, and at last follow-up (Figure 1)—that is, the last available blood sample during natalizumab treatment before discontinuation or database closure via standard vena puncture—and centrifuged at 1800*g* for 10 minutes at room temperature. Serum was aliquoted and stored at −80°C until analysis. NfL quantification was performed using an in-house developed Simoa assay.[11] The samples of each individual patient were analyzed within one run and the personnel performing the analyses was blinded for the clinical data.

Magnetic Resonance Imaging

MRI protocols included proton density/T2-weighted and postcontrast T1-weighted images. Slice thickness was 3 mm with an in-plane resolution of 1 mm^2. Brain MRI scans were performed on a 1.5T or a 3.0T scanner in the VU University Medical Center Amsterdam. Image acquisition differed among patients (i.e., magnetic field strengths, pulse sequences, head coils, and spatial resolution), which was taken into consideration by the raters in the radiologic analyses. MRI acquisition followed the Magnetic Resonance Imaging in Multiple Sclerosis (MAGNIMS) expert panel guidelines. MRI scans were performed yearly and evaluated by experienced neuroradiologists for inflammatory activity, defined as new T2 lesions or GE (Figure 1).

Statistical Analyses

Statistical data analysis was performed using SPSS for Windows, version 22. Median comparisons were assessed using the Mann-Whitney *U* test. Proportion differences were assessed using the χ^2 test. Mean age differences were assessed using analysis of variance. To compare NfL levels between EP and ENP or E^+P and E^+NP, age, sex, disease duration, relapse activity, and MRI disease activity–corrected univariate analyses of variance were performed on log-transformed NfL values. Binary logistic regression was used to identify predictors for clinical progression at last follow-up, with EDSS progression or $EDSS^+$ progression as dependent variables, and sex, age, disease duration, and log-transformed baseline and year 1 NfL as covariates. A p value < 0.05 was considered statistically significant. The graphs in Figure 2 were constructed in GraphPad Prism version 7.02.

Standard Protocol Approvals, Registrations, and Patient Consents

This study received approval from the local ethics committee on human experimentation. All patients provided written informed consent.

Data Availability

The raw data can be obtained upon reasonable request by contacting the corresponding author.

Results

Patient Characteristics

Eighty-nine natalizumab-treated patients with RRMS were selected, with a follow-up period of at least 3 years (median follow-up time of 5.2 years, interquartile range [IQR] 4.3–6.7, range 3.0–11.0) after natalizumab initiation (Table 1). Data for the $EDSS^+$ endpoint were available for 73 out of the 89 patients (Table 1). Mean age of the entire cohort (n = 89) at time of natalizumab treatment was 36.9 years (SD 8.5) and median disease duration at time of natalizumab initiation was 7.4 years (IQR 3.8–12.1) (Table 1). A total of 14.6% of patients had 1 relapse or more during the follow-up time excluding the first 3 months and 10.1% of patients had MRI disease activity during the follow-up time excluding the first year (Table 1). These numbers are in accordance with the high efficacy of natalizumab to prevent acute focal inflammatory disease activity.[12,13] Between year 1 and the last follow-up visit, 28/89 patients (31.5%) showed confirmed EDSS progression and 35/73 (47.9%) showed confirmed $EDSS^+$ progression (Table 1). Accordingly, median EDSS at last follow-up was higher in EP vs ENP (5.8 [IQR 3.6–6.0] vs 3.5 [IQR 2.0–4.5], $p < 10^{-5}$) and in E^+P (5.0 [3.5–6.0] vs E^+NP (3.5 [2.4–4.0], $p < 10^{-5}$) (Table 1). At baseline, median

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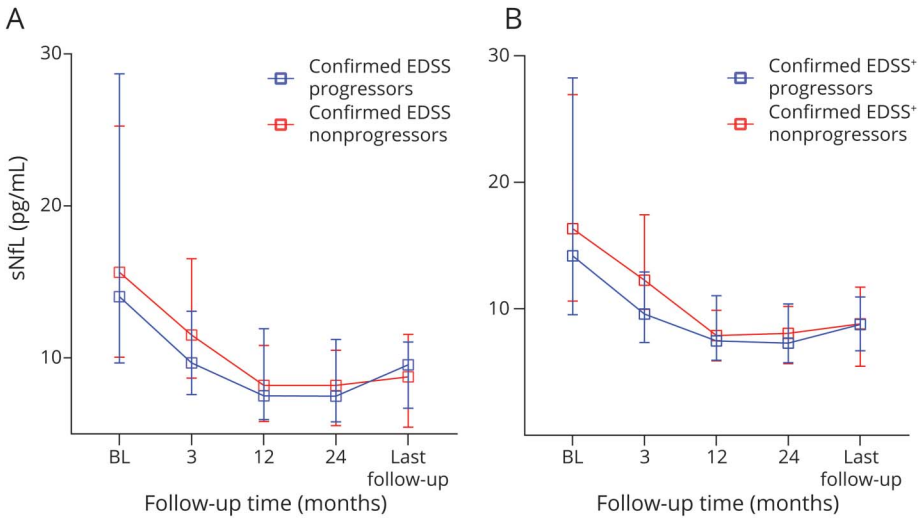
Figure 2 Longitudinal Dynamics of Neurofilament Light (NfL)



(A) Longitudinal dynamics of NfL in Expanded Disability Status Scale (EDSS) progressors (red), nonprogressors (blue), and the entire cohort (black) over time. (B) Longitudinal dynamics of NfL in EDSS$^+$ progressors (red), nonprogressors (blue), and the entire cohort (black) over time. sNfL = serum neurofilament light.

age was higher in EP compared to ENP (40.0 vs 35.4, p = 0.019) and in E$^+$P compared to E$^+$NP (39.5 vs 34.9, p = 0.011) (Table 1). Median disease duration was longer in EP compared to ENP (8.2 [IQR 4.4–16.5] vs 6.9 [IQR 3.2–10.9], p = 0.047), but not between E$^+$P and E$^+$NP (7.9 [IQR 4.3–15.7] vs 7.4 [4.4–11.9], p = 0.480) (Table 1). The percentage of individuals with 1 relapse or more during the follow-up period excluding the first 3 months after natalizumab initiation was low and did not differ between EP and ENP or between E$^+$P and E$^+$NP (Table 1). Similarly, the percentage of individuals with new T2/GE lesions during the follow-up period excluding the first year after natalizumab initiation did not differ significantly between EP and ENP or between E$^+$P and E$^+$NP (Table 1).

Longitudinal Dynamics of Serum NfL Levels After Natalizumab Treatment Initiation

NfL was measured in serum sampled on the day of natalizumab initiation (baseline), 3 months, 1 year, and 2 years after baseline, and on the last follow-up visit (Figure 1). Median NfL decreased significantly from 14.8 pg/mL at baseline to 11.1 pg/mL at 3 months, and reached its nadir of 7.9 pg/mL at year 1, remaining low thereafter (Table 1). Mean baseline and follow-up levels of NfL did not differ between EP and ENP (Table 1 and Figure 2A) or between E$^+$P and E$^+$NP (Table 1 and Figure 2B).

NfL as a Predictor of Future Disability Progression

NfL at baseline or at year 1 did not predict EDSS or EDSS$^+$ progression at last follow-up visit; neither did sex, age at natalizumab onset, or disease duration (data not shown).

Sensitivity Analysis

In this study, the follow-up time was heterogeneous, and patients with a longer follow-up period had a higher chance to progress than those with shorter follow-up periods, thereby introducing a possible classification bias. In order to assess the robustness of our findings, we performed a sensitivity analysis including only those patients who were followed for the same time period of 4 years. Confirmed EDSS and EDSS$^+$ progression were assessed between year 1 and year 4 for all patients. We obtained results similar to those of the primary analysis; that is, no difference in the longitudinal NfL dynamics between progressors and nonprogressors (Table 2).

Discussion

Highly effective therapies such as natalizumab have dramatically changed the short-term and possibly long-term neurologic prognosis of MS.[14] These therapeutic breakthroughs have also revealed that disability worsening can occur in treated patients with RRMS, even in the absence of clinical and MRI signs of focal inflammatory disease activity.[3] While the evidence supporting serum NfL as a biomarker of neuroaxonal damage arising in the context of acute inflammatory disease activity is unequivocal, its potential to capture disability progression is less clear.[8,15]

In this study, we take advantage of a cohort of natalizumab-treated patients with RRMS to study progression largely independent of acute focal inflammation, and how it reflects on serum NfL levels. We find that clinical and radiologic acute inflammatory disease activity is abrogated in a majority of patients, in accordance with the high efficacy of this drug reported in clinical trials.[12,13] About 30% of the patients show confirmed EDSS progression during the follow-up time of the study, and about 45% confirmed EDSS$^+$ progression. None of the patients fulfilled the criteria for transition towards secondary progressive MS during the follow-up period under natalizumab treatment.[16]

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Table 1 Participant Characteristics

	Total cohort EDSS outcome	EDSS nonprogressors	EDSS progressors	*p* Value	Total cohort EDSS$^+$ outcome	EDSS$^+$ nonprogressors	EDSS$^+$ progressors	*p* Value
N (% of whole cohort)	89 (100)	61 (68.5)	28 (31.5)	—	73 (100)	38 (52.1)	35 (47.9)	—
% Female	74.2	72.1	78.6	0.519	74.0	71.1	77.1	0.554
Age at baseline, y	36.9 (8.5)	35.4 (8.5)	40.0 (7.8)	0.019	37.1 (8.1)	34.9 (8.1)	39.5 (7.6)	0.011
Disease duration at baseline, y	7.4 (3.8–12.1)	6.9 (3.2–10.9)	8.2 (4.4–16.5)	0.047	7.6 (4.4–12.6)	7.4 (4.4–11.9)	7.9 (4.3–15.7)	0.480
Follow-up time from baseline to last follow-up visit, y	5.2 (4.3–6.7)	5.0 (4.0–6.4)	5.8 (5.1–8.7)	0.004	5.2 (4.3–6.9)	5.0 (4.0–6.3)	5.4 (5.0–7.2)	0.043
% Individuals with relapse during the follow-up time of the study, excluding the first 3 months after natalizumab initiation	14.6	16.4	10.7	0.481	16.4	21.1	11.4	0.268
% Individuals with new T2 or GE lesions during the follow-up time of the study, excluding the first year after natalizumab initiation	10.1	9.8	10.7	0.898	9.6	10.5	8.6	0.777
EDSS at 12-month follow-up	3.5 (2.4–4.5)	3.0 (2.5–4.0)	3.8 (2.6–5.0)	0.390	3.5 (2.5–4.5)	3.0 (2.5–4.0)	3.5 (3.0–5.0)	0.095
EDSS at last follow-up	4.0 (3.0–5.75)	3.5 (2.0–4.5)	5.8 (3.6–6.0)	$<10^{-5}$	4.0 (3.0–5.8)	3.5 (2.4–4.0)	5.0 (3.5–6.0)	$<10^{-5}$
sNfL at baseline, pg/mL	14.8 (10.0–27.1)	15.2 (10.1–25.3)	14.0 (9.7–28.7)	0.912	15.6 (10.2–27.1)	16.3 (10.6–26.9)	14.2 (9.5–28.3)	0.719
sNfL at 3-month follow-up, pg/mL	11.1 (8.4–16.0)	11.5 (5.8–16.5)	9.7 (7.6–13.1)	0.480	11.1 (8.4–15.6)	12.1 (10.0–17.4)	9.6 (7.4–12.9)	0.185
sNfL at 12-month follow-up, pg/mL	7.9 (5.9–11.0)	8.2 (5.8–10.8)	7.5 (6.0–11.9)	0.926	7.6 (5.9–10.5)	7.9 (5.9–9.9)	7.5 (5.9–11.0)	0.816
sNfL at 24-month follow-up, pg/mL	7.9 (5.7–10.5)	8.2 (5.6–10.5)	7.5 (5.8–11.2)	0.429	7.7 (5.7–10.2)	8.1 (5.5–10.2)	7.3 (5.7–10.4)	0.623
sNfL at last follow-up, pg/mL	8.9 (5.6–11.3)	8.8 (5.5–11.6)	9.6 (6.7–11.1)	0.334	8.8 (5.8–11.3)	8.8. (5.5–11.7)	8.8 (6.7–10.9)	0.344

Abbreviations: EDSS = Expanded Disability Status Scale; EDSS$^+$ = a composite endpoint including the EDSS, the 9-hole peg test, and the timed 25-foot walk test; GE = gadolinium-enhancing; sNfL = serum neurofilament light.
Values are mean (SD) or median (interquartile range).

The percentage of individuals with relapses or new T2/GE lesions did not differ significantly between progressors and nonprogressors, although small differences between the groups may have been missed due to the relatively small size of the cohort. This supports the hypothesis that the mechanisms driving progression are distinct from those underlying acute focal inflammatory disease activity. We find that individuals who progressed either according to the confirmed EDSS or the confirmed EDSS$^+$ outcome were slightly older and their disease duration at baseline was slightly longer compared to those who did not, suggesting an age and disease duration threshold before progression becomes clinically manifest.

We observe a reduction in NfL levels of almost 50% of baseline levels 1 year after natalizumab initiation, in accordance with other studies.[17,18] Furthermore, we find that NfL remains low for the entire follow-up period under natalizumab treatment. We observe no differences in the longitudinal dynamics of NfL levels between EP and ENP or between E$^+$P and E$^+$NP, correcting for age, sex, disease duration, relapses, and MRI signs of acute focal inflammatory disease activity. Although the cohort size is relatively limited, the absence of even a trend towards significance suggests NfL does not capture progression occurring largely independently of relapse or MRI activity in natalizumab-treated patients.

Median follow-up time was slightly longer in EP and E$^+$P compared to ENP and E$^+$NP, and in order to evaluate the effect of a possible classification bias, we performed a sensitivity analysis with a fixed follow-up time of 4 years. We found similar results, suggesting the heterogeneity in follow-up periods does not introduce a large bias, although the cohort investigated in the sensitivity analysis was smaller than the initial cohort.

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Table 2 Characteristics of Participants With a 4-Year Follow-up Period

	Total cohort EDSS outcome	EDSS nonprogressors, year 1–year 4	EDSS progressors, year 1–year 4	*p* Value
N	65	57	8	—
% Female	72.3	70.2	87.5	0.305
Age at baseline, y	38.1 (8.3)	37.9 (8.4)	39.3 (7.9)	0.649
Disease duration at baseline, y	7.6 (4.3–12.1)	8.5 (4.3–12.6)	6.5 (3.1–9.8)	0.231
% Individuals with relapse during the 5-year follow-up, excluding the first 3 months after natalizumab initiation	10.8	15.8	12.5	0.809
% Individuals with new T2 or GE lesions during the 5-year follow-up, excluding the first year after natalizumab initiation	15.4	12.3	0.0	0.294
EDSS at year 1 follow-up	3.5 (2.5–4.5)	3.5 (2.5–4.5)	3.5 (3.0–5.6)	0.755
EDSS at year 4 follow-up	4.0 (2.5–5.0)	3.5 (2.5–4.5)	5.3 (4.0–6.5)	0.025
sNfL at baseline, pg/mL	13.9 (9.4–21.5)	14.2 (9.5–22.2)	12.4 (9.3–16.6)	0.771
sNfL at 3-month follow-up, pg/mL	10.3 (5.8–13.0)	10.3 (7.8–14.4)	10.1 (7.1–12.8)	0.981
sNfL at 12-month follow-up, pg/mL	7.3 (5.8–10.1)	7.4 (5.8–10.1)	6.5 (5.3–10.8)	0.426
sNfL at 24-month follow-up, pg/mL	7.1 (5.3–10.1)	7.5 (5.3–10.5)	6.5 (4.8–9.1)	0.298

Abbreviations: EDSS = Expanded Disability Status Scale; GE = gadolinium-enhancing; sNfL = serum neurofilament light.
Values are mean (SD) or median (interquartile range).

Few studies have investigated the potential of NfL to reflect disease progression or neurodegeneration in MS. Ibudilast, a molecule currently investigated as a treatment to slow progression in MS, is associated with a dose-dependent reduction in whole brain atrophy progression in patients with progressive MS.[19] In a recent study, it was reported that this reduction in brain atrophy is not reflected in NfL levels, as serum NfL levels did not differ between individuals with or without brain atrophy progression.[20] These data suggest NfL may not capture neurodegeneration, which is thought to underlie disability progression. However, in a phase 3 randomized controlled trial of natalizumab in SPMS, NfL levels at week 96 were higher in E^+P vs E^+NP.[21] In this poster, it is not reported whether E^+P and E^+NP differed in terms of acute inflammatory disease activity, which may account, at least partially, for the differences in NfL levels.

NfL levels increase most substantially in neurologic conditions characterized by a high rate of neuroaxonal loss, such as amyotrophic lateral sclerosis and stroke, while in conditions characterized by a lower yet sustained rate of neuroaxonal loss such as Alzheimer disease, the increase in NfL levels is more subtle.[4] We may thus hypothesize that while a powerful tool to capture the massive increase in acute neuroaxonal damage that occurs over the relatively short time period of a relapse, NfL probably lacks the sensitivity to reflect the lower rate of sustained neurodegenerative axonal damage that underlies progression in RRMS.

Our data do not support a prognostic value for baseline or year 1 NfL in terms of EDSS or $EDSS^+$ progression prediction at last follow-up, when focal acute inflammatory disease activity is largely suppressed. This finding suggests the prognostic value of NfL reported in other studies may rather be related to its ability to reflect acute neuroaxonal damage due to focal inflammatory disease activity than progression.[6,7,22-25.]

A limitation of our study is the use of EDSS worsening as a clinical outcome measure of disability progression. Despite being the most widely used outcome measure for disability progression in MS, this metric has several limitations. First, it is based on neurologic examination, which is intrinsically subjective, and EDSS scoring has been reported to have high intra- and interrater variability.[26] We mitigated measurement variability by having EDSS assessments made exclusively by trained medical personnel. Second, EDSS worsening occurs not only in the context of progression, but also transiently in the context of a relapse. To reduce the contribution of relapses to EDSS worsening, we used confirmed EDSS as an outcome. Confirmation of the EDSS was obtained at least 1 year apart, in order to reduce the likelihood of capturing events that would subsequently regress. Third, the EDSS may lack sensitivity to capture progression, especially in individuals with higher baseline EDSS score. To increase the sensitivity for identifying progression in SPMS, the $EDSS^+$ endpoint was developed, which includes measures of short-distance ambulatory function (T25W) and upper-extremity function (9-HPT).[10] The $EDSS^+$ was reported to be more sensitive than the EDSS to detect progression in SPMS.[10] Although not validated as a measure of progression in RRMS, we reasoned that it is the rate rather than the nature of progression that

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differs between RRMS and SPMS, and the EDSS$^+$ may thus be an interesting alternative disability outcome measure in RRMS as well. The proportion of progressors according to the EDSS$^+$ outcome was higher compared proportion of progressors according to the EDSS outcome, suggesting a higher sensitivity for detection of progression in RRMS as well. Finally, the EDSS score is nonlinear, and the rate of EDSS progression varies as a function of the EDSS score at baseline.[27] We thus used a definition of EDSS worsening adjusted to baseline EDSS to lessen this limitation. Other limitations of the EDSS include an underrepresentation of cognitive function in disability scoring, which we did not address in this study.

Using confirmed EDSS or EDSS$^+$ worsening as clinical outcomes of disability progression, this study identifies progression in a significant proportion of patients with RRMS unmasked by treatment with natalizumab, and reveals NfL trajectories do not vary between progressors and nonprogressors, suggesting NfL may not be a well-suited biomarker to monitor or predict this process.

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Cyra E. Leurs, MD, PhD	Department of Neurology, Amsterdam UMC, the Netherlands	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data
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Zoé L.E. van Kempen, MD, PhD	Department of Neurology, Amsterdam UMC, the Netherlands	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data
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Bernard M.J. Uitdehaag, MD, PhD	Department of Neurology, Amsterdam UMC, the Netherlands	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data
Joep Killestein, MD, PhD	Department of Neurology, Amsterdam UMC, the Netherlands	Major role in the acquisition of data; study concept or design; analysis or interpretation of data
Charlotte E. Teunissen, PhD	Clinical Chemistry Laboratory, Amsterdam UMC, the Netherlands	Drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data

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Serum Neurofilament Light Association With Progression in Natalizumab-Treated Patients With Relapsing-Remitting Multiple Sclerosis

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Manuscript 4

Inge MW Verberk, Marleen Koel-Simmelink, Harry Twaalfhoven, Hugo Vrenken, Carsten Korth, Joep Killestein, Charlotte E Teunissen, Claire Bridel. *Ultrasensitive immunoassay allows measurement of serum neurofilament heavy in multiple sclerosis.* Mult Scler Relat Disord 2021 May; 50:102840.

This study aimed at evaluating and comparing 3 novel highly sensitive immunoassays, including 2 in-house developed assays, to measure candidate biomarker of progression NfH in serum of PwMS and controls. We found that the commercially available single molecule assay (Simoa) was most sensitive, allowing robust measurement of serum NfL both in PwMS and controls.

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Original article

Ultrasensitive immunoassay allows measurement of serum neurofilament heavy in multiple sclerosis

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ABSTRACT

Background: Neurofilament heavy (NfH) is a promising biomarker for neuro-axonal damage in Multiple Sclerosis (MS). We compared the performance of high-sensitivity serum-NfH immunoassays, with as aim to investigate the value of serum-NfH as biomarker for MS.
Methods: We measured serum-NfH in 76 MS patients with Simoa (one commercial, one in-house) or Luminex assays. Serum-NfH measured by the immunoassay with greatest sensitivity was related to clinical and radiological outcomes with age and sex-adjusted linear regression analysis, and to biological outcomes cerebrospinal fluid (CSF)-NfH, serum neurofilament light (NfL) and CSF-NfL with Spearman's correlation analysis.
Results: With the commercial Simoa assay, we obtained 100% serum-NfH detectability (in-house Simoa: 70%, Luminex: 61%), with lowest coefficient of variation (CV) between duplicates of 11%CV (in-house Simoa: 22%CV, Luminex: 30%CV). Serum-NfH quantified with the commercial Simoa assay was associated with disease duration (standardized beta (sβ) = 0.28, $p = 0.034$), T2 lesion volume (sβ = 0.23, $p = 0.041$), and tended to associate with black hole count (sβ = 0.21, $p = 0.084$) but not with Expanded Disease Disability Score (EDSS) or normalized brain volume (all: $p>0.10$). Furthermore, serum-NfH showed correlations with CSF-NfH (rho = 0.27, $p = 0.018$) and serum-NfL (rho=0.44, $p < 0.001$), but not with CSF-NfL.
Conclusions: Serum-NfH can be quantified with high-sensitivity technology. Cross-sectionally, we observed some weak correlations of serum-NfH with MS disease burden parameters, suggesting there might be some utility for serum-NfH as biomarker for MS disease burden.

1. Introduction

Multiple Sclerosis (MS) is a chronic inflammatory and degenerative disease of the central nervous system (Thompson et al., 2018). Converging evidence indicates that neuronal damage occurs from MS disease onset and most likely underlies the progressive, irreversible accumulation of disability. A number of treatment options are available to reduce relapse rate in relapsing-remitting MS (RRMS) (Thompson et al., 2018; Ziemssen et al., 2015). However, those drugs have little, if any, efficacy in preventing disability progression during secondary progressive MS (SPMS). Clinical trials targeting prevention of disability progression require tools to dynamically quantify axonal damage over a relatively short time period. Such tools would be useful in the clinical setting as well, to monitor disease progression and to assist therapeutic decisions. Neurofilaments are the major components of the axonal cytoskeleton (Khalil et al., 2018; Petzold, 2005), and are promising biomarker candidates to fulfill these roles.

Neurofilaments are heteropolymers composed of the light (NfL), medium and phosphorylated heavy (NfH) subunits (Khalil et al., 2018). NfL and NfH levels are elevated in the CSF of MS patients compared to controls (Bridel et al., 2019; Khalil et al., 2018; Kuhle et al., 2011, 2013; Trentini et al., 2014), and levels correlate with focal inflammatory

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disease activity measures such as relapse rate and new T2 lesions (Sellebjerg et al., 2018; Disanto et al., 2017; Barro et al., 2018; Damasceno et al., 2019; Kuhle et al., 2019; Dalla Costa et al., 2019; Varhaug et al., 2018; Kuhle et al., 2013). Neurofilament levels may also capture progressive neuronal damage in MS, which is thought to underlie disability progression. Indeed, CSF-NfH levels were reported to correlate with clinical disease severity measures (Khalil et al., 2013; Kuhle et al., 2011; Petzold et al., 2016) and to relate to neurodegeneration measures on MRI (Khalil et al., 2013; Petzold et al., 2016).

The development of an ultrasensitive NfL immunoassay on Simoa (Quanterix) (Rissin et al., 2010), has allowed robust measurement of serum NfL (Kuhle et al., 2016). Consequently, it became possible to demonstrate that serum-NfL has clear relationships with measures of acute disease activity and decreases upon effective treatment (Khalil et al., 2018; Barro et al., 2018; Kuhle et al., 2019; Novakova et al., 2017). The assessment of NfH in blood has not yet led to reproducible results in MS (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017). So far, studies reporting NfH values in blood in MS applied traditional immunoassays (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017). Conflicting results are likely due to poor assay sensitivity hampering reliable sample measurement. In this study, we first aimed to compare the analytical sensitivity of three bead-based immunoassays developed on high-sensitive analytical platforms, comprising two Simoa assays (commercially available and in-house developed) and one Luminex assay (in-house developed). Subsequently, we selected the best performing assay to explore the potential of serum-NfH as a biomarker for inflammatory disease burden, neuronal damage and clinical disease severity in MS. As a comparative analysis, we investigated the relationships of CSF-NfH, serum-NfL and CSF-NfL with these severity measures as well.

2. Materials and methods

2.1. Subjects

We included 76 MS patients, who visited the Amsterdam UMC MS center between 2000 and 2004. Patients were diagnosed with MS according to McDonald criteria (McDonald et al., 2001) and subtyped according to Lublin and Reingold criteria (Lublin and Reingold, 1996) ($n = 64$ relapse-onset MS ($n = 38$ RRMS; $n = 26$ SPMS) and $n = 12$ primary progressive MS (PPMS)). Clinical disease severity was assessed by neurological examination and quantified with the Expanded Disability Status Scale (EDSS). Serum was collected by vena puncture and CSF ($n = 75$) was collected by lumbar puncture within three weeks of the clinical visit. Two patients experienced a relapse in the month preceding body fluid collection. Body fluids were biobanked until use according to consensus guidelines (Teunissen et al., 2009). The local medical ethical committee consented with this research and all patients gave written informed consent for use of biomaterials and medical data. The study was conducted in accordance with the ethical principles of the Helsinki Declaration of 1975.

2.2. MRI

MRI was performed in 73 (96%) patients using a 1.0 Tesla scanner (Siemens Magnetom Impact, Erlangen, Germany), mostly within three weeks of the serum and CSF collection ($n = 72$, 99%; one patient had a MRI scan 362 days prior to blood and CSF collection). Both T1-weighted axial pre- and post-contrast images (repetition time (TR) = 700 ms, echo time (TE = 15 ms) and T2-weighted (TR = 2700 ms, TE = 90 ms) were acquired with 5.0 mm slice thickness and 0.5 mm inter-slice gap. Using in-house developed semi-automated seed growing software, gadolinium enhanced (Gd+) lesions, black hole lesions (T1 hypo-intense lesions) and T2 lesions were quantified based on a local thresholding technique. Normalized brain volume was assessed as a measure of brain atrophy from the pre-contrast T1-weighted images using the 'Structural Image Evaluation, using Normalization, of Atrophy Cross-sectional' (SIENAX) technique (Smith et al., 2002).

2.3. Serum neurofilaments analysis

Prior to serum analysis, samples were thawed at room temperature and centrifuged at 14,000xg for 10 min to remove any debris from the samples. All samples were measured in duplicates.

2.3.1. In-house Luminex serum-NfH assay

We measured serum-NfH with an in-house developed SinglePlex xMAP® assay (Luminex), following the procedure described previously (Koel-Simmelink et al., 2014). Samples were diluted 40-fold.

2.3.2. In-house Simoa serum-NfH assay

An in-house developed Simoa (Quanterix) serum-NfH assay was developed by transferring the Luminex NfH assay set-up (Koel-Simmelink et al., 2014) onto the Simoa HD-1 platform, using the homebrew assay development kit (Quanterix). We measured serum-NfH with this automated assay with onboard 4-fold sample dilution. In short, paramagnetic carboxylated beads (Quanterix) were activated using 0.3 mg/mL EDC (ThermoScientific) and coated with 0.3 mg/mL anti-NfH monoclonal 9C9 antibody (shared by Carsten Korth; Schmitz et al., 2014). Detector antibody N4142 (Sigma Aldrich) was biotinylated using NHS-PEG4-Biotin (Thermo Scientific) in antibody to biotin challenge ratio of 40x. Reagent and sample diluent consisted of TBS + 6 mM EDTA + 1% BSA + 0.1% Tween + 10 μg/mL Rabbit IgG (DAKO, Agilent Pathology Solutions). In the first step, 25μl of 250 K assay beads with 250 K Simoa dye-encoded helper beads (Quanterix) were incubated for 30 min with 20μl 0.3 μg/mL biotinylated detector antibody and 100μl of 4-fold diluted sample or calibrator (bovine NfH protein; Neurofilament 200 kD; Progen). After a wash cycle, in step two, 100 μl 100pM enzyme streptavidin β-galactosidase (Quanterix) was added and incubated for 5 min 15 sec. After a last wash cycle, 25 μl of Resorufin β-D-galactopyranoside (Quanterix) was added and beads were pulled directly onto the imaging disk for time-lapsed fluorescent imaging.

2.3.3. Commercial Simoa serum-NfH assay

We measured serum-NfH with the commercially available Simoa™ pNF-Heavy Discovery kit (Quanterix) on the Simoa HD-1 analyzer according to manufacturer's instructions, with onboard automated 4-fold dilution.

2.3.4. Simoa serum-NfL

We measured serum-NfL with an in-house assay on the Simoa HD-1 analyzer, with onboard automated 4-fold dilution. The Uman Diagnostics antibodies (capture: mAb 47:3, detector: biotinylated mAb 2:1) were transferred onto the Simoa HD-1 platform using the homebrew assay development kit (Quanterix), as described previously (Kuhle et al., 2016).

2.4. CSF-NfH and CSF-NfL measurements

CSF-NfH was measured with the in-house NfH Luminex assay (Koel-Simmelink et al., 2014) and CSF-NfL was measured with the commercially available NF-light® ELISA assay (Uman Diagnostics).

2.4.1. Assay performance comparison and statistical analyses

We used SPSS for windows (version 24.0) for data analysis, and R (version 3.4.2) to construct graphs. $p<0.05$ was considered statistically significant, and $p<0.10$ a statistical trend. When duplicate serum-NfH values were at or below blank reading (i.e. non-detectable), we assigned the concentration 0. When at least one of the duplicate measurements gave a detectable serum-NfH concentration, we counted the

measurement as detectable and used the monoplo concentration for statistical analysis. Coefficients of variation (CV; standard deviation divided by the mean) of the detectable duplicate serum-NfH measurements were calculated. We plotted %CV against the detected concentrations to visualize precision for each assay. A local regression line (LOESS) was fitted to the precision plots, and lower limit of quantification (LLOQ) was extracted at the point where the fitted line crossed 20%CV. We did not exclude serum-NfH values below LLOQ or values with a duplicate %CV above 20% from statistical analysis. We conducted age and sex-adjusted linear regression analyses between natural log-transformed neurofilament levels and clinical or MRI measures Additionally, we conducted Spearman's correlation analysis between neurofilament levels. Analyses were performed both on the total cohort and restricted to the relapse-onset subset (i.e. RRMS + SPMS).

3. Results

3.1. Comparison of the analytical performance of the three serum-NfH assays

The commercially available Simoa assay could detect serum-NfH in all samples, of which serum-NfH level was below LLOQ for 10/76 (13%) samples. The in-house developed Simoa assay could detect serum-NfH in 53/76 (70%) samples, of which 31 were below the LLOQ. The Luminex assay could detect serum-NfH in 46/76 (61%) samples, of which 21 were below the LLOQ. Average intra-assay precision of detectable duplicate values was 11%CV for the commercial Simoa assay (calculated on 74/76 samples; 11/74 samples had%CV>20%), 22%CV for the in-house Simoa assay (calculated on 30/76 samples; 9/30 samples had%CV>20%) and 30%CV for the Luminex assay (calculated on 46/76 samples; 21/46 samples had%CV>20%). Precision profiles (supplemental figure 1) show a notably steeper increase in %CV at lower serum-NfH values for the Luminex (supplemental figure 1A) and in-house Simoa (supplemental figure 1C) assays, as compared to the commercial Simoa assay (supplemental figure 1E).

Correlations between the serum-NfH levels generated by the three assays showed a strong correlation of Spearman's rho = 0.90 ($p < 0.001$) between both Simoa serum-NfH measurements, whereas the serum-NfH Luminex measurement correlated with rho = 0.44 ($p < 0.001$) and rho = 0.48 ($p < 0.001$) with the commercial Simoa and in-house Simoa assays respectively (supplemental figure 2).

Because of its highest sensitivity (i.e. maximal detectability) and highest robustness (i.e. lowest mean duplicate %CV) among the three assays investigated, we selected the commercial Simoa assay for further clinical validation.

3.2. Neurofilament levels in relation to MS clinical features and MRI measures

The MS patients had a median (interquartile range (IQR)) age of 46 (38 – 53) years and median disease duration prior to serum sampling of 11 (IQR: 4 – 20) years (Table 1). Measured by the commercially available Simoa assay, the cohort's median serum-NfH level was 48 pg/mL (IQR: 26 – 111). In RRMS, median serum-NfH was 37 pg/mL (IQR: 23 – 78), in SPMS this was 67 pg/mL (IQR: 29 – 163) and in PPMS this was 72 pg/mL (IQR: 32 – 111).

Relationships between serum-NfH measured by the commercially available Simoa assay and MRI and clinical outcome measures are presented in Table 2 and Fig. 1. Adjusted for age and sex, in the total study cohort we observed positive moderate associations between serum-NfH and T2 lesion volume (standardized (s)β = 0.23, $p = 0.041$) but not T2 lesion count, a trend for a positive association with black hole count (sβ = 0.21, $p = 0.084$) but not black hole volume and a positive association with disease duration (standardized beta (sβ) = 0.28, $p = 0.034$). When focusing on the relapse-onset subset, serum-NfH tended to positively associate with T2 lesion volume (sβ = 0.24, $p = 0.053$) and remained

Table 1
Demographics and clinical characteristics of the MS cohort.

	Total cohort	Relapse-onset subset
	$n = 76$	$n = 64$
Characteristics		
Age, years	46 (38 – 53)	46 (36 – 53)
Female (%)	36 (47%)	32 (50%)
MS subtype: RRMS/SPMS/PPMS	38 / 26 / 12	38 / 26 / 0
Disease duration, years	11 (4 – 20) (	11 (4 – 20)
EDSS	4 (3 – 6)	4 (3 – 6)
Interferon-beta used (%)	24 (32%)	23 (36%)
MRI measures		
Normalized brain volume, cm^3	-	1197 (1134 – 1281)
Black hole count	3 (2 – 14)	3 (2 – 12)
Black hole volume, mm^3	226 (58 – 1189)	188 (55 – 1188)
T2 lesion count	25 (11 – 41)	25 (11– 44)
T2 lesion volume, mm^3	3736 (939 – 9639)	4011 (1100 – 9981)
Gd+ lesions present	10 (13%)	9 (14%)
Serum measures		
NfH, pg/mL, Commercial Simoa	48 (26 – 111)	46 (25 – 111)
NfH, pg/mL, In-house Simoa	1.7 (0 – 18)	1.8 (0 – 20)
NfH, pg/mL, Luminex	18 (0 – 61)	21 (0 – 61)
NfL, pg/mL	13 (9 – 19)	13 (9 – 18)
CSF measures		
NfH, pg/mL,	605 (453 – 796)	574 (453 – 790)
NfL, pg/mL,	665 (480 – 925)	665 (480 – 925)

Demographic features of the total cohort (RRMS, SPMS, PPMS), and for the relapse-onset subset (RRMS and SPMS). Features are presented as median (interquartile range: 25th – 75th percentile) or n(%). Normalized brain volume was unavailable for the PPMS group. Serum-NfL was available for 71 patients. CSF-NfH and CSF-NfL were available for 75 patients. When the immunoassay could not detect serum-NfH, we assigned the value 0 pg/mL. RRMS=relapsing-remitting multiple sclerosis, SPMS=secondary progressive multiple sclerosis, PPMS=primary progressive multiple sclerosis, EDSS=Expanded Disability Status Scale, Gd+=Gadolineum enhanced, NfH=Neurofilament heavy, Simoa=Single Molecule Array, NfL=Neurofilament light, CSF=Cerebrospinal fluid.

Table 2
Neurofilaments levels in relation to MS clinical features and MRI measures.

		Serum-NfH sβ	CSF-NfH sβ	Serum-NfL sβ	CSF-NfL sβ
Normalized brain volume	Total cohort	–	–	–	–
	Relapse-onset subset	−0.19	−0.42 **	0.00	−0.05
T2 lesion count	Total cohort	0.08	0.22	0.23	0.20
	Relapse-onset subset	0.05	0.28 *	0.21	0.26 *
T2 lesion volume	Total cohort	0.23 *	0.17	0.17	0.07
	Relapse-onset subset	0.24	0.17	0.16	0.09
Black hole count	Total cohort	0.24	0.12	0.15	0.12
	Relapse-onset subset	0.22	0.10	0.15	0.07
Black hole volume	Total cohort	0.12	0.13	0.06	0.09
	Relapse-onset subset	0.12	0.12	0.07	0.07
EDSS	Total cohort	0.13	0.33 **	0.12	−0.02
	Relapse-onset subset	0.20	0.29 *	0.15	−0.06
Disease duration	Total cohort	0.28 *	0.06	0.15	−0.07
	Relapse-onset subset	0.41 **	−0.03	0.20	−0.10

Results are presented as standardized betas, obtained from linear regression analysis adjusted for relevant confounders age and sex, with natural log-transformed neurofilament data as independent and disease activity and MRI parameters as dependent variables. Normalized brain volume was available only for the relapse-onset subset. Analysis was performed both on the total dataset and on the relapse-onset subset (RRMS and SPMS cases) * $p<0.05$ ** $p<0.01$.

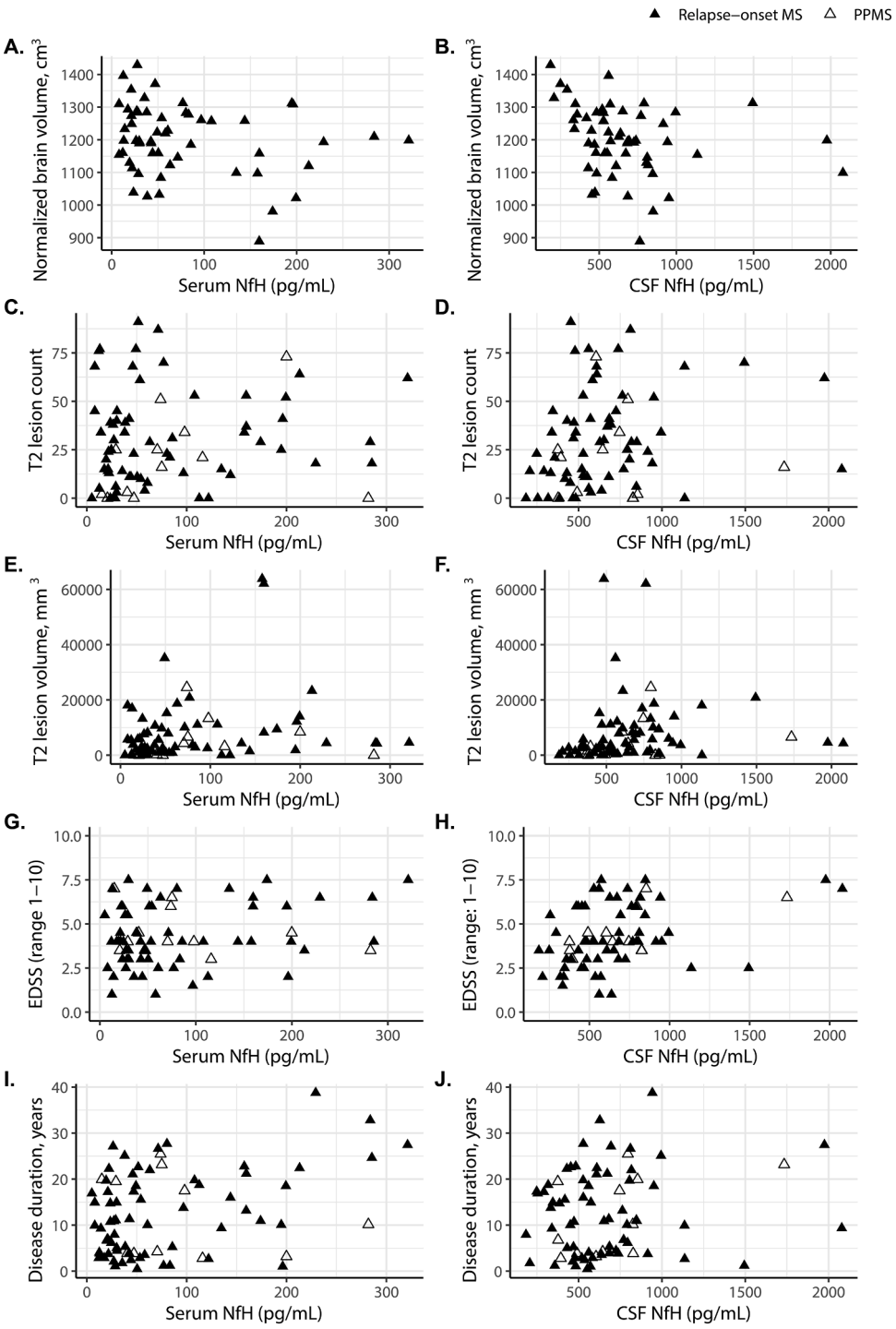

Fig. 1. Correlation of serum-NfH and CSF-NfH with MS clinical features and MRI measures
Scatterplots for the total study cohort ($n = 76$). Closed triangles represent the relapse-onset subset (RRMS and SPMS cases), open triangles are the PPMS cases. Serum NfH was measured by the commercial Simoa assay. NfH=Neurofilament heavy, CSF=Cerebrospinal fluid, EDSS=Expanded Disability Status Scale.

positively associated with disease duration ($s\beta = 0.41$, $p = 0.006$). No relationship of serum-NfH with normalized brain volume or EDSS was observed.

As comparative analysis, we investigated relationships of CSF-NfH, serum-NfL and CSF-NfL with clinical features and MRI outcome measures (Table 2, Fig. 2). In contrast to serum-NfH, in the total study cohort CSF-NfH tended to positively associate with T2 lesion count ($s\beta = 0.22$, $p = 0.053$) and positively associated with EDSS ($s\beta = 0.33$, $p = 0.007$) (Table 2, Fig. 2). In the relapse-onset subset, CSF-NfH showed a positive association with T2 lesion count ($s\beta$=0.28, $p = 0.024$), a negative association with normalized brain volume ($s\beta$=−0.42, $p = 0.002$), and a positive association with EDSS ($s\beta = 0.29$, $p = 0.033$). Serum-NfL and CSF-NfL showed a trend to a moderate positive association with T2 lesion count in the total study cohort (serum-NfL: $s\beta$=0.23, $p = 0.064$; CSF-NfL: $s\beta = 0.20$, $p = 0.085$), but were not associated with any of the other MRI or clinical measures. In the relapse-onset subset, this relationship with T2 lesion count disappeared for serum-NfL ($s\beta = 0.21$, $p = 0.113$) whereas it became significant for CSF-NfL ($s\beta = 0.26$, $p = 0.041$).

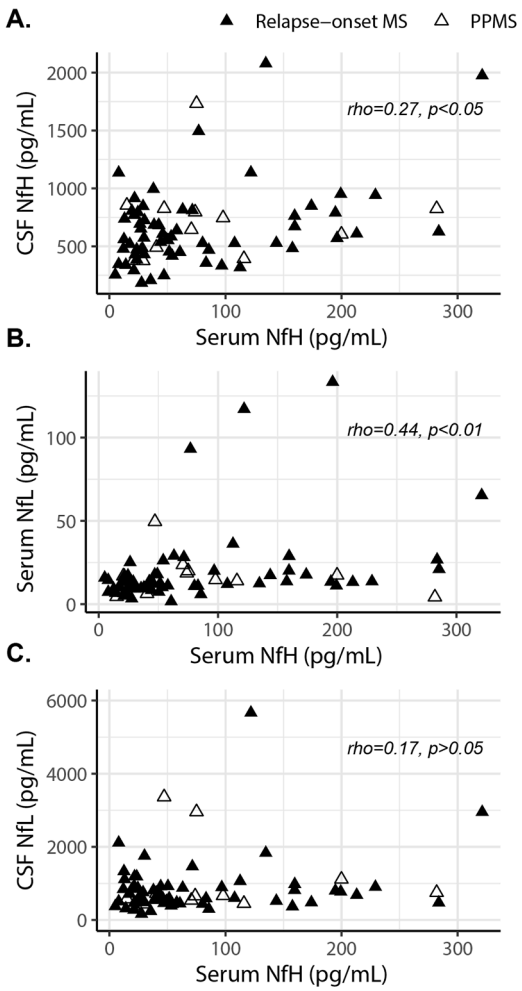

Fig. 2. Correlation of serum-NfH (commercial Simoa assay) with CSF-NfH, serum-NfL and CSF-NfL

Scatterplots including spearman's rho correlation coefficients for the total study cohort ($n = 76$). Closed triangles represent the relapse-onset subset (RRMS and SPMS cases). Serum-NfH was assessed using the commercial Simoa assay. CSF neurofilament data was missing for one subject. NfH=Neurofilament heavy, CSF=Cerebrospinal fluid, NfL=Neurofilament light.

3.3. Correlations of serum-NfH with CSF-NfH, serum-NfL and CSF-NfL

Serum-NfH assessed by the commercial Simoa assay correlated with CSF-NfH (Table 3, Fig. 2A), with comparable Spearman's correlation coefficients in the total study cohort (rho = 0.27, $p = 0.018$) and in the relapse-onset subset (rho = 0.26, $p = 0.036$). Serum-NfH correlated with serum-NfL as well (Table 3, Fig. 2B), again with comparable correlation coefficients in the total study cohort (rho = 0.44, $p < 0.001$) and the relapse-onset subset (rho = 0.49, $p < 0.001$). No correlation between serum-NfH and CSF-NfL levels was observed (Table 3, Fig. 2C).

Table 3

Spearman's correlation of serum-NfH (commercial Simoa assay) in relation to CSF-NfH, serum-NfL and CSF-NfL.

Total cohort	CSF-NfH	Serum-NfL	CSF-NfL
Serum-NfH	0.27 *	0.44 **	0.17
Relapse-onset subset			
Serum-NfH	0.26 *	0.49 **	0.11

Spearman's rho correlation coefficients for the total study cohort ($n = 76$) and for the relapse-onset subset only ($n = 64$; RRMS and SPMS), between serum-NfH measured by the commercially available Simoa assay and CSF-NfH, serum-NfL and CSF-NfL. CSF neurofilament data was missing for one subject. * $p<0.05$, ** $p<0.01$. NfH=Neurofilament heavy, NfL=Neurofilament light, CSF=Cerebrospinal fluid.

4. Discussion

We found that NfH levels in serum of MS patients can be detected with an ultrasensitive immunoassay. With this assay, serum-NfH levels were associated with T2 lesion volume and disease duration, and there was a tendency for an association with black hole count. These findings suggest that serum-NfH might have some utility as biomarker of disease burden in MS.

Analytical sensitivity was an issue in four out of five previously published studies on blood-based NfH as biomarker for MS (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017). These studies all applied conventional ELISA immunoassays from different vendors. We reasoned that high-sensitive technology is needed for reliable serum-NfH measurement. We first performed a comparison of three different phosphorylated serum-NfH immunoassays, on two different platforms: a Luminex assay, an in-house developed Simoa assay using the same antibodies and comparable buffers as the Luminex assay, and a commercially available Simoa assay. All three assays were bead-based, thus capture-antibodies are coupled to paramagnetic beads instead of coated on the surface of 96-well plates. In bead-based assays, incubation steps are more effective compared to ELISAs, because the capture beads are in suspension with samples and as such can easier encounter antigens. Additionally, the washing steps in bead-based assays are more effective compared to ELISA, by using the magnetic properties of the beads resulting in reduced background signals thus increased signal to noise ratios. Simoa has an additional advantage over Luminex, because in Simoa the beads are pulled into femtoliter-sized reaction chambers that are sized to fit no more than one bead (Rissin et al., 2010). Due to this extremely low reaction volume a minimal increase in fluorescent signal can be detected, whereas in Luminex this minimal increase in signal would diffuse in the larger reaction volume and consequently remain below detection limit. In our assay comparison, we found that the correlation of serum-NfH levels between the Luminex assay and both Simoa assays was substantially weaker (rho<0.48), as compared to the between-Simoa assays correlation (rho = 0.90), even though the Simoa assays employed different antibodies. This weaker correlation suggested that the Luminex results were more hampered by lack of measurement reliability compared to Simoa, which fits with the technological benefits Simoa has over Luminex (Rissin et al., 2010). Still, only the commercial Simoa assay yielded 100% serum-NfH detectability in the samples, indicating higher analyte-affinity of the antibodies as compared to the in-house Simoa assay. It is to note that still not all samples were measured above LLOQ and with a reliable%CV of duplicate measurements however, showing that serum-NfH measurement remains challenging. A recent study applied a similar design as our study, and compared serum-NfH assay performance in patients with amyotrophic lateral sclerosis and frontotemporal dementia (Wilke et al., 2019). Serum-NfH levels are generally higher in those patients as compared to MS patients as included in our study. Yet, their conclusion that serum-NfH can be most reliably measured by a Simoa assay and not by lower-sensitivity alternatives supports our conclusion.

Upon exploration of the possible clinical value of serum-NfH in MS, we observed positive relationships of serum-NfH with T2 lesion volume, and a trend for a positive relationship with black hole count. The correlations were moderate, which could be due to the fact that the T2 lesions and black holes were not aged precisely, and a proportion of these lesions may be old and inactive. We hypothesized that serum-NfH reflects neuronal damage, which increases with MS disease duration. Similarly, T2 and black hole lesion load increases with disease duration. The positive relationships we observed between serum-NfH and T2 lesions and black holes support this hypothesis. We did not find significant

associations of serum-NfH with normalized brain volume and EDSS, although the direction of the effect sizes were in line with expectations. Since previous studies on serum-NfH in MS were hampered by analytical sensitivity (Fialova et al., 2013; Ljubisavljevic et al., 2016; Gnanapavan et al., 2013; Gresle et al., 2014; Kuhle et al., 2017), and there were notable cohort differences between our study and those previous studies (e.g. inclusion of only SPMS patients (Gnanapavan et al., 2013), additional inclusion of unaffected controls (Gresle et al., 2014; Ljubisavljevic et al., 2016) or patients with one clinically isolated event of MS (Ljubisavljevic et al., 2016) and/or larger sample sizes (Gresle et al., 2014)), we are limited in comparing our findings against findings of previous studies. We however did a comparative analysis with other neurofilament measures in both CSF and serum in this cohort. We made seemingly contrasting observations, with serum-NfH being associated with disease duration, T2 lesion volume and black hole count (trend), and CSF-NfH being associated with EDSS, T2 lesion count and normalized brain volume. Also, we only observed a moderate correlation between serum-NfH and CSF-NfH levels (rho = 0.27). Our results for the relation of CSF-NfH with clinical parameters are in line with several previous studies (Trentini et al., 2014; Kuhle et al., 2011; Khalil et al., 2013; Fialova et al., 2013). Relations between CSF-NfH and MRI parameters have been less studied. The one study that explored such relationships investigated clinically isolated syndrome (CIS) patients who have lower amounts of axonal loss compared to clinically definite MS, and in contrast to our study they did not observe any relationships between CSF-NfH and T2 lesions or normalized brain volume (Khalil et al., 2013). Not all patients with CIS will develop definite MS and according to two previous studies, CSF-NfH levels were not elevated yet in CIS patients that eventually convert to MS (Khalil et al., 2013; Arrambide et al., 2016), which could potentially explain our discrepancy in findings.

Based on previous literature on NfL, we expected that we would observe relationships between increased NfL and worse MS disease severity scores (Barro et al., 2018; Dalla Costa et al., 2019; Disanto et al., 2017; Kuhle et al., 2013; Sellebjerg et al., 2018) or MRI outcomes (Disanto et al., 2017; Barro et al., 2018; Damasceno et al., 2019; Kuhle et al., 2019; Dalla Costa et al., 2019; Varhaug et al., 2018). However, we only observed a trend for a relationship of serum-NfL and CSF-NfL with T2 lesion count, while for serum-NfH and CSF-NfH various relationships were shown. Taken together, these data suggest that NfH measurement in MS, in addition to NfL, could be of added clinical value.

The strength of our study lies in the selection of several high sensitive serum-NfH assays that were compared head-to-head in a relevant cohort of MS patients with paired serum and CSF samples available. With the emergence of more treatment possibilities for MS with potential beneficial effect on disability progression, the need for easy-to-use dynamic tools to monitor focal inflammatory activity and neurodegeneration becomes stronger, making the current study timely. Among the limitations is that we conducted the assay comparison only for the serum-NfH measurement. As a consequence, different assays and platforms were used for the CSF and the serum measurements, which might have influenced our comparisons of the observed relationships of the various neurofilament measures with the clinical and MRI measures. Although we could detect serum-NfH in all samples with the commercial Simoa assay, still some samples were measured below LLOW or with a duplicate %CV higher than 20%. Since the selected cohort was not very large, statistical analyses for clinical validity were regarded as exploratory and therefore not corrected for multiple testing. Furthermore, we did not include healthy controls, since it was our aim to investigate associations with MS disease burden and not investigate diagnostic utility for differentiating MS patients and controls. It is recommended to repeat the comparative serum (and CSF) NfH and NfL analysis in independent cohorts, preferably with data on relapses and medication as well as longitudinal serum samples and longitudinal clinical data. Longitidudinal analysis is recommended, because likely, neurofilament biomarkers NfH and NfL are most useful in therapeutic effectiveness monitoring (i.e. having increased or decreased levelslevels compared to previous visits).

To conclude, serum-NfH can be assessed with a high-sensitivity assay and showed some potential as biomarker for neuro-axonal disease burden in MS.

5. Author's contributions

IV conceptualized the study, curated and formally analysed the data and wrote the original draft. CT and CB conceptualized the study, and reviewed and edited the text. MK, HT, HV, CK and JK curated the data and reviewed and edited the text. All authors read and approved the final manuscript.

Declaration of Competing Interest

IV, MK, HT, CK, CB have nothing to disclose. HV has received research grants from Pfizer, MerckSerono, Novartis and Teva, speaker honoraria from Novartis, and consulting fees from MerckSerono; all funds were paid directly to his institution. JK has accepted speaker and consulting fees from Merck, Biogen, TEVA, Sanofi, Genzyme, Roche and Novartis. CT has a collaboration contract with ADx Neurosciences, performed contract research or received grants from Probiodrug, AC Immune, Biogen-Esai, CogRx, Toyama, Janssen prevention center, Boehringer, AxonNeurosciences, Fujirebio, EIP farma, PeopleBio and Roche.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2021.102840.

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Discussion and Outlook

Biomarkers of progression are critically needed to assess novel therapeutic molecules targeting this process. NfL is to date the body fluid biomarker with highest promise for this endeavor. NfL is increased in the CSF of patients with many neurological conditions, as shown in manuscript 2. It is thus not a specific biomarker for MS, but rather a biomarker of neuroaxonal damage, regardless of its cause. While evidence supporting the utility of NfL as a biomarker of acute focal inflammatory disease activity in MS is unequivocal, data regarding its potential to predict or monitor progression independent of clinical and/or radiological acute focal inflammatory disease activity is inconsistent. Recent technical developments have enabled robust measurement of serum NfL, which correlates highly with CSF levels, allowing repetitive assessments of this biomarker in blood. In manuscript 3, we find that serum NfL is not well suited to monitor progression in people with RRMS treated with natalizumab, as the longitudinal changes in serum NfL do not differ between progressors and non progressors. Similar findings were recently reported in a large clinical trial of patients with secondary progressive MS treated with natalizumab.[122] Both our study and this study investigated a population of PwMS in which acute focal inflammatory disease activity was virtually silenced by natalizumab, allowing to uncouple progression and acute focal inflammatory disease activity. In contrast, a large study reporting prognostic potential for future disability accumulation investigated PwMS treated with sipominod, which is less efficient in reducing focal inflammatory disease activity than natalizumab. Accordingly, 43% of the siponimod-treated patients of the study had new or enlarging T2 lesions during the 24 months follow-up, and the prognostic value of sNfL may thus be attributable to its ability to capture residual inflammatory disease activity.[123]

The mechanisms driving neurodegeneration and progression are probably manifold, and it is unlikely a single biomarker will be able to capture all these processes. Additional biomarkers are thus needed, underscoring the importance of continued efforts towards the discovery of novel candidate biomarkers. In manuscript 1, we describe an unsuccessful effort using an unbiased proteomic approach applied on CSF. I will now discuss 2 projects, both aiming at identifying novel body fluid biomarkers of progression. The first project is supported by the *Fondation privée des Hôpitaux Universitaires de Genève* and *the Fondation Schmidheiny,* and performed in collaboration with the laboratory of Professor Jean-Charles Sanchez. In contrast to the project presented in manuscript 1, this project uses both unbiased proteomic and transcriptomic tools, in order to enhance the probability of identifying robust candidates. Moreover, it uses extracellular vesicles enriched from the CSF rather than whole CSF as substrate, in an attempt to focus on molecules deriving from cells of the CNS rather than cell-free molecules of uncertain origin. The second project is hypothesis-driven, and proposes to use a targeted approaches to measure synaptic proteins in the CSF to evaluate their potential as markers of neuronal dysfunction and progression.

Extracellular vesicles as a source of biomarkers of progression in multiple sclerosis

Extracellular vesicles (EVs) are lipid bilayer membrane-delimited vesicles containing proteins, lipids, and nucleic acid cargoes. They are naturally released into the extracellular milieu by virtually all eukaryotic cells, through exocytosis or membrane budding.[124,125] EVs are thought to be important mediators of intercellular communication, although the mechanisms by which signaling occurs are still under investigation.[126] EVs are present in many body fluids, including blood and CSF, and expose cell-specific protein markers on their surface which allow to identify their cellular origin. CSF-derived-EVs have been reported to contain a high proportion of neuron-derived proteins, but also microglia and oligodendrocyte-specific proteins.[127,128] In MS, a few recent studies have provided initial proof of concept that the protein cargo of CSF-derived EVs is modified in MS. Lee et al. identified potential EV protein biomarkers assisting the differential diagnosis of MS and neuromyelitis optica,[129] Welton et al. found significant differences in the proteome of CSF-derived EVs of MS and controls,[130] and Geraci et al. found an increased number of CSF-derived EVs in MS patients compared to controls.[131] In collaboration with the laboratory of Professor Jean-Charles Sanchez, Geneva Medical Faculty, we are currently testing the hypothesis that the molecular cargo of CSF-derived EVs differs between people with RRMS and SPMS, reflecting the pathological mechanisms that predominate at the two extremes of the disease evolution spectrum. Specifically, we hypothesize that neuronal dysfunction and death, which is most conspicuous in SPMS, will lead to modifications in the molecular cargo of neuron-derived EVs in SPMS, and may be a source of molecular markers of disease progression. In order to limit the influence of EVs derived from CSF lymphocytes on our results and focus on neuron-derived EVs, we compare the molecular cargo of CSF-derived EVs of recently diagnosed RRMS individuals treated with highly active DMTs with that of SPMS individuals. Highly active DMTs allow to silence acute inflammatory disease activity in the CNS and CSF of RRMS patients.[132] Studying this population will allow us to investigate disease mechanisms occurring independently of acute inflammatory disease activity, and their impact on the transcriptome and proteome of CSF-derived EVs. RRMS and SPMS are distinct not only in terms of disease stage and progression rate, but also in terms of age. The composition of the CSF changes with age,[133] and in order to capture only those changes associated with the disease stage and rate of disease progression, two groups of age-matched HC are analyzed in parallel to the MS groups. While most studies so far investigated either the transcriptome or the proteome of EVs, we will combine both approaches in order to maximize the identification of candidate biomarkers. We plan to compare the proteome and transcriptome (messenger RNAs and/or small non coding RNAs) of EVs enriched from CSF of RRMS and SPMS patients, aiming to identify modifications in sets of proteins and RNA molecules which reflect disease progression, which occurs most conspicuously during the later stages of the disease. This objective will be reached through 4 steps. Step 1 consists of enriching, quantifying, and characterizing CSF-enriched EVs from a discovery cohort of 40

participants. The discovery cohort includes MS patients (RRMS, n=10, SPMS, n=10) and HC (n=20, age-matched to MS patients). Step 2 and 3 explore the EV proteome and transcriptome respectively, to identify candidate biomarkers of disease progression. This will be done by analyzing the proteome and transcriptome and selecting proteins or RNA molecules that are differentially regulated in EVs of RRMS and SPMS patients. Step 4 consists of validating our findings in CSF of an independent validation cohort (n=150 MS patients), including RRMS and SPMS patients, selected from a well characterized large cohort of PwMS with biobanked CSF (Swiss MS cohort, https://dkf.unibas.ch/en/competencies/registries-cohorts/swiss-ms-cohort/). The validation of molecular candidate biomarkers will be performed using targeted immunoassays or RT-PCRs. Association of candidate molecular biomarkers with clinical characteristics, MRI parameters and NfL levels, will then be evaluated, to assess their clinical pertinence.

Synaptic proteins as promising biomarkers of progression

Synaptic dysfunction and loss are pathological features of many neurological and psychiatric diseases.[134] Synaptic proteins that are detectable in the CSF are thus actively investigated as candidate biomarkers of these processes, in particular in Alzheimer's disease where synaptic loss correlates strongly with cognitive decline.[135,136] Comparatively, much less is known about synaptic loss in MS, but recent post mortem studies reported widespread reduced synaptic density in the cortex of PwMS, both within demyelinating lesions and in the surrounding NAGM.[137–139] Synaptic loss was also identified in the hippocampus, thalamus, and most recently in the spinal cord of PwMS compared to controls.[140–144] . A study which reconstructed single cortical projection neurons showed that synaptic density was reduced in NAGM where axonal density was preserved, indicating synaptic loss is not secondary to neurodegeneration but rather precedes it.[138] The mechanisms leading to synaptic loss remain to be elucidated, but complement activation and deposition is observed in MS cortical and hippocampal grey matter in relation to synaptic loss and is thus thought to be a critical mediator of this process.[142,145,146] Biomarkers of synaptic loss would allow to identify neuronal dysfunction at an early stage, possibly before irreversible damage, and to monitor the effect of candidate drugs targeting neuronal dysfunction and degeneration. An exhaustive literature search resulted in the identification of 42 individual pre- and post-synaptic proteins (or peptides thereof) that can be quantitatively assessed in the CSF of patients with a variety of neurological diseases and healthy controls (Table). Synaptic proteins were assessed either by ELISA, solid-phase extraction monitoring (SRM) or parallel reaction monitoring mass spectrometry (PRM-MS).[147–149] Few studies have evaluated the potential of synaptic proteins as biomarkers in MS. Growth Associated Protein 43 (GAP43), highly expressed in neuronal growth cones during development and axonal regeneration, was not significantly different between recently diagnosed PwMS and healthy controls.[150] In contrast, GAP-43 was lower in people with progressive MS than in healthy controls, suggesting potential as a marker of synaptogenesis which is preserved in the earliest stages of MS while impaired in later stages.[151]

Neurogranin is a small protein expressed in pyramidal cells of the hippocampus and cortex involved in synaptic plasticity, regeneration, and long-term potentiation mediated by the calcium- and calmodulin-signaling pathways. In one study, CSF concentration of neurogranin was not different between PwMS and healthy controls, while in a second it was lower in PwMS compared to controls.[152,153] Additional data of larger cohorts are thus needed to clarify the potential of neurogranin as a biomarker of synaptic loss and neurodegeneration in MS. Measure of alpha synuclein (α-syn), which regulates synaptic vesicle trafficking and subsequent neurotransmitter release, yielded conflicting results in PwMS. Indeed, both increased and decreased concentrations were reported in MS compared to controls.[154,155]

In this study, we plan to measure synaptic proteins in CSF of people with relapsing and progressive MS, and assess associations with disability scores and MRI measures of atrophy. The subjects will be selected from a well characterized large cohort of PwMS with biobanked CSF (Swiss MS cohort, https://dkf.unibas.ch/en/competencies/registries-cohorts/swiss-ms-cohort/). We will measure 2 panels of synaptic proteins. A first panel of proteins will be quantified by ELISAs after extensive in house validation of the commercially available immunoassays (orange shade, Table). A second panel of proteins which are not measurable in CSF by ELISA will be assessed through collaborations with groups who developed specific SRM and PRM-MS protocols (blue shade, Table). The peptides with most potential as biomarkers of progression in MS will be selected and specific immunoassays for these peptides will be developed, as SRM and PRM-MS are not techniques used in routine clinical practice.

Table Synaptic proteins or peptides measurable in cerebrospinal fluid

Protein (or peptide)	Type of assay with reference publication
alpha-synuclein	ELISA[156]
beta-synuclein	ELISA 33380492
chromogranin A	ELISA[157]
growth associated protein 43	ELISA[158,159]
neurogranin	ELISA[159]
synaptosomal-associated protein 25	ELISA[160]; immunoassay singulex erenna[161,162]
synapsin I	ELISA[163]
synaptophysin	ELISA[163]
visinin-like protein 1	immunoassay singulex [164]
alpha-synuclein peptide	MRM[165]
AP-2 complex subunit beta peptide	SRM[149]
calsyntenin-1 peptide	SRM[148]
complexin-2 peptide	SRM[149]
14-33 epsilon	SRM[149]
14-33 eta	SRM[149]
14-33 theta	SRM[149]
14-33 zeta/delta	SRM[149]
gamma-synuclein	MRM,[165] SRM[149]
gluR2 subunit peptide	SRM[149]
gluR4 subunit peptide	SRM[149]
neurexin 1	PRM[147,166]
neurexin 1a	PRM[167]
neurexin 1b	PRM[147,167]
neurexin 2	PRM,[147] SRM[149]
neurexin 2a	PRM,[167] SRM[148,149]
neurexin 3	MRM,[168] PRM [147]
neurexin 3a	PRM,[167] SRM[148]
Neurocan core protein precursor	PRM[147]
neurofacin	PRM[147 166]
neuroligin-1	PRM[167]
neuroligin-2	PRM,[167] SRM[148]
neuroligin-3	PRM[167]
neuroligin-4	PRM[167]
neuronal pentraxin 1	PRM,[147,166] SRM[149]
neuronal pentraxin 2	SRM[149]
neuronal pentraxin receptor	SRM[149]
Rab GDI alpha	SRM[149]
secretogranin I	MRM[169]
secretogranin II	PRM[147,166]
syntaxin-1B	SRM [148]
syntaxin-7	SRM[149,170]
vesicle-associated membrane protein2	SRM[148]

Conclusion

The advent of highly efficient drugs to prevent relapses and acute focal inflammatory disease activity now means that a majority of PwMS can live without relapses, and the mid-term neurological prognosis of PwMS has greatly improved. These recent therapeutic developments are also a powerful drive for research because they highlight, in contrast, aspects of MS care which lag. One major unmet clinical need is the treatment of progression. In contrast to relapses and acute focal inflammatory disease activity whose immunopathological features are well characterized, the mechanisms underlying progression remain elusive. Treatments specifically targeting this characteristic feature of MS are not available, yet progression contributes overwhelmingly to long-term disability. Evaluating new drugs to reduce progression rate requires robust tools to quantify it over the relatively short time frame of clinical trials. Whether NfL is a good candidate for this endeavor is still matter of debate, but given the complexity of the disease, no single biomarker will likely suffice to reflect the different mechanisms underlying progression, and additional research is needed to identify novel candidates.

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