Investigations into the role of Epstein-Barr virus in the pathogenesis of multiple sclerosis

Daniel Jons

Department of Clinical Neuroscience

Institute of Neuroscience and Physiology

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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daniel.jons@vgregion.se

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“I have approximate answers and possible beliefs and different degrees of uncertainty about different things, but I am not absolutely sure of anything and there are many things I don't know anything about…”

— Richard P. Feynman

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Department of Clinical Neuroscience,
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Sahlgrenska Academy, University of Gothenburg
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ABSTRACT

Epstein-Barr virus (EBV) infection may be a prerequisite for the development of MS. Virtually all MS patients have antibodies to Epstein Barr virus nuclear antigen 1 (EBNA1), compared to 90-95% of healthy individuals. This antibody response is increased both in pre-symptomatic and manifest MS. Infectious mononucleosis (IM), the symptomatic variant of primary EBV infection of adolescence, doubles the risk of future MS. This thesis investigates the EBV-MS connection both by studying individuals with the risk factor for MS of previous IM, and by investigating samples acquired prior to MS onset. It aims at determining the temporal relationship between neuroaxonal damage and EBV antibody response before MS onset and searches for an immunological residual state after IM.

The first study examined B cell populations in bone marrow from MS patients. The following two studies were follow-up studies that investigated individuals for persistent immunological activity a decade after IM. We here assayed seven selected cytokines and chemokines in the CSF (Study II), and antibody reactivity to EBV, Measles and Varicella Zoster in sera and CSF (study III). The last two nested case–control studies (IV and V), of 669 pre-symptomatically acquired blood samples from individuals who later developed MS, investigated the marker of neuroaxonal damage, serum neurofilament light (sNfL), and several anti-EBV antibodies.

No deviations in early B cell lineages were found in MS bone marrow. SNfL concentrations were increased in pre-MS compared to matched controls (p <

0.0001). The increase started approximately 10 years before MS onset and was significant from 5-10 years before onset ($p = 0.02$), with increasing difference over time. Anti-EBNA1 reactivity showed an increase in pre-MS compared to controls from 10–15 years before onset ($p = 0.001$) and did not increase over time. In the pre-MS group, the percentage of samples with an increased sNfL were concentrated to the EBV positive group compared to the EBV negative group ($p = 0.038$). Anti-gp350 was elevated 10 years after IM ($p = 0.007$), while no significant increase in CSF cytokines was detectable, with low power, after IM.

In conclusion, neuroaxonal damage was detectable 10 years before MS onset but still preceded by an EBV serological response. We observed less neuroaxonal damage in the small group of EBV negative samples acquired before MS. This strengthens the connection between a previous EBV infection and the start of neuroaxonal damage in pre-symptomatic MS.

Keywords: Multiple sclerosis, Epstein-Barr virus, Serology, Neurofilament light,

Sammanfattning på svenska

Orsaken till multipel skleros (MS) är okänd. Flertalet studier visar att det finns ett starkt samband mellan MS och Epstein-Barr virus (EBV), ett virus om infekterar merparten av mänskligheten. I princip alla MS patienter har antikroppar mot EBV, jämfört med 90-95% av friska, dessutom i högre nivåer. Körtelfeber, den symptomgivande variant av EBV infektion som man ofta insjuknar i om man smittas i tonåren, dubblar risken för framtida MS. Hur sambandet mellan EBV och MS ser ut, och om det är kausalt eller ej är okänt.

Den här avhandlingen undersöker sambandet mellan EBV och MS på två sätt. Dels genom undersökningar av individer som insjuknade i körtelfeber, som är en riskfaktor för MS, för tio år sedan, dels genom undersökningar av blodprover tagna flera år innan de första symptomen på MS. Målsättningen är att undersöka hur långt innan MS debut som stegring i antikroppar mot EBV inträffar och hur långt innan MS debut som nervskada börjar. Sedan kan vi relatera dessa två tidsperioder och undersöka vilken förändring som inträffar tidigast. Vi letar också efter ett immunologiskt resttillstånd efter körtelfeber.

Den första studien undersökte fördelningen av cellpopulationer i benmärgsprov från MS patienter. De två följande studierna (**II** och **III**) var uppföljningsstudier av individer som hade körtelfeber för ca 10 år sedan, för att se om en del, trots att de är friska, hade utvecklat inflammatoriska förändringar i ryggmärgsvätskan eller ett högre antikroppssvar mot EBV och andra virus, jämfört med individer som inte haft körtelfeber. Det här är förändringar som ses hos flertalet MS patienter. Studie **IV** och **V** var case-control studier av sparade blodprov som individer som senare har insjuknat i MS har lämnat i samband med tidigare sjukvårdskontakter. Vilka jämförs med matchade kontroller som inte insjuknat i MS. De här sparade proven ger alltså en möjlighet att undersöka blodprov från individer innan de insjuknar i MS. Här undersöks hur långt innan de första symptomen på MS som dessa individer utvecklar tecken på nervskada (mätt med neurofilament). Samt hur lång tid innan MS debut som antikroppar mot EBV stiger.

Resultaten visar att, på gruppnivå startar nervskadan i centrala nervsystemet ca 10 år innan MS debut och ökar fram till det kliniska insjuknandet. En ökning i EBV antikroppar sker från ca 15 år innan MS debut, dvs före den subtila nervskadan börjar. Om man jämför EBV positiva med EBV negativa individer i den gruppen som kommer att insjukna i MS, så förefaller de som har börjat få en nervskada vara koncentrerade till den EBV positiva gruppen. Dock är den EBV negativa gruppen liten och jämförelsen därför svår. Vi såg

ingen säkerhetsställd påverkan på inflammatoriska markörer i ryggmärgsvätskan efter körtelfeber. Antikroppar mot ett yt-antigen (gp350) på EBV var ökade 10 år efter körtelfeber jämfört med efter en mildare EBV infektion. Antikroppar mot mässling och varicella-zoster (vattkoppor) var ökade i samband med körtelfeber.

Den ökade reaktiviteten hos MS patienter mot EBV börjar innan den subtila nervskada som är starten på MS patologin, börjar. Hos de individer som kommer att få MS men ännu inte fått en EBV infektion finns det indikationer på att nervskada inte har startat. Det här stärker argumenten för, men kan inte bevisa, att en del av MS kan orsakas av EBV infektion och att en vaccination mot EBV eventuellt skulle minska MS-frekvensen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Jons D**, Kneider M, Fogelstrand L, Jeppsson A, Jacobsson S, Andersen O. *Early hematopoiesis in multiple sclerosis patients.* Journal of Neuroimmunology. 2016;299:158-63.

II. **Jons D**, Zetterberg H, Malmeström C, Bergström T, Axelsson M, Blennow K, Thulin M, Sundström P, Andersen O. *Intrathecal immunoreactivity in people with or without previous infectious mononucleosis.* Acta Neurologica Scandinavica. 2020;142(2):161-8.

III. **Jons D**, Persson Berg L, Sundström P, Haghighi S, Axelsson M, Thulin M, Bergström T, Andersen O. *Follow-up after infectious mononucleosis in search of serological similarities with presymptomatic multiple sclerosis.* Multipel Sclerosis and Related Disorders. 2021;56:103288.

IV. **Jons D**, Zetterberg H, Biström M, Alonso-Magdalena L, Gunnarsson M, Vrethem M, Blennow K, Nilsson S, Sundström P, Andersen O. *Axonal injury in asymptomatic individuals preceding onset of multiple sclerosis.* Annals of Clinical and Translational Neurology. 2022;9(6):882-7.

V. **Jons D**, Bergström T, Zetterberg H, Biström M, Alonso-Magdalena L, Gunnarsson M, Vrethem M, Brenner N, Butt J, Blennow K, Nilsson S, Huang J, Kockum I, Olsson T, Waterboer T, Sundström P, Andersen O. *Increase in Epstein-Barr virus sero-reactivity precedes neuroaxonal damage in pre-symptomatic multiple sclerosis*. Manuscript.

Table of Contents

LIST OF PAPERS I

ABBREVIATIONS VI

1 INTRODUCTION 1

2 MULTIPLE SCLEROSIS 2

2.1 Clinical course of multiple sclerosis 2

2.2 Diagnostic criteria 3

2.3 Pathology and immunopathogenesis 3

2.4 Epidemiology 4

2.5 Hereditary risk 5

2.6 Combination of genes and environmental factors 6

2.7 Hypothesis regarding the role of EBV in MS pathogenesis 7

2.8 Hematogenesis and MS – examinations of the bone marrow 8

2.9 MS prodrome 9

2.10 Neurofilament Light 11

3 EPSTEIN BARR VIRUS 12

3.1 General 12

3.2 Life cycle 13

3.3 Epidemiology 14

3.4 Infectious mononucleosis 14

3.5 Serology 15

4 AIMS 16

5 SUBJECTS, STUDY DESIGN AND METHODS 17

5.1 Study design 17

5.2 Study **I** Early haematopoiesis in multiple sclerosis patients 17

5.2.1 Analyses 18

5.3 Studies **II** and **III** Follow-up after infectious mononucleosis in search of similarities with pre-symptomatic multiple sclerosis 19

5.3.1 Analyses 21

5.4 Studies **IV** and **V** 22

5.4.1 Analyses 23

5.5 Analytical Methods 25

5.5.1 Fluorescence-activated single cell sorting 25

5.5.2 Serology – viral antibody quantification 26

5.5.3 ELISA 27

5.5.4 Luminex bead based immunoassay 28

5.5.5 Cytokine and chemokine detection 29

5.5.6 Neurofilament Light 32

5.5.7 SIMOA 32

5.6 Lumbar puncture 32

5.7 Statistical methods 32

5.8 Ethics 34

5.8.1 Ethical approvals: 34

6 RESULTS 35

6.1 STUDY **I** haematopoiesis in multiple sclerosis patients.................... 35

6.1.1 The proportion of major leukocyte subsets did not differ between patients and controls 35

6.1.2 The proportion of B cells precursors did not differ between patients and controls 35

6.1.3 The proportion of NKT cells was lower in bone marrow of MS patients.. 35

6.2 STUDY **II** Intrathecal immunoreactivity in people with or without previous infectious mononucleosis.. 37

6.2.1 Neurofilament was not increased in the post-IM group 37

6.2.2 CSF cytokine levels showed a possible trend towards increase in the post-IM group 37

6.2.3 Power calculation 37

6.3 STUDY **III** Follow-up after infectious mononucleosis in search of serological similarities with pre-symptomatic multiple sclerosis.............. 39

6.3.1 Antibody reactivity at Infectious mononucleosis and at follow-up 39

6.3.2 CSF analysis of post-IM group 40

6.3.3 Antibody reactivity in MS group.. 40

6.4 STUDY **IV** Axonal injury in asymptomatic individuals preceding onset of multiple sclerosis 42

6.5 STUDY **V** Increase in Epstein-Barr virus sero-reactivity precedes neuroaxonal damage in pre-symptomatic multiple sclerosis 44

6.5.1 EBV sero-reactivity in pre-symptomatic samples. 44

6.5.2 EBV negative samples – Neurofilament Light........................... 46

7 DISCUSSION 47

7.1 A possible pathway from infectious mononucleosis to MS or an MS endophenotype 47

7.2 No deviation in early B-cell lineages from MS patients 49

7.3 EBV infection precedes the first recognizable signs of MS 49

7.3.1 Neuroaxonal damage starts approximately 10 years before clinical onset 49

7.3.2 EBV sero-reactivity in pre-symptomatic MS 51

7.3.3 Increase in EBV sero-reactivity occurs before neurofilament increase in pre-symptomatic MS 51

7.3.4 Individuals with high neurofilament accumulate in the EBV positive pre-symptomatic MS group 52

7.3.5 No correlation between sNfL and EBV serologies in EBV positive samples 52

8 CONCLUSION 54

9 FUTURE PERSPECTIVES 55

10 ACKNOWLEDGEMENT 57

11 REFERENCES 59

12 APPENDIX 74

ABBREVIATIONS

a.a.	Amino acid
ANOVA	Analysis of Variance
BBB	Blood brain barrier
CCL	Chemokine (C-C motif) ligand
CD	Cluster of Differentiation
CHO	Chinese hamster ovary
CI	Confidence interval
CIS	Clinically isolated syndrome
CNS	Central nerve system
CO	Cut-off
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
EBNA1	Epstein-Barr virus antigen 1
EBNA 2	Epstein-Barr virus antigen 2

EBV	Epstein–Barr virus
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FSC	Forward scatter
FU	Follow-up
GC	Germinal center
GP	Glycoprotein
GP350	Glycoprotein 350
HLA	Human leukocyte antigen
IFNB	Inteferon B
IM	Infectious mononucleosis
IQR	Inter quartile range
LMP1	(EBV) latent membrane protein 1
LMP2	(EBV) latent membrane protein 2

LOESS	Locally estimated scatterplot smoothing
MANOVA	Multivariate Analysis of Variance
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MRZ	Measles, rubella and varicella zoster virus
MS	Multiple sclerosis
NCORE	N-terminal portion of the measles nucleocapsid protein
sNFL	Serum Neurofilament
NK	Natural killer (cell)
NKT	Natural killer T (cell)
OR	Odds Ratio
PHAS	Public Health Agency of Sweden
PIRA	Progression independent of relapse activity

PPMS	Primary progressive multiple sclerosis
RIS	Radiologically isolated syndrome
RNA	Ribonucleic acid
RRMS	Relapsing remitting multiple sclerosis
SIMOA	Single-molecule enzyme-linked immunosorbent assay
SPMS	Secondary progressive multiple sclerosis
SSC	Side scatter.
VCA	(EBV) viral capsid antigen
VZV	Varicella Zoster virus
VZVgE	Varicella glycoprotein E

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic disease which, according to classical description, damages the white matter of the central nervous system (CNS). It affects approximately 2.8 million individuals globally (1). Although the cause of MS is unknown, the prevailing view is that it is an autoimmune disease.

Since Charcot's original clinical and neuropathological description of MS, or *sclerose en plaque disséminées*, as a unified disease in 1868 (2), numerous theories regarding the aetiology of MS have been proposed. For example, as early as 1894, Charcot's student Pierre Marie had argued for an infectious cause (3); this received increased attention after a 1950 report (4) that the cerebrospinal fluid (CSF) levels of gamma globulin were increased in MS, as well as in a chronic CNS infection, neurosyphilis. Intense research over more than a century has culminated in the modern era of effective MS treatment, with the first drug, interferon beta-1b, showing positive effects in a randomised trial in 1993 (5). This was soon followed by more effective treatments, all of which target the immune system. The risk of developing MS is influenced both by genetics and environmental risk factors and by a combination thereof. Epstein-Barr virus (EBV), smoking and vitamin D deficiency all play important roles as environmental risk factors (6). A model of disease development, consisting of a stepwise progression that includes genetics and several potential risk factors has been proposed (6).

A substantial amount of evidence for the involvement of EBV, primarily as a trigger for MS, has been reported over the last 20 years (7), but the question of possible causality has been difficult to determine. In other words, it is hard to determine whether the increased immunoreactivity to EBV in MS patients is a cause or an effect of the disease or a coinciding factor.

The studies in this thesis primarily concern EBV as a risk factor and a potentially obligatory pre-condition for MS development. Herein we take two approaches: the first is to study the effects of infectious mononucleosis (IM), the clinical acute EBV infection of adolescence, in healthy individuals, with the hope of finding long-term effects on the immune system or serological responses (i.e., that IM in some way leads to an alteration in individual immune response; Studies **II** and **III**). The other approach is to study pre-symptomatic MS patients, examining the appearance, temporal order, and amplitude of EBV serological response and serum neurofilament light (sNfL) levels, a biomarker of axonal damage, before or in the very first stages of disease development (Studies **IV** and **V**).

2 MULTIPLE SCLEROSIS

2.1 CLINICAL COURSE OF MULTIPLE SCLEROSIS

A diagnosis of multiple sclerosis is usually suspected when a person experiences a first mono- or polysymptomatic typical neurological symptom from the central nervous system (CNS). Although symptoms vary widely, optical neuritis, brainstem and spinal cord-focal symptoms are common in relapses, which usually develop over hours to days before reaching a plateau and then gradually subsiding. A first clinical episode is known as clinically isolated syndrome (CIS). New relapses occur with varying frequency, and over time, the recovery from these relapses is less complete, giving rise to persistent disability. This phenotype of MS, known as relapsing remitting multiple sclerosis (RRMS), is experienced by 80–90% of patients (8, 9). For every clinical relapse, studies with magnetic resonance imaging (MRI) show 5–10 new asymptomatic white matter lesions (9), and many patients already have several visible MRI lesions at the time of first symptoms.

Gradually over time, typically 10–20 years from MS onset, untreated individuals develop secondary progressive MS (SPMS) with a slowly progressive disease and worsening disability (8). No strict distinction is made, so relapses may occur on top of this secondary progressive state. Recently, the concept of progression independent of relapse activity (PIRA) has been introduced, and studies have shown that a large part of disability accumulation in RRMS appears independently of relapse activity (10).

Ten to fifteen percent of patients experience a different phenotype, where the disease is progressive from onset, known as primary progressive MS (PPMS). The most common presentation of PPMS is progressive spastic paraparesis, but other variants are also frequent, such as cerebellar or sensory ataxia (9).

Several definitions have been used for these three clinical phenotypes of MS. In 1996 the U.S. National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis presented consensus criteria to aid in clinical studies (11). Criteria have later been refined to include MRI findings, and the concepts of CIS and radiologically isolated syndrome (RIS) have been introduced (12). RIS identifies individuals with incidentally found MRI abnormalities suggestive of demyelination but without neurological symptoms.

2.2 DIAGNOSTIC CRITERIA

The basic clinical criterion for MS as defined by Poser (13) is a dissemination in time and space, meaning that to be defined as clinically definite MS the disease must affect multiple sites in the CNS at several times (at least 30 days apart) and have no other better explanation. The most recent McDonald criteria for MS diagnosis from 2017 (14) have evolved over several revisions, from purely clinical to allowing the possibility of including MRI findings and oligoclonal bands in the CSF as possible proof of dissemination in time and space.

2.3 PATHOLOGY AND IMMUNOPATHOGENESIS

MS is an immune-related disease and is considered by most researchers an autoimmune disease. However, this is still debated, since MS does not fulfil the criteria for a classic autoimmune disease, a primary reason being that there still is no known specific autoantigen (15). However, some studies indicate cross-reactivity and molecule mimicry (discussed below) (16). The hallmark of MS is inflammatory lesions leading to demyelinating plaques in the CNS. Macroscopically, these lesions are more obvious in the white matter. The dominating cell types in these lesions are MHC class I restricted CD8+ T cells and macrophages, but B cells and plasma cells are also present in lower numbers (9).

The lesions are typically located around small venules and are characterized by breakdown of the blood-brain-barrier (BBB)(17, 18). Active lesions are thus visible on MRI by gadolinium contrast enhancement. Demyelination occurs as an effect of inflammation and axons can be relatively spared initially but as the disease progresses irreversible axonal damage occurs (9). The classic inflammatory lesion is prevalent in RRMS and also seen in progressive disease, but less commonly, here chronic active plaques with macrophages in the periphery are more prominent (9, 19).

Evidence also exists of extensive grey matter (GM) pathology in MS that is present from the earliest stages of the disease (20). Less is known about the nature of GM pathology or atrophy, but inflammation seems to be less prominent than in white matter lesions and often occurs in the vicinity of germinal centre-like lesions in the meninges (21). In older patients at late stages of the disease, inflammation declines (22).

Another distinct feature of MS is the presence of oligoclonal bands in the CSF. These bands represent the synthesis of immunoglobulins by the clonal expansion of plasma cells in the CNS. When CSF from MS patients is applied to an electrophoretic gel, the antibodies form bands related to different mobilities in the gel. The specificities of the oligoclonal IgG have been extensively studied but remain largely unknown. About 2% of the intrathecally synthesized IgG consist of antibodies to measles, rubella and varicella-zoster (MRZ) (23). Intrathecal response against the MRZ index can be used to support an MS diagnosis (24). The MRZ reaction is positive in the majority of MS patients and absent in other inflammatory neurological diseases (25). Paradoxically, the intrathecal anti-EBV response is relatively low compared to the MRZ viruses (26). Some of the B cells producing antibodies have recently been shown to target myelin producing glial cells (16).

From being seen as primary a T cell mediated disease, the prevailing view of MS immune pathogenesis has expanded to include interactions, where immune cells in the periphery, including T, B and myeloid cells, can enter the CNS, where they interact with resident cells such as microglia and astrocytes (27).

The presence of oligoclonal bands is one argument for B cell involvement in MS pathogeneses; a second is the effects of anti-CD20 treatment (for example by Rituximab), which depletes several B cell subsets including memory B cells. The third argument is the involvement of these B cells in EBV infection (28).

2.4 EPIDEMIOLOGY

The prevalence of MS varies between countries, from 2 per 100,000 in some Asian countries to 100 per 100,000 in northern Western countries (17). Sweden has an MS prevalence of approximately 200 per 100,000 (29), amongst the highest in the world. The prevalence of MS has increased over recent decades, especially in women, which could represent either a true increase or better diagnostic methods (30). The female to male ratio is approximately 3:1 (17).

The incidence of MS can generally be said to increase with latitude (31), in other words, the prevalence increases with distance north or south of the equator. This latitude effect was proposed to be related to sunlight and associated vitamin D status (32), but the timing of infections have also been

suggested. The latitude effect, which persists after adjusting for HLA-DRB1 allele frequencies, strongly supports an environmental factor (32)

Classic migration studies show that individuals who migrate from a high-risk region to one with low risk (e.g. from Great Britain to South Africa) retain the risk of their original country but that their children have the risk of the new region (33). Individuals who migrate from a low to a high endemic region are less well studied but still demonstrate the same pattern. This strongly supports the importance of environmental factors. Further studies have also proposed a susceptible age when individuals who migrate before adolescence acquire the risk of the new region, while those who migrate after adolescence retain the original risk of the region where they grew up (6). This epidemiological pattern could be consistent with early sunlight exposure but was also proposed early on to be consistent with delayed early infection in countries with higher hygienic standards (hygiene hypothesis) (34). However, a systematic study of late infection with common childhood infections (measles, rubella, mumps, varicella, pertussis and scarlet fever) showed no increased risk for MS (35).

2.5 HEREDITARY RISK

The risk of MS in white northern Europeans is approximately 0.3% (8). This is increased to approximately 3% in first-degree relatives, with siblings having a 5% risk and children of one affected parent a risk of 2% (36). MS risk is higher in monozygotic (25%) than in dizygotic twins (5%) in the UK and Canada (37). Twin studies estimate the hereditary contribution to MS risk at about 50% (38). A comprehensive study based on Swedish MS and population registers estimated heritability at 64% and a shared environmental factor at 1% (39). These results are compatible with the existence of an important ubiquitous environmental factor, such as potentially EBV infection.

Much, but not all, of this heritability has been explained by genetic studies: risk alleles are confined almost entirely to genes involved in the immune response. The primary, and first discovered, genetic risk factor is located in the MHC class II HLA-DRB1 gene (HLA-DRB1*15:01 and absence of HLA-A*02:01) involved in antigen presentation (40). HLA-DRB1*15:01 increases the risk of MS by about three times in heterozygotes and six times in homozygotes (41).

More than 200 non-HLA genes, generally expressed within the immune system, are involved in MS pathogenesis, each with a low risk. These genetic

risk factors interact with several environmental factors. Many of these genes are targets for the EBV transcription factor EBNA2 (42).

2.6 COMBINATION OF GENES AND ENVIRONMENTAL FACTORS

Known environmental risk factors for MS are Epstein-Barr virus infection, smoking, low serum levels of vitamin D and obesity during adolescence. Additive interaction between these risk factors and genetics on MS risk has been reported for several of the risk factors, including smoking, EBV infection and obesity (43). In other words, the risk of developing MS when combing these risk factors is higher than the risk expected by the sum of the individual risks. These interactions point at a common pathological pathway, possibly through adaptive immunity (43). For EBV, the risk of MS was shown to have a 10-fold increase, from approximately a 2‰ prevalence, in DRB1*15-positive individuals with a history of IM (44). Combining the risk factors of Epstein-Barr nuclear antigen 1 (EBNA1) antibodies and HLA DRB1*15 increased the risk more than the sum of each risk factor alone (45).

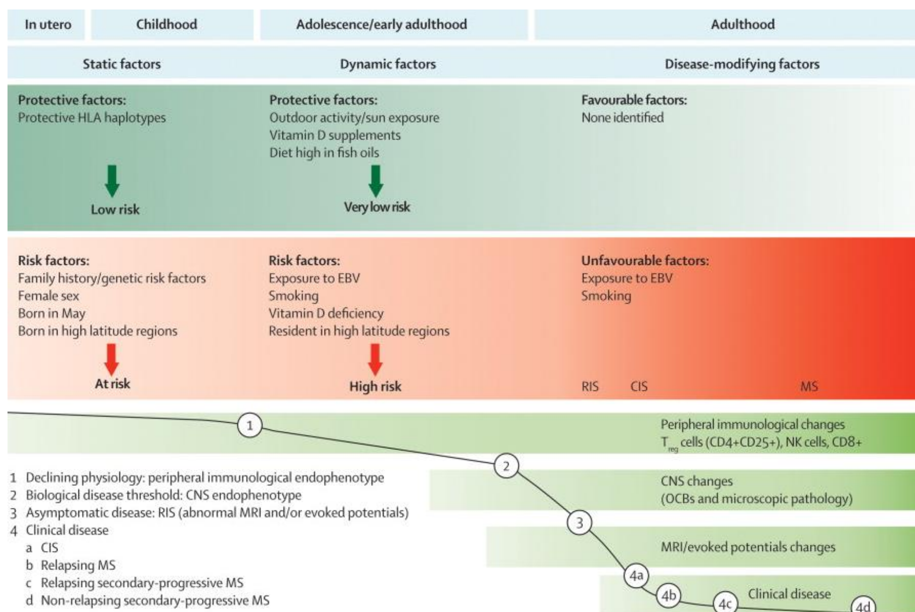

Figure 1. Potential causal pathways for MS development. Reprinted with permission from Ramagopalan SV, et al. "Multiple sclerosis: risk factors, prodromes, and potential causal pathways". Lancet Neurol. 2010;9(7):727-39 (6).

2.7 HYPOTHESIS REGARDING THE ROLE OF EBV IN MS PATHOGENESIS

An infectious aetiology of MS has been suspected for more than a century, and several viruses and other infections have shown an association to the disease. Association does not imply causation, and most of these suspicions have not been confirmed (46). An example is that measles infection was strongly linked to MS, but follow-up investigations after the introduction of vaccinations showed no effect on MS incidence (47).

In recent years, a substantial body of evidence has linked EBV to MS. EBV seropositivity in MS patients is virtually 100% (48), compared to 90–95% in the general population; being sero-negative for EBV thus protects against MS (49). Studies using material stored in biobanks on pre-symptomatic MS mimicking a prospective approach show that EBNA1 antibodies are increased before MS onset (50, 51). In an influential case–control study by Levin et. al. that examined pre-symptomatic samples, 10 of 305 samples were EBV sero-negative at first sampling. These 10 individuals all sero-converted to EBV positive on average 5 years before MS onset (52).

Infectious mononucleosis (IM), the clinically symptomatic primary EBV infection of adolescence, increases risk of future MS more than twofold (53, 54). Descriptive studies show similarities in the geographical distribution between IM and MS on both local (55) and global (56) levels.

A recent study by Björnevik et. al. in a large sample of pre-symptomatic MS patients showed that 34 of 35 EBV-negative individuals sero-converted before MS onset, compared to 57% of EBV-negative matched controls. When comparing within the pre-MS group they reported 32 times increased risk of future MS in individuals that sero-converted to EBV positive compared to the one individual that did not (57). This risk estimation is strongly dependent on the results of individual samples. Furthermore, sNfL concentrations increased on a group level after, and not before, EBV primary infection in pre-symptomatic samples.

Several mechanisms by which EBV could influence MS pathogenesis have been proposed. Efforts have been made to locate the actual existence of EBV or its DNA in the CNS of MS patients. However, results have been mixed or less convincing (58). Several studies have reported the absence of EBV DNA and RNA in the brain and CSF of MS patients (59, 60). At the same time, one group documented EBV-infected B cells in post-mortem brain tissue from

both PPMS and RRMS patients (61). It remains unproven whether EBV infects the MS brain.

Alternative mechanisms by which EBV could mediate an immune attack in MS have been investigated. The most studied possibility is molecular mimicry, wherein EBV viral proteins could mimic human myelin proteins. Several studies show examples of this in parts of MS populations. Recently, common epitopes were demonstrated between a peptide in EBNA1 and the CNS proteins anoctamin 2 and glial cell adhesion molecule in a small portion of multiple sclerosis patients (GlialCAM) (16, 62). EBV infection and transformation that allows the survival of B cells that recognize autoreactive antigens of the CNS, as first proposed by Pender (63), is another possibility (64). The third possible mechanism is EBV reactivation and bystander damage in the CNS (65).

2.8 HEMATOGENESIS AND MS – EXAMINATIONS OF THE BONE MARROW

The immunologic events that lead to MS are thought to begin in the periphery and then lead to immune cells infiltrating the CNS, where particularly autoreactive T cells attack myelin (6). The source of these primary events is unknown; peripheral sites as gut microbiota (66) have been proposed. Cell lines involved in this CNS infiltration originate and are renewed from the bone marrow, as well as macrophages and possibly microglia.

Previous studies on bone marrow have been few and small. Some older studies suggested a role of the bone marrow as a reservoir of virus (67, 68). Only one study before the study included in this thesis (study **I**) examined the immunophenotypes of BM cells; it showed a reduced cell count, which was interpreted as an effect of ongoing immunosuppressant treatment (69). However, a recent study showed increased myelopoiesis but not lymphopoiesis in treatment-naïve MS patients, with the increased production of myeloid cells accompanied by the clonal expansion of T cells (70).

Natural killer T (NKT) cells are cells that express both T cell receptors of adaptive immune defence and NK-cell surface receptors associated with innate immune response (71). They have been implicated in multiple sclerosis (72) and have a role in defending against viruses, including EBV(73) .

In Study **I**, we hypothesize that disturbances in early haematopoiesis, and especially in early B cell development, are important in the development of MS.

2.9 MS PRODROME

Given the definition of MS, with dissemination in time and space, CNS damage begins before a diagnosis. Inclusion in the Swedish MS register (used in study **IV** and **V**) requires the first typical neurological debut symptom. The possibility of a clinical MS prodrome with early signs or symptoms prior to this first neurological symptom has been addressed in several ways (74). To study this, an entirely prospective study tracking populations of healthy individuals would be hard to accomplish, given a follow-up time of several decades and high costs. Instead, studies using retrospective patient-reported data and prospective population-based health administrative data and health records have been performed. These studies show alterations in health behaviour in the 5–10 years prior to MS onset, consisting of increased frequency of health care visits and prescriptions issued for diverse complaints, such as fatigue, sleep disorders, pain and depressive symptoms (74-76).

Another approach is to study individuals with a higher-than-normal risk of future MS in search of subtle signs in otherwise healthy individuals. Examples of such studies include findings that 20% of first-degree relatives of individuals with MS, who had passed the age of expected MS onset, had CSF-enriched oligoclonal bands (77), that 5% of asymptomatic first-degree relatives fulfilled MRI criteria of MS (78) and of decreased vibration sensation in asymptomatic family members (79). This kind of "intermediate phenotype" has been dubbed by some as an endophenotype, a concept that is aimed at describing an "at risk" population for a complex disease with both genetic and environmental contributions (6). For some individuals, these findings may be prodromal to disease, while for some, they are present in individuals who do not develop the disease. A similar "shadow" of specific subclinical autoimmunity exists in close relatives to patients with other autoimmune disorders, such as systemic lupus erythematosus (80)

One high-risk population of future MS is individuals with RIS, around 30–45% of whom convert to a first clinical event or CIS in 5-year follow-up (81), reaching up to 50% after 10 years (82). Male sex, younger age and the presence of spinal cord lesions are risk factors for conversion, as are the presence of oligoclonal bands, neurofilament light (NfL) or some pro-inflammatory cytokines (YKL-40 and Il-8) in the CSF (81, 83-85). Studies **II**

and **III** in this thesis ask the question of whether there may be such a possible endophenotype in a follow-up after IM.

Regarding biomarkers of the MS prodrome or pre-symptomatic MS, one study showed an increase in sNfL in 30 MS cases, compared to 30 controls, in samples drawn a median of 6 years before MS onset (86). In Study **IV**, we extend this to a larger sample and longer timeframe, aiming to determine how long before clinical MS onset this neuronal damage starts. Study **V** compares these findings of pre-symptomatic sNfL with studies of different EBV antibodies.

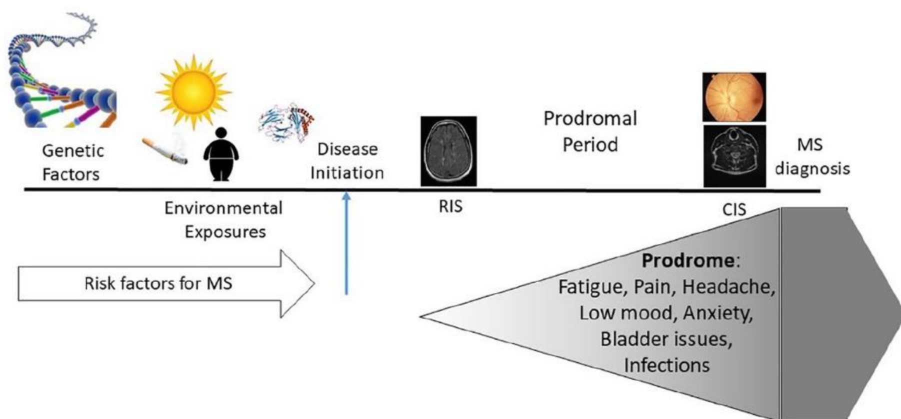

Figure 2. Time period from risk factors for MS to the prodromal phase, MS onset, and finally MS diagnosis. Reprinted with permission from Tremlett H, et al. "The multiple sclerosis prodrome: Emerging evidence, challenges, and opportunities." Mult Scler. 2021;27(1):6-12.(74) .

2.10 NEUROFILAMENT LIGHT

Neurofilament light chain (NfL) is a protein expressed in myelinated axons. It is a sensitive biomarker of acute and chronic axonal damage in neurological disease, including MS (87). Studies in MS show that serum neurofilament light (sNfL) levels are associated with disability progression, relapses, MRI lesions and grey matter atrophy (88-90).

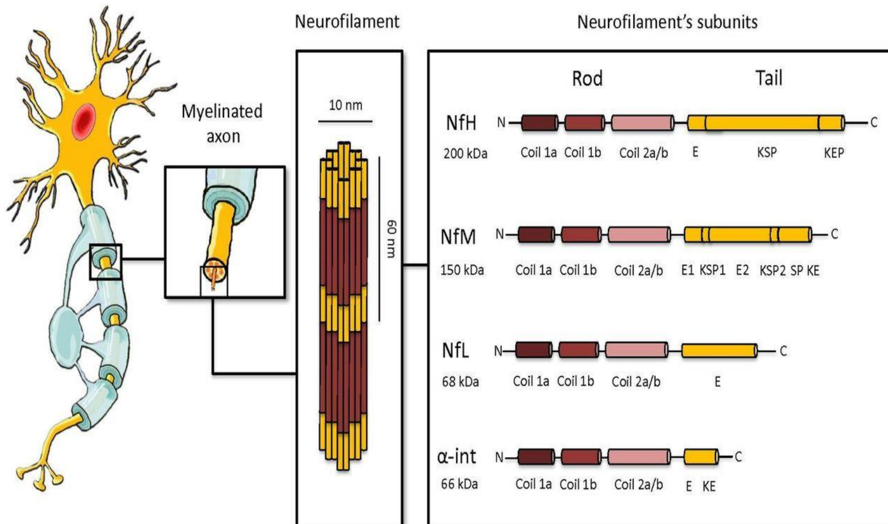

Figure 3. Overview of structure of neurofilaments and neurofilament light chains. Reprinted with permission from Gaetani L, et. al. "Neurofilament light chain as a biomarker in neurological disorders". J Neurol Neurosurg Psychiatry. 2019;90(8):870-81 (87).

3 EPSTEIN BARR VIRUS

3.1 GENERAL

Epstein-Barr virus (EBV), also known as human herpes virus 4, is a herpesvirus belonging to the gamma herpes viral subfamily. It is one of the most successful human viruses and infects 90–95% of the human population. The virus was first discovered in tumour cells from Burkitt lymphoma and was the first discovered human oncovirus (91). EBV has a linear dsDNA genome which encodes more than 85 genes (92). The virus has a long history of co-existing with mankind; for example, EBV DNA has been found by sequencing samples from the mouth of a human that lived 5,700 years ago in Denmark (93). Comparative genome studies indicate that EBV probably came as interspecies transfer from old world monkeys to hominids (94).

Human herpes viruses have four primary components: core, capsid, tegument and envelope. The core is dsDNA packed inside a capsid which is surrounded by the tegument, a layer of viral proteins that connects the capsid to the envelope. The envelope is derived from the host cell but incorporates glycoproteins, coded by the virus, which can attach to a new host cell (95).

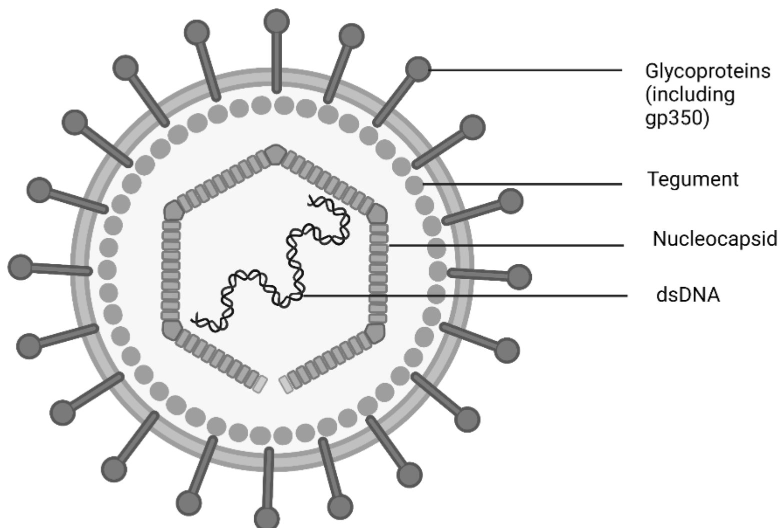

Figure 4. Schematic illustration of EBV virus.

3.2 LIFE CYCLE

Herpes viruses give rise to a persistent latent infection of the host. The goal of the viruses is to evade the host's immune regulation and persist within a small number of cells with minimal effects on the host (95).

EBV infects B cells and endothelial cells (lytic phase); it establishes and maintains a benign latent infection that is persistent for the host's lifetime, by exploiting various aspects of normal B cell biology (96). According to the germinal centre (GC) model, currently the most accepted for EBV infection and persistence (96), the virus is spread through saliva and enters through the epithelium that lines the nasopharynx (lytic phase). There, it infects naïve B cells, which it can drive to become a proliferating blast using the growth transcription program. Normally, antigen-activated B cell blasts migrate into the GC, where the survival of the B cell requires signals from cognate antigen and antigen-specific helper T cells. EBV-infected B cells can trick this system by the expression of viral proteins LMP1 and LMP2, which provide surrogate antigen and T helper cell survival signals (96). The latently infected B cell can then leave the GC and enter the pool of resting memory B cells. Proliferation here is driven by the normal cellular mechanisms for cell division. Here, EBV persists at a very low stable viral load, expressing only EBNA1. Approximately half a million memory B cells are infected in the human body, one percent of which circulate in the peripheral blood (96). From here, if a latently infected memory B cell is signalled to differentiate to a plasma cell, the virus will be released to infect new naïve B cells or the epithelium (lytic reactivation).

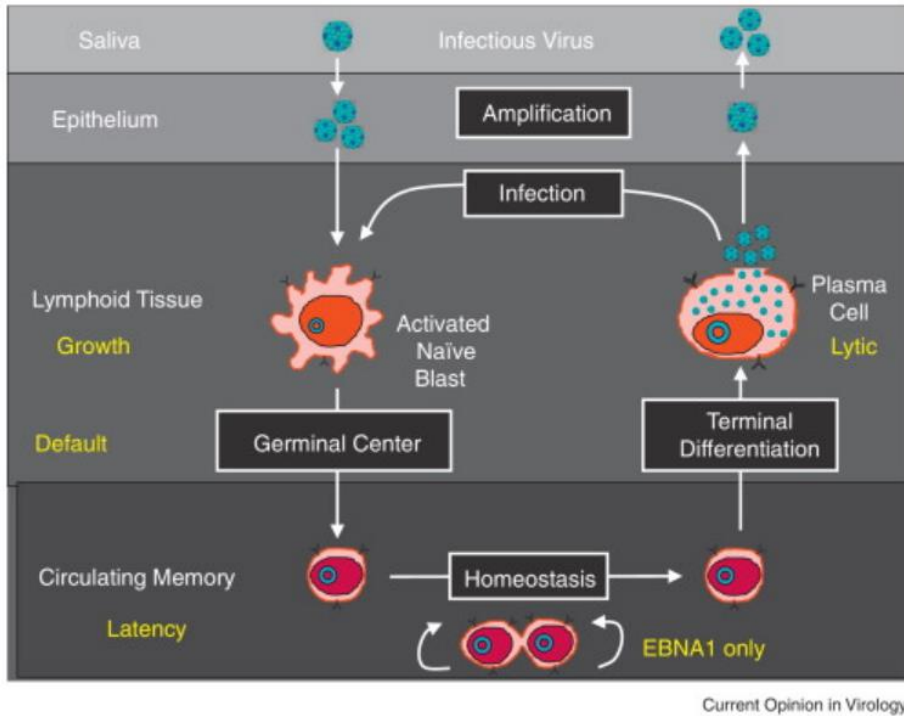

Figure 5. Life cycle of EBV infection. Reprinted with permission from Thorley-Lawson DA. "EBV Persistence--Introducing the Virus." Curr Top Microbiol Immunol. 2015;390(Pt 1):151-209. (96)

3.3 EPIDEMIOLOGY

As stated above, most of humanity is infected with EBV, however the age of primary infection varies with geographical region. While the absolute majority of individuals in developing countries are infected in childhood, developed countries show a bimodal pattern where primary infection in adolescence in many cases leads to infectious mononucleosis (97).

3.4 INFECTIOUS MONONUCLEOSIS

In childhood, primary EBV infection is mild and often goes unnoticed. In adolescence, primary EBV infection gives rise to IM in a proportion of the population, with a peak in the ages of 15–24 years (98). IM can last for

several weeks and is characterized by lymphocytosis, sore throat, lymphadenopathy and fatigue. Approximately 25–65% of individuals who experience a primary EBV infection in adolescence develop clinically symptomatic IM (99, 100). Although uncommon, primary EBV infection may still occur in middle age, although with modified symptoms, dominated by fever (101). IM is a dramatic event, with an expansion of the peripheral memory B cell population, during which up to 50% of these cells may carry the EBV genome (102). The quantity of EBV virus is associated with severity of illness in IM (103) and with the level of specific and neutralizing antibodies with gp350 reactivity. Sera from patients with IM contain heterophile antibodies to erythrocytes of various mammalian species (Paul-Bunnel antibodies) (104). In addition, some studies have suggested the presence of increased IgG antibody levels against measles and varicella-zoster during IM (105)

3.5 SEROLOGY

Serological diagnosis of EBV infection relies on antibodies against EBNA1 and viral capsid antigen (VCA), where the presence of IgM and IgG antibodies to VCA without EBNA1 IgG marks acute infection, while IgG antibodies to EBNA1 and VCA mark past, latent infection (106). EBNA1 is intranuclear and expressed during the division of the B memory cell (latency 1) (96).

EBVgp350 is an immuno-dominant lytic surface glycoprotein that mediates cellular entry upon infection. Gp350 antibodies are thought to be the predominant EBV neutralizing antibody in humans (107).

VCA, as originally defined by immunofluorescence and microscopy, contains several antigens, including the small capsid protein VCAp18, the major capsid protein p160, the tegument protein p23 and immunoreactive glycoproteins such as gp350 (108). In the studies in this thesis, we analyse antibodies to EBNA1 and VCA and to the single-protein antigens VCAp18 and gp350.

4 AIMS

The aim of the work included in this thesis was to investigate the relationship between EBV infection and subsequent multiple sclerosis before the clinical onset of MS. This was done by studying the potential long-term immunological consequences of infectious mononucleosis in healthy individuals and by examination of pre-symptomatic MS samples.

The specific objectives for each included paper are as follows:

I. To investigate whether B cell alterations are already present during cytogenesis in the bone marrow in MS patients compared to controls?

II. To investigate whether the increased risk of developing MS after IM means that IM gives rise to a subclinical long-term increase of inflammatory cytokines in the CSF with similarities to that found in MS.

III. To investigate whether IM give rise to long-term increases in serological reactivity to EBV and other viruses with similarities to the MRZ index found in MS.

IV. To investigate how long before the clinical onset of MS, the neuroaxonal damage begins.

V. To investigate how long before the clinical onset of MS, increases in lytic and latency EBV antibodies begin and whether we can relate these increases to the start of neuroaxonal damage.

5 SUBJECTS, STUDY DESIGN AND METHODS

5.1 STUDY DESIGN

Study **I** was an exploratory pilot study assessing the distribution of cell types, primarily the B cell lineage in the bone marrow and blood of patients with MS and controls.

Study **II** was a pilot study of levels of prospectively selected cytokines and chemokines in the CSF approximately 10 years after IM.

Study **III** was a follow-up study of the longitudinal serological response 10 years after IM.

Studies **IV** and **V** were nested case–control studies assessing the temporal development of different EBV antibodies and sNfL prior to developing MS.

5.2 STUDY **I** EARLY HAEMATOPOIESIS IN MULTIPLE SCLEROSIS PATIENTS

This exploratory pilot study assessed the distribution of cell types in the bone marrow and peripheral blood of MS patients and controls, focusing on the proportions of stem cells, B cell precursors and NKT cells. The acquisition of these bone marrow samples was initially motivated by older studies indicating the presence of virus, especially paramyxovirus, in bone marrow from MS patients (67). At that point, emerging studies (50, 51, 109) were shifting the focus of MS viral research to pinpoint EBV as a major factor, and viral quantification in bone marrow was postponed. We thus decided to present this pilot study of basic cell proportions.

Nine MS patients (six women, three men) were recruited and neurologically examined at the MS Centre, Department of Neurology, Sahlgrenska University Hospital, Gothenburg, Sweden. Crista puncture was performed at the Department of Haematology, Sahlgrenska University Hospital. Median age was 51 years (range 41–56 years). Six had relapsing remitting MS (RRMS) and three secondary progressive MS (SPMS). The patients had been clinically stationary for at least a year. The RRMS cases had ongoing therapy with Glatiramer acetate, and patients with other disease-modifying therapies

were excluded due to concerns that these could potentially be associated with bone marrow suppression. SPMS cases were untreated.

As controls, bone marrow was obtained from 11 neurologically healthy patients (four women, seven men), who underwent a sternum splitting thoracotomy for non-inflammatory cardiac disorders at the Department of Haematology, the Sahlgrenska University Hospital. The median age for controls was 40 years (range 22–59 years).

Table 1. Characteristics of cases and controls, Study 1.

	Age, median, years (range)	Female N (%)
Multiple sclerosis	51 (41–56)	6 (66%)
RRMS (n=6)	49 (41-53)	4 (66%)
SPMS (n=3)	52 (45-56)	2 (66%)
Controls	40 (22–59)	4 (36%)

5.2.1 ANALYSES

Flow cytometry was used to sort cell populations in peripheral blood and bone marrow to determine the proportions of early hematogones, total hematogones, mature B cells, T cells, NKT cells granulocytes and monocytes. For the definitions of examined cell types, see the methods section.

5.3 Studies II and III FOLLOW-UP AFTER INFECTIOUS MONONUCLEOSIS IN SEARCH OF SIMILARITIES WITH PRE-SYMPTOMATIC MULTIPLE SCLEROSIS

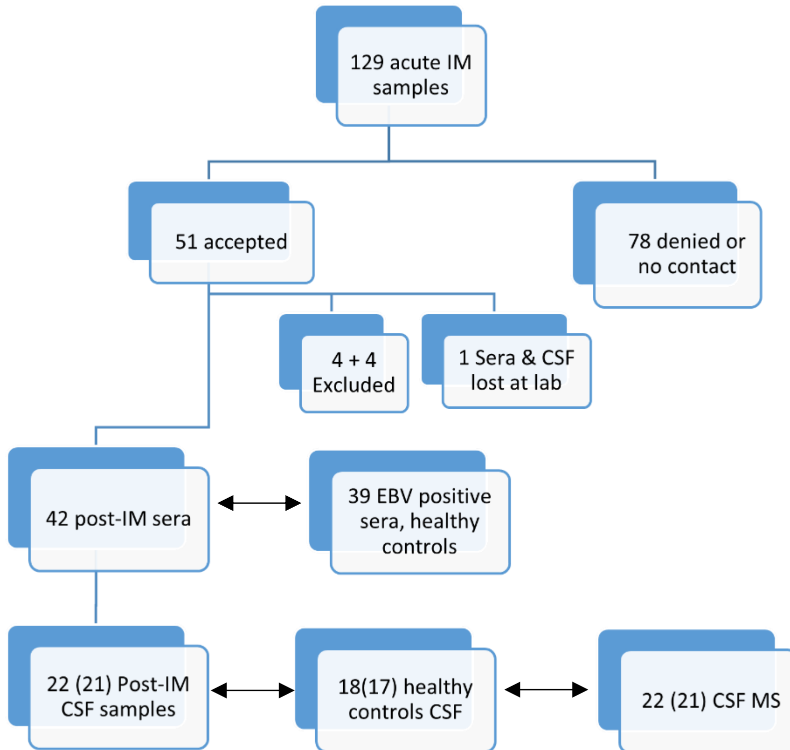

Figure 6. Flow chart of selection of cases and controls, studies II and III.

Studies **II** and **III** explore the potential role of IM in the acquisition of a risk to further develop MS. In Study **II**, we hypothesize that this endophenotype is mediated by a modification of the innate immune profile within the CNS and measure the concentrations of cytokines and chemokines in the CSF. Study **III** explores differences in humoral response after IM compared to individuals with a less symptomatic primary EBV infection.

Samples from individuals with serologically verified acute IM (anti-VCA IgM titres > 1:160) between 2003 and 2007 were identified in the database of the Department of Virology at Sahlgrenska University Hospital. This resulted

in 331 samples from 316 individuals. Of these individuals, 129 met the inclusion criteria of age 7–30 years at time of sampling and residency in the Gothenburg area. Letters were sent to these 129 individuals asking if they were interested in participating in our follow-up investigation. Participation was offered either with only a serum sample or, optionally, also with a CSF sample acquired by lumbar puncture.

Of the 51 individuals who originally agreed to participate in our studies, four were excluded (three for other diseases and one for having developed MS). Twenty-two of the remaining individuals were also willing to contribute a CSF sample and were thereby included in Study **II** (post-IM group).

For Study **III**, using sera and CSF, four additional individuals were excluded due to insufficient material of original IM sera or that the participant opted out. One sample from Study **II** was lost in the laboratory and could not be retrieved. This resulted in the total number of samples to be analysed in the post-IM group of Study **III** being 42 sera and 21 CSF samples.

All participants completed a health questionnaire with standardized questions concerning previous IM (see appendix), and all confirmed memory of the clinical episode. The median follow-up time for the CSF group was 10 years (range 9-13 years).

Healthy controls completed the same health questionnaire as post-IM individuals, where all included denied having any memory of previous IM, any autoimmune disorder, or any other serious disorder. In the heathy control group that contributed CSF, 15 were EBV positive, as defined by positive EBNA1 and VCA serologies. To improve the size of the healthy control group, sera from 24 EBV-positive blood donors were also included. The total EBV-positive healthy control group in Study **III** thus consisted of 39 serum donors, including 15 individuals who contributed CSF. For the 24 blood donors without clinical data, IM prevalence was estimated based on cumulative IM prevalence in a Danish population (13% for males and 22% for females) (110). This resulted in an estimated prevalence of past IM of approximately 13% in the EBV-positive healthy control group.

For reference, sera and CSF samples from untreated patients with MS from a previous study (77) was used. In Study **II,** 23 CSF samples for neurochemical assays (NfL and YKL-40) and 17 for the immunochemical assays. Sera and CSF from 22 of these were included as reference group in Study **III**. These samples were acquired in 1996–97 and stored at −80°C. These were selected from a larger sample to be optimally age-matched with the post-IM group and included 13 persons with RRMS and nine with SPMS.

Table 2. Participant gender and age by study group, study III.

	N	Age, median, years (range)	Female N (%)
Infectious mononucleosis (serum)	42	18 (11–34)	24 (57%)
Post-IM (serum)	42	28 (22–43)	24 (57%)
POST-IM (CSF)	21	27 (22–40)	13 (62%)
EBV pos. Healthy controls (serum)	39	23 (18–34)	21 (54%)
EBV pos. Healthy controls (CSF)	15	25 (20–46)	11 (65%)
Multiple sclerosis (serum and CSF)	22	35 (18–45)	16 (73%)

5.3.1 ANALYSES

For Study **II**, the selection of cytokines and chemokines was based on a literature search of previous studies showing their potential relevance with early MS or RIS when measured in the CSF. Table 5 summarizes the arguments for selecting these molecules. Five cytokines (IL-1b, IL-6, YKL-40, TNF-alpha) and three chemokines (IL-8, CCL2, IP-10) were selected before the study began. CSF was also analysed for cell count, albumin, oligoclonal bands and NfL.

5.4 STUDIES **IV** AND **V**

Paper **IV** and **V** were nested case-control studies that used 669 pre-symptomatically collected blood samples from persons who later received a RRMS diagnosis, and 669 matched controls. This national pre-symptomatic MS case-control material was used in previous studies of risk factors for MS (111-113). We here examined pre-symptomatic sNfL and the humoral response to EBV consisting of several antibodies.

Data from individuals with an MS diagnosis was retrieved from the Swedish MS register (114) and from a local MS database in Umeå in 2012. The MS register then contained 11,146 patients, and the Umeå database 2,887 patients. This data was cross-linked with five biobanks containing remainders of serum samples stored after microbiological analysis or screening at the university hospitals in Umeå, Örebro, Linköping, Skåne and Gothenburg and one biobank at the Public Health Agency of Sweden (PHAS).

For each sample found, drawn before the age of 40 years from an individual who later developed RRMS, a control sample was chosen from the same biobank. Controls were matched for biobank, sex, date of blood sampling and date of birth in decreasing priority. The matching had a mean difference of 6 days for date of sampling and 152 days for age at sampling. The full material is described in detail in Figure 7.

The date of onset of MS was extracted from the Swedish MS registry and medical records in Umeå and is defined as the date of the first symptoms suggestive of MS. The Swedish MS registry (www.neuroreg.se) has a good coverage of individuals with MS diagnosis in Sweden (80% in 2021), but in 2009, the coverage was lower in northern Sweden, so the local Umeå database was created for other projects.

Cases were excluded firstly based on inclusion criteria in the registry and secondly due to missing samples in freezers or missing registry data. If several samples from the same individual were found, the oldest sample was chosen. Study subjects were informed, with an opt-out option; a total of 10 individuals declined participation.

A scarcity of sample material after previous studies were completed resulted in additional exclusions for the various examinations performed in the present studies. Six-hundred and sixty-nine case−control pairs were available for anti-EBNA1 antibodies and anti-VCA antibodies (one was excluded from original study based on registry data), and 570 case−control pairs were available for gp350 and 519 for sNfL. For the total sample, median sampling

age was 25 years (range 4–39 years), and median time from sampling until MS onset was 9 years (range 1–32 years, see Table 3).

An estimation of the indication for sampling shows that for the five microbiological biobanks, approximately 38% of the samples were drawn for hepatitis or HIV screening, 30% for pregnancy screening, 24% for an unspecified serological analysis and 8% for an unknown reason. For the PHAS samples, the reason for collection is unknown; however, it is likely that the majority were originally collected for diagnostic microbiology (115).

Handling and storage of the samples varied and is to a large extent unknown. Long-term storage was at −20°C for all biobanks.

5.4.1 ANALYSES

Sera were analysed for sNfL (Study IV), EBNA1, VCAp18 and gp350 antibodies (Study **V).**

Table 3. Participants gender and age (median, IQR) for pre-symptomatic MS group, divided by strata of 5 years to clinical onset of MS. Studies IV and V. Reprinted with permission from study IV.

Time from sampling to onset of MS (total)	1-33 years, n=669	<5 years, n=220	5-10 years, n=190	10-15 years, n=147
Sex (F) (%)	82	82	85	77
Age at sampling, years	25 (21–29)	27 (23–32)	25 (20–29)	25 (21–29)
Age at MS onset, years	35 (29 -41)	30 (26–34)	32 (28–37)	37 (32–42)

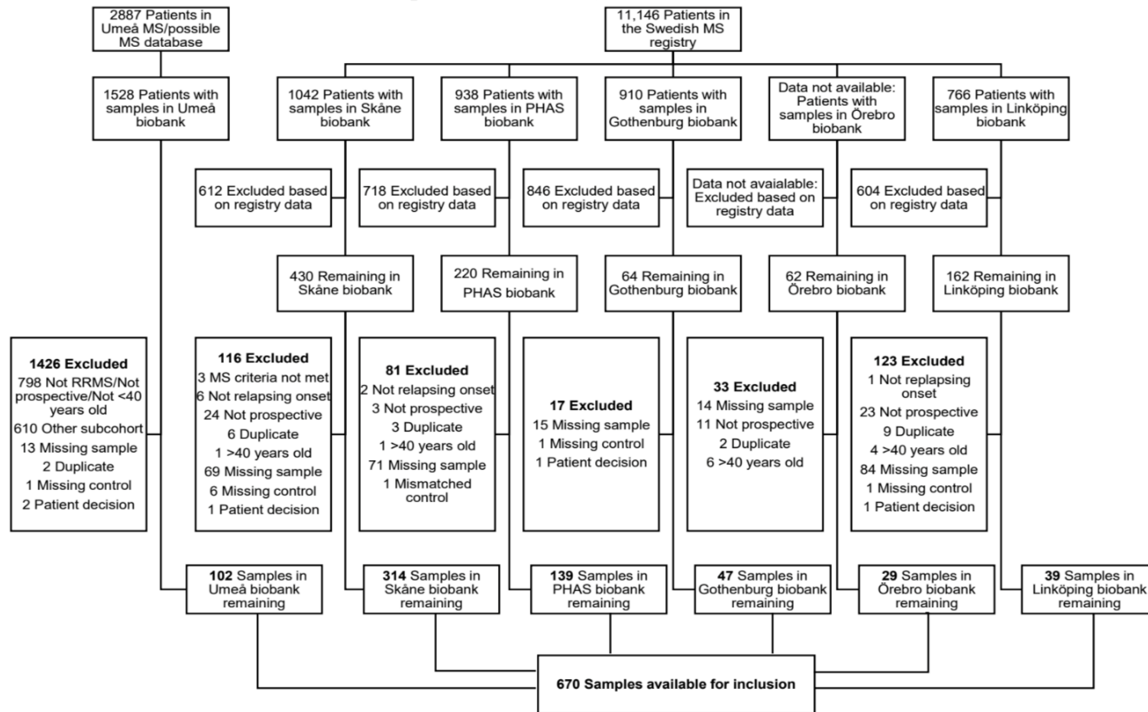

Figure 7. Flowchart of samples collected before MS onset identified through cross-linking the Swedish MS register and a local Umeå MS/possible MS database with six microbiological biobanks. Reprinted from Bistrom M. "High serum concentration of vitamin D may protect against multiple sclerosis"(111). 670 case-control pairs were eligible for inclusion in the study, but due to low sample volumes for the various analyses, additional case-control sets were excluded. The resulting total number of case-control pairs n=669 for EBNA1, VCA, n= 570 for gp350 and n=519 for sNfL.

5.5 ANALYTICAL METHODS

5.5.1 FLUORESCENCE-ACTIVATED SINGLE CELL SORTING

Fluorescence-activated single cell sorting (FACS) was used in Study **I** to examine different cell populations in bone marrow (BM) and peripheral blood.

Flow cytometry and FACS analysis are methods for separation of cell populations. Light is applied from a laser beam on a flow of single cells, and the scattering of light reveals characteristics of the cells. Forward scatter (FSC) is proportional to size, while side scatter (SSC) is proportional to cell granularity (internal complexity). Furthermore, cells can be labelled with fluorescent markers that bind to one or several molecules on their plasma membrane. Specific fluorescence-conjugated antibodies are added to the bone marrow or blood, where they will bind to any complementary antigen on the cell surface. A flow cytometer can excite and detect various light signals, and the presence of a light signal represents the expression of a certain molecule (116). A cell population is selected (gated) in a scatter plot based on parameters (e.g., FSC and SSC), then new analysis and gating are performed within the selection made. Therefore, results are here expressed as percentages of parent population.

Peripheral blood and BM aspirates were collected, erythrocytes were lysed, and cells were stained with monoclonal antibodies. All cells were analysed on a FACS Canto II, with at least 30,000 acquired events per tube.

Table 4. Applied definitions of cell types based on the expression of surface molecules. SSC= side scatter.

Cell type	Defined by:
Myeloblasts	SSC, CD45 + D, CD34-, and CD117+.
Myeloid progenitors	low SSC, CD19+, CD38+, and CD45 + D
Hematogones (117)	low SSC, CD19+, CD38+, and CD45 + D
Early hematogones (stage 1)	very low SSC, CD45 + D, CD34+, and CD117-
Mature B cells	low SSC, CD45 + B, CD19+, and CD20+ and confirmed expression of light chains (kappa/lambda).
T cells	CD4, CD8, CD27, CD45RA, CD45R0, and CD56
T cells subdivision	low SSC, CD45 + B, CD3+, and CD19- a
NKT cells	CD3+ CD56+

5.5.2 SEROLOGY – VIRAL ANTIBODY QUANTIFICATION

For the detection of IgG antibodies against EBVgp350 (Studies **III** and **V**), measles nucleocapsid protein (Ncore) and Varicella glycoprotein E (VZVgE) single-protein antigens were used (118, 119). These are single, immunodominant viral proteins that serve to increase the specificity and sensitivity of the assay, compared to the crude whole virus antigens commonly used. This minimizes possible contributions from autoantibodies (119). As described elsewhere (118, 120), Chinese hamster ovary (CHO) cells were used to express the viral glycoproteins for EBVgp350 and VZVgE. This was done by collaboration between the core facility Mammalian Protein Expression and the virology department, both at the University of Gothenburg, Sweden. The Ncore antigen was produced by collaborators at Centre National de la Rechereche Scientifique, Université Aix-Marseille, Marseille, France.

5.5.2.1 Epstein-Barr virus glycoprotein 350 (EBVgp350)

We analysed anti-gp350 IgG antibodies using an ELISA with a recombinant single-protein EBVgp350 antigen. The EBV envelope protein gp350 consists of 907 a.a., with an 860 a.a.-long extracellular part. As described elsewhere (118), three EBV DNA constructs (a.a. 1-506, 751-860 and 502-860) were synthesized and expressed in CHO cells. The first and third constructs could then be combined to encode the whole extracellular domain of gp350 (a.a. 1-860) and expressed in CHO cells. This protein was then purified and used as antigen in our ELISA.

5.5.2.2 Varicella-zoster virus glycoprotein E

The protein used for a specific VZV antigen was the predominant viral cell envelope molecule glycoprotein E. The protein (a.a. 1-539) was synthesized and expressed in CHO cells (120), purified and used as antigen in the present ELISA.

5.5.2.3 Measles virus nucleocapsid protein

The Ncore fragment (a.a. 1-392) of the immunodominant measles nucleocapsid protein was used. The core part was chosen as serological antigen since it is stable and conserved while the carboxyterminal is variable between different genotypes(121) .

5.5.3 ELISA

Indirect Enzyme-linked immunoassay (ELISA) was used to analyse the presence of IgG antibodies to EBVgp350, measles Ncore and VZVgE. In Study **III**, EBV gp350, measles Ncore and VZVgE antibodies were analysed in sera and CSF. In Study **V**, EBVgp350 antibodies was analysed in sera.

Antigens diluted in carbonate buffer were coated on Maxisorb™ 96-well ELISA plates, incubated and washed, and blocked with dry milk diluted in PBS to avoid nonspecific binding. Diluted sera or CSF was added to the wells, allowing any complementary IgG to the viral antigen to bind to the antigen. Plates were incubated and washed, and a second anti-human antibody conjugated with alkaline phosphate AffiniPure added. After washing, phosphatase substrate was added to the wells, and if any binding of the secondary antibodies had occurred, the alkaline phosphate enzyme would induce a colour change to yellow. The relative levels of detectable anti-

EBVgp350, anti-VZVgE and Ncore IgG antibodies were measured as optical density with the use of a spectrophotometer.

In Studies **II** and **III**, anti-EBNA1 IgG and anti-VCA IgM and IgG analyses were performed using Architect ELISA (Abbott, Illinois, USA).

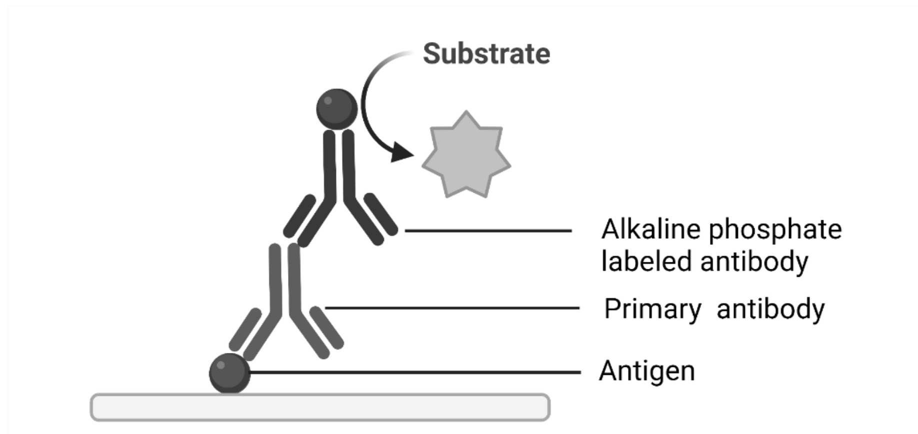

Figure 8. Illustration of indirect ELISA.

5.5.4 LUMINEX BEAD BASED IMMUNOASSAY

In Study **V**, IgG antibody levels against EBNA1 (a.a. 325-641) and VCAp18 (a.a. 1-175) proteins were measured in plasma with a multiplex Luminex bead-based immunoassay (122). This was performed at the Infections and Cancer Epidemiology Centre in Heidelberg, Germany. This method uses colour-coded beads coated with antigen. The beads are originally coated with glutathione-casein (GC), and the viral proteins (antigens) are expressed fused with glutathone s-transferas (GST), which binds to GC. Antigen-coated beads are then incubated with the subject's plasma binding the complementary antibodies. These antibodies are detected by an anti-IgG secondary antibody and form an antibody sandwich. Phycoerythrin-streptavidin is added and binds to the biotinylated detection antibodies.

Finally, a dual-laser flow-based detection analyser (Luminex 200) was used to quantify antibodies as median fluorescent intensity (MFI). Single beads are excited by laser beams. A first laser excites the bead, identifying the antibody that is bound to the bead, and a second laser excites the Phycoerythrin, which emits light in proportion to the amount of antibody bound.

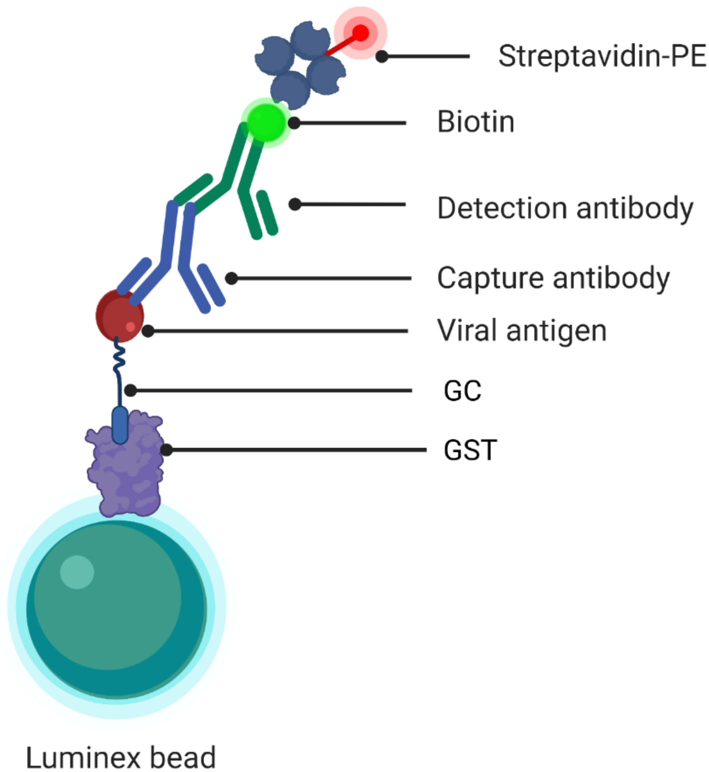

Figure 9. Illustration of Luminex bead-based immunoassay.

5.5.5 CYTOKINE AND CHEMOKINE DETECTION

In the follow-up after IM conducted in Study **II**, 10 cyto-chemokines were originally selected based on a literature search of findings in early MS/RIS which identified possible biomarkers with connections to early MS. This selection was pre-determined and did not change during the study. However, three analyses had to be cancelled due to low analytical sensitivity. The remaining seven cytokines and chemokines (IP-10, CCL2, TNF-alpha, IL-1b, IL-6, IL-8, and IL-23) were analysed by selecting these assays from the V-plex Neuroinflammation Panel (MesoScale Discovery). These cyto-/chemokines are essentially pro-inflammatory, except for CCL-2, which is reported to have a reversed mechanism in MS (123). The Immunology

Department at Sahlgrenska University Hospital performed the analysis. Table 5 summarizes the basis for selection of relevant cytokines and chemokines.

Table 5. CSF cytokines and chemokines pre-selected for analysis as factors potentially associated with IM and MS.

Cytokines, chemokines	Main cellular production and effect sites	Proposed relationship to multiple sclerosis	Ref
YKL-40 (CHI3L1)	Upregulated in inflammation, mostly in infections; synthesized in macrophages.	Increased in all phases of MS. Higher in PPMS than RRMS and CIS. Predictive of course after CIS diagnosis.	(124-126)
IP-10	Produced in several viral infections; discriminates infectious from non-infectious CNS disorders; induced by interferon-gamma; promotes Th1 immunity.	Associated with CIS to MS conversion. Associated with new T2 lesions.	(127-129)
CCL2	Produced by Th1-type cells; mediator of endothelial transmigration and leucocyte accumulation	Inversely related to IgG in the CSF; levels vary inversely with number of gadolinium-positive MRI lesions, probably due to cytokine consumption; EBV induces CCL2 expression	(123, 130, 131)
TNF-alpha	Major cytokine in infections; produced mainly by macrophages after antigen contact; induces fever from hypothalamus	Significantly increased in CSF of individuals with MS B cell lines from PPMS produced more TNF-alpha than control B cell lines	(77, 132, 133)
IL-1beta	Major endogenous pyrogene; multiple effects; induces IL-2 in lymphocytes; T- and B-cell activation	CSF IL-1b was associated with neurodegeneration and disease progression	(134)
IL-6	Induces acute phase response; crosses BBB; induces fever; secreted by macrophages.	Significantly increased in CSF of individuals with MS. B cell lines from PPMS produced more TNF-alpha than control B cell lines	(123, 135)
IL-8	Regulates migration of neutrophils and macrophages across the endothelium; also produced intrathecally	CSF IL-1b was associated with neurodegeneration and disease progression	(84, 135)

5.5.6 NEUROFILAMENT LIGHT

For Study **II**, the NfL concentrations in CSF were measured with an in-house ELISA, as previously described (136). CSF YKL-40 levels were measured with solid-phase sandwich ELISA (R&D Systems, Minneapolis, MN, USA). Analyses were performed by board-certified laboratory technicians in the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital.

5.5.7 SIMOA

NfL in sera was analysed in Studies **IV** and **V**. For measurements of NfL in sera, a higher sensitivity is needed than for analyses in CSF. Single-molecule array (SIMOA) is similar to a sandwich ELISA but uses magnetic beads coated with antibodies that capture the analyte and are then detected in microwells, where only one bead can fit. The SIMOA assay can improve the sensitivity of a normal ELISA 100- to 1,000-fold (137). In short, the analyte (NfL) from the investigated sera, is bound to antibody coated paramagnetic microbeads, washed, and incubated with a detector antibody. β-galactosidase-labelled streptavidin (SBG) is added, and beads placed in individual microwells containing substrate. The concentration of sNfL is determined by the number of wells that contain a bead compared to the number of wells with a bead that emits a fluorescent signal.

5.6 LUMBAR PUNCTURE

For collecting the CSF from healthy volunteers used in Studies **II** and **III**, we performed a lumbar puncture with the participant in the lateral position.
20 ml of CSF was collected using an atraumatic needle.

5.7 STATISTICAL METHODS

Non-normally distributed data were analysed using non-parametric tests (Studies **I**–**III**), the Mann–Whitney U test for unpaired data and the Wilcoxon signed rank test and a non-parametric ANOVA for matched paired data (Study **III**). In Study **II**, we used a one-sided test, given the pro-inflammatory hypothesis of each biomarker. To perform multiple testing between the two groups in Study **II**, a Hotelling's T2 test (138) was used. This avoided the problem of multiple testing and could potentially detect

differences between the three groups, with small differences between groups for each biomarker. The test calculates a score for each subject, where the seven dimensions from the seven biomarkers can be plotted on a two-dimensional plot. Principal component analysis and a linear discriminant analysis were used to visualise differences between groups. In Study **III**, quantile regression was used to model group differences adjusted for age. For correlations, Spearman's rank correlation test was used in Study **III**, and Pearson correlations in Study **V**.

Studies **IV** and **V** were nested case–control studies and therefore used slightly different statistical methods. Individual sNfL concentrations were log-transformed, and ratios (pre-MS/matched controls) plotted against time to MS onset. The relationship was estimated with smoothed regression analyses (LOESS), and a paired t-test was used to compare log-transformed sNfL values between groups for the total material, for 5-year time strata until MS onset (Studies **IV** and **V**) and to compare sNfL in subgroups with and without sero-reactivity against EBV. Odds ratios were estimated using conditional logistic regression with MS as outcome and log sNfL as predictor. In Study **V**, delta values (pre-MS – matched control) were plotted against time to MS onset and analysed with the LOESS function in R. Cohen's d was calculated for each group. The difference in distribution of sNfL dependent on EBV status within the pre-MS group was tested by comparing the number of higher sNfL values in the EBV-positive than in the EBV-negative group, with an expected number calculated from the hypothesis of equal distribution.

Statistical analysis was performed with IBM SPSS, Graphpad and R Statistics software. Statistical tests were performed at a 5% level of significance.

5.8 ETHICS

All study subjects and controls participated voluntarily in the studies. For Studies I–III, all participants provided written consent after oral and written information was provided. For Studies IV and V, written consent was not required for participation. All potential study participants (n = 1,365) were provided information through a letter wherein they were presented with the possibility to ask questions or opt out if they did not wish for their stored sample to be used in the study. Data was anonymized and kept in the university's locked computer to ensure no unauthorized access.

5.8.1 ETHICAL APPROVALS:

Study **I**: The study of bone marrow analysis of MS patients and controls was approved by the Research Ethics Committee of Gothenburg 2010 (Dnr 188-10).

The national virological biobank study used as a basis for Studies **II–V** was originally approved by the research committee of Umeå in 2011 (Dnr Umeå 2011-198-31M).

Study **II**: The study of infectious mononucleosis individuals and controls from the region of greater Gothenburg was approved as supplemental to the original application by the research committee of Umeå (Dnr 2013-226-32M and 2017-484-32M).

Study **III**: The same approval as in study II. The examination of samples from MS patients used as a reference group was originally approved by the research committee of Gothenburg in 1997 (Dnr S361-96 and S8-97) and supplemental Dnr 216-18.

Studies **IV** and **V** were based on the original application for the national studies described above. Amendment Dnr Umeå 2017-104-32M described the opt-out option for participants. The analysis of serum neurofilament was approved by the research committee in Umeå in amendment Dnr 2019-03402.

6 RESULTS

6.1 STUDY I HAEMATOPOIESIS IN MULTIPLE SCLEROSIS PATIENTS

This pilot study assessed the distribution of cell types in the bone marrow and blood of patients with MS and healthy controls.

6.1.1 THE PROPORTION OF MAJOR LEUKOCYTE SUBSETS DID NOT DIFFER BETWEEN PATIENTS AND CONTROLS

No significant difference was seen for major leukocyte subsets in patients compared to controls. The median percentages of lymphocytes of total white blood cells in BM from MS patients were 19.8% and 14.3% for healthy controls (p=0.15). Of these lymphocytes 15.7% were B cells in the MS group and 21.6% in the control group (p=0.37). The frequencies of T cells, monocytes, granulocytes and NK cells were similar between the groups (table 6). Myeloblasts were slightly lower in patients, but not significantly so.

6.1.2 THE PROPORTION OF B CELLS PRECURSORS DID NOT DIFFER BETWEEN PATIENTS AND CONTROLS

No significant difference was seen for the proportions of mature B cells between MS patients and controls. The groups also had the same proportions of both total hematogones and stage 1 (early) or stages 2–3 (late) hematogones in the BM. The proportion of CD5+ B cell subsets did not differ between MS patients and controls.

6.1.3 THE PROPORTION OF NKT CELLS WAS LOWER IN BONE MARROW OF MS PATIENTS

The T cell population proportions of CD4+ and CD8+ cells and subsets of these cells (CD27+, CD45R0, CD45RA) were similar between the MS and control groups. NKT cells (CD3 + CD56 +) were lower in MS patients than in controls (3.5% and 7.1%, respectively), with borderline significance that would not withstand a Bonferroni correction (P = 0.046).

Table 6. Proportions of basic cell types in MS bone marrow. Expressed in percentages of parent cell type. IQR = inter quartile range. Reprinted and adjusted with permission from study I.

	Patients, median (IQR)	Controls, median (IQR)	P- value
White blood cells (% of all events)	89.60 (82.70–90.80)	85.50 (78.70–87.10)	0.37
Lymphocytes (% of white blood cells)	19.80 (16.10–23.50)	14.30 (9.70–21.40)	0.15
B cells (% of lymphocytes)	15.70 (14.90–20.80)	21.60 (11.30–26.20)	0.37
T cells (% of lymphocytes)	63.60 (56.10–70.80)	58.80 (52.00–68.50)	0.71
NK cells (% of parent)	16.40 (13.50–21.00)	14.00 (9.10-25.80)	0.71
NKT cells (% of parent)	3.50 (1.80-7.00)	7.10 (3.60-13.00)	0.046
Monocytes (% of white blood cells	4.30 (3.10-4.40)	4.30 (3.80-5.30)	0.29
Granulocytes (% of parent)	79.10 (71.80-82.60)	82.10 (73.00-83.90)	0.60
Myeloblasts (% of parent)	13.40 (8.80-18.70)	18.50 (13.90-27.30)	0.13

6.2 STUDY II INTRATHECAL IMMUNOREACTIVITY IN PEOPLE WITH OR WITHOUT PREVIOUS INFECTIOUS MONONUCLEOSIS

6.2.1 NEUROFILAMENT WAS NOT INCREASED IN THE POST-IM GROUP

The NfL level in the MS group was significantly increased compared to healthy controls ($p < 0.0001$). All individuals in the MS group had at least two CSF-enriched oligoclonal bands. No increases in NfL or oligoclonal bands were detected in the post-IM group compared to controls.

6.2.2 CSF CYTOKINE LEVELS SHOWED A POSSIBLE TREND TOWARDS INCREASE IN THE POST-IM GROUP

In the MS group, levels of YKL-40 (also known as CHI3L1), IL-1b, IL-8 and TNF-alpha were significantly increased, and the levels of CCL-2 decreased, as predicted for pro-inflammatory (all but CCL2) and anti-inflammatory (CCL2) cytokines and chemokines. Together, using a multivariate test, the seven cyto-/chemokines showed significantly higher levels in the MS group than in the control group (p=.00001).

For the post-IM group, levels of IP10, YKL-40 and CCL2 showed significantly increased median values compared to the HC group (p=.021, .0495, .028). Median values for each of the seven cytokines and chemokines were higher for the post-IM group than for controls (binomial ratio 7/7, one-tailed P = .008, figure 10). However, this potential overall trend could not be confirmed by a multivariate test of the cytokines and chemokines levels (p=0.22).

6.2.3 POWER CALCULATION

The study did not reach the dimension required according to our power calculations before the study. Based on the present results, sample sizes for a possible future study were calculated. Testing the hypothesis that IM induces long-term increases in the CSF concentrations of five selected chemokines would require between 20 and 75 samples, including correction for mass significance.

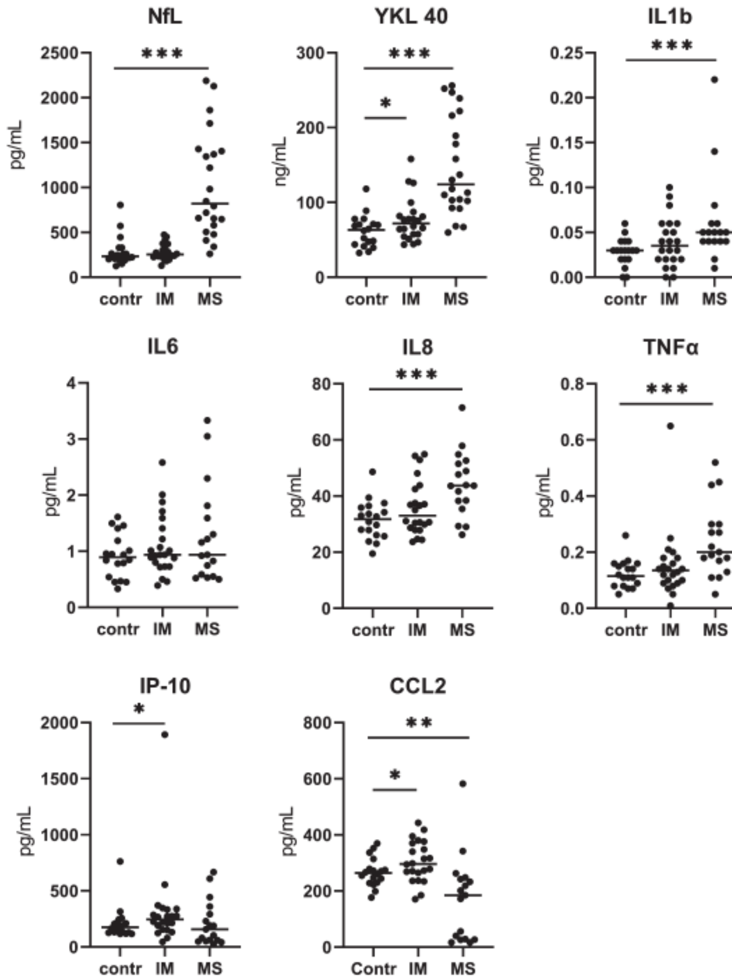

*Figure 10. NfL and seven selected CSF cytokines/chemokines in the control, post-IM, and MS groups. Significance was tested between the control (contr) and post-IM groups (short bar), and between the control and MS groups (long bars). NFL: neurofilament light chain; IM: infectious mononucleosis; MS: multiple sclerosis; *p <0.05, **p <0.01, ***p <0.001. Reprinted and adapted with permission from Study II*

6.3 STUDY III FOLLOW-UP AFTER INFECTIOUS MONONUCLEOSIS IN SEARCH OF SEROLOGICAL SIMILARITIES WITH PRE-SYMPTOMATIC MULTIPLE SCLEROSIS

6.3.1 ANTIBODY REACTIVITY AT INFECTIOUS MONONUCLEOSIS AND AT FOLLOW-UP

Anti-VCA IgM were high at IM while anti-EBNA1 IgG, anti-gp350 IgG and anti-VCA IgG values, were lower in the IM group than in controls and increased to follow-up (post-IM).

Antibodies to measles (Ncore) and varicella (VZVgE) both had a higher reactivity in serum during IM than in the control group ($p < 0.001$ and $p < 0.0001$, respectively). This reactivity decreased at follow-up.

Comparing the post-IM group with EBV-positive healthy controls with a low historical incidence of IM, the post-IM group had higher anti-gp350 reactivity ($p = 0.007$). No significant difference was seen for anti-VCA or anti-EBNA1 IgG, although anti-VCA showed a non-significant trend towards an increase at follow-up ($p = 0.08$).

After age adjustment, anti-Ncore IgG reactivity was higher in the post-IM group than in controls ($p = 0.014$). No difference was observed for anti-VZVgE levels between post-IM and healthy controls.

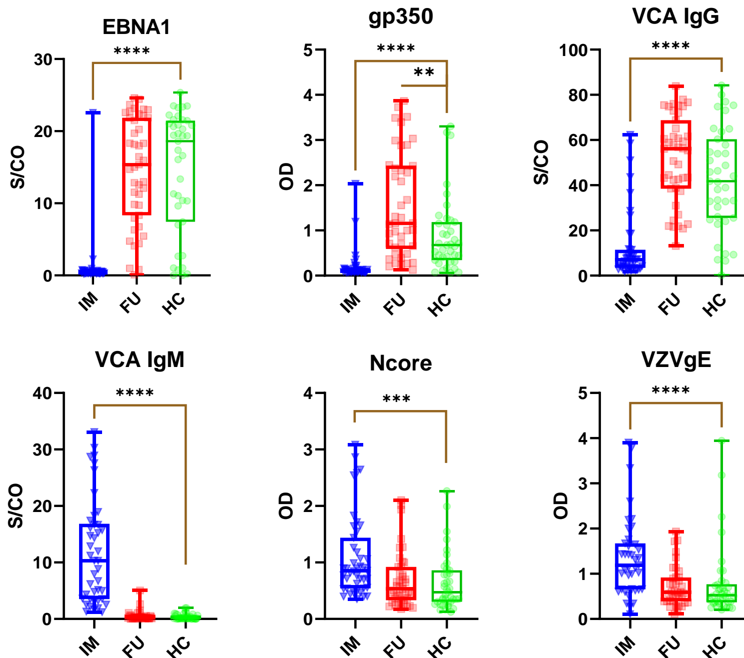

Figure 11. Antibody reactivity for groups IM = acute infectious mononucleosis, FU = post-IM, 10-year follow-up after IM and HC = healthy controls. Elevated gp350 reactivity was shown 10 years after IM compared to EBV-positive controls, and increased reactivity to Ncore and VZVgE during IM. EBNA1: Epstein-Barr nuclear antigen 1; gp350: glycoprotein 350; VCA: viral capside antigen; Ncore: N-terminal portion of the measles nucleocapsid protein; VZVgE: varicella-zoster glycoprotein E; OD: optical density; S/CO: sample to cut-off. Reprinted and adapted with permission from Study III.

6.3.2 CSF ANALYSIS OF POST-IM GROUP

For CSF, no significant differences were seen for anti-gp350 IgG, anti-Ncore IgG or anti-VZVgE when comparing the FU group with controls using the EBV-positive group only.

6.3.3 ANTIBODY REACTIVITY IN MS GROUP

Gp350 reactivity was higher in the sera of individuals with MS than in the EBV-positive control group (OD 1.98 vs 0.68, $p = 0.006$) (Table 7). For CSF, gp350 reactivity was higher in individuals with MS, but with borderline significance ($p=0.048$). Measles anti-Ncore reactivity was clearly elevated in

individuals with MS compared to controls, both in sera and CSF (Table 7). VZV gE was increased in CSF but not in sera. There was no difference for the CFS/sera ratio between MS patients and controls for anti-gp350, while the CFS/sera ratio was increased for anti-Ncore and anti-VZVgE.

Table 7. Antibody reactivity (OD values) for MS patients compared to EBV positive controls. Showing increased reactivity in persons with MS for all three antibodies in sera and CSF except for VZVgE in sera. Gp350: glycoprotein 350; Ncore: N-terminal portion of portion of the measles nucleocapsid protein; VZVgE: varicella-zoster glycoprotein E; OD: optical density; IQR: interquartile range.

	MS, median (IQR)	Controls, median (IQR)	P- value
GP 350 IgG, Sera	1.98 (1.24)	0.68 (0.84)	0.0063
Ncore IgG, Sera	2.5 (1.2)	0.48 (0.56)	<0.0001
VZVgE IgG, Sera	0.75 (1.0)	0.53 (0.4)	n.s.
GP 350 IgG, CSF	0.31 (0.41)	0.18 (0.22)	0.048
Ncore IgG, CSF	1.78 (1.41)	0.17 (0.17)	<0.0001
VZVgE IgG, CSF	0.46 (0.81)	0.15 (0.13)	0.01

6.4 STUDY **IV** AXONAL INJURY IN ASYMPTOMATIC INDIVIDUALS PRECEDING ONSET OF MULTIPLE SCLEROSIS

Serum neurofilament light (sNfL) concentrations were higher in pre-symptomatic MS individuals compared to matched controls, with a geometric mean value of 7.1 pg/ml (CI 6.8–7.4) in the total pre-MS group and 6.2 pg/mL (CI 5.9–6.5) in the control group (p = 0.00001). Ratios between log sNfL for pre-MS and matched controls plotted against time until MS onset, using the LOESS function, showed an increasing difference between the groups, starting at approximately 10 years before MS onset (Figure 12).

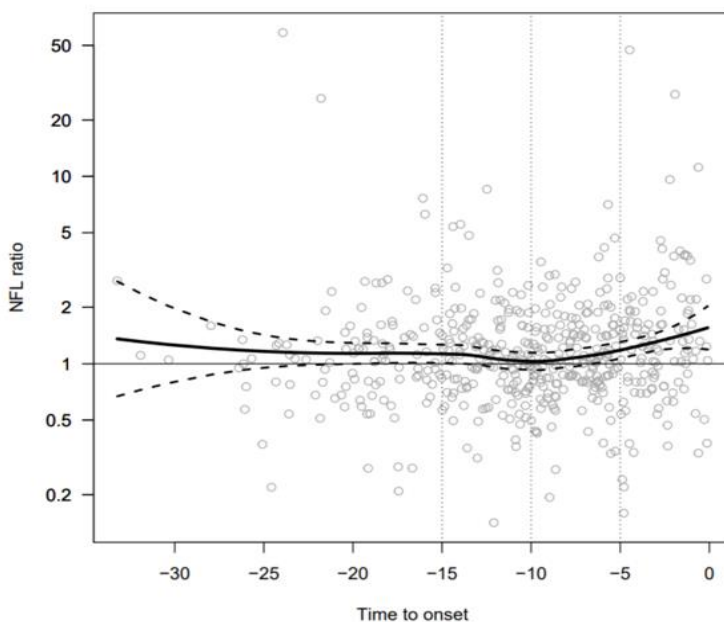

Figure 12. sNfL ratios for pre-MS/controls plotted over time to MS onset (years). Line shows LOESS regression. Adapted and reprinted with permission from Study IV.

After dividing the material by 5-year strata leading to MS onset, significant differences were seen between pre-MS and controls for the groups between 0–5 years to onset, geometric mean 9.6 pg/ml (CI 8.4–10.9) in the pre-MS group and 7.4 pg/ml (CI 6.7–8.3) in the control group (p=0.002), and for 5-10

years before onset, pre-MS 6.4 pg/mL (CI 5.9–7.0) and controls 5.8 pg/ml (CI 5.4–6.2, p = 0.02). No significant differences were seen for the groups 10–15 years and 15–32 years to MS onset, but when analysed together (10–32 years to onset), a significant difference was seen (p = 0.026; Figure 13).

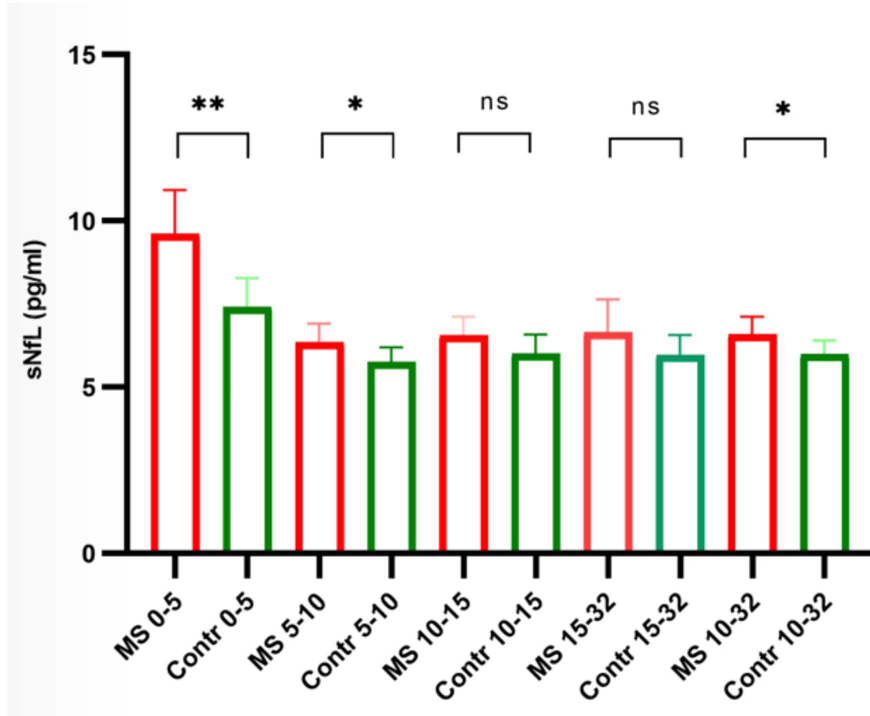

*Figure 13. Geometric mean levels pre-symptomatic MS patients and controls, 5-year strata with time leading to MS onset. Adapted and reprinted with permission from study IV. sNfL = serum neurofilament, MS = pre symptomatic MS individual, Contr = matched control, Error bars show 95% CI. *p ≤ 0.05; **p ≤ 0.05*

Odds ratios for increase in sNfL and subsequent MS showed that a tenfold increase in sNfL was associated with an increased risk of MS, with OR 4.1 (CI 2.1–8.0) for the whole sample, OR 6.6 (CI 1.8–23) for 0–5 years before onset and OR 4.8 (CI 1.2–19) for 5–10 years to MS onset. No significance was found for the other years-before-onset strata; however, similar to results above using t-tests, when merged (10–32 years to onset), the OR was 2.8 (CI 1.1–7.3).

6.5 STUDY **V** INCREASE IN EPSTEIN-BARR VIRUS SERO-REACTIVITY PRECEDES NEUROAXONAL DAMAGE IN PRE-SYMPTOMATIC MULTIPLE SCLEROSIS

6.5.1 EBV SERO-REACTIVITY IN PRE-SYMPTOMATIC SAMPLES.

Ninety-four percent of the total pre-MS group and 93% of controls were EBV positive (EBNA1 and/or VCAp18 IgG positive), and 87% in the pre-MS group were positive for both EBNA1 and VCAp18. The total pre-MS group had higher antibody reactivity to EBNA1, gp350 and VCAp18 than controls (p from 0.005 to < 0.0001).

Comparing only EBV-positive case-controls, the pre-MS group had an increase in anti-EBNA1 IgG reactivity compared to controls, which appeared between 15–20 years before MS onset and was significant in the time strata 10–15, 5–10 and 0–5 years to MS onset. The difference in reactivity between pre-MS and controls was approximately equal in these time strata, meaning that the samples acquired from individuals with less time to MS onset did not show higher reactivity than those from individuals acquired 10-15 years before onset. Figure 14 illustrates the pre-symptomatic increase in EBNA1 (pre-MS case), plotted against time to MS onset. As described in Study **IV**, in the same material, an increase in sNfL was detectable in the pre-MS group from approximately 10 years before MS onset. Thus, increased reactivity to EBNA1 was detectable longer before MS onset than the increase in sNfL concentrations. Figure 15 visualises this by calculating the z-score for individual delta values ($z = [\text{raw score-mean}]/SD$), enabling a visualisation of the two parameters in the same figure.

Anti-gp350 IgG showed a similar pattern to EBNA1. No significant difference was seen between groups for VCA antibodies. There was no correlation between anti-gp350 reactivity and anti-EBNA1 reactivity in EBV-positive patients ($r = -0.001$; $p = 0.98$).

The increase in sNfL for the pre-MS group was not correlated with any increase in EBV antibodies in the EBV-positive patients ($r = -0.05$, $p = 0.26$ for EBNA1, $r = -0.04$, $p = 0.39$ for VCAp18, and $r = -0.05$, $p = 0.34$ for gp350).

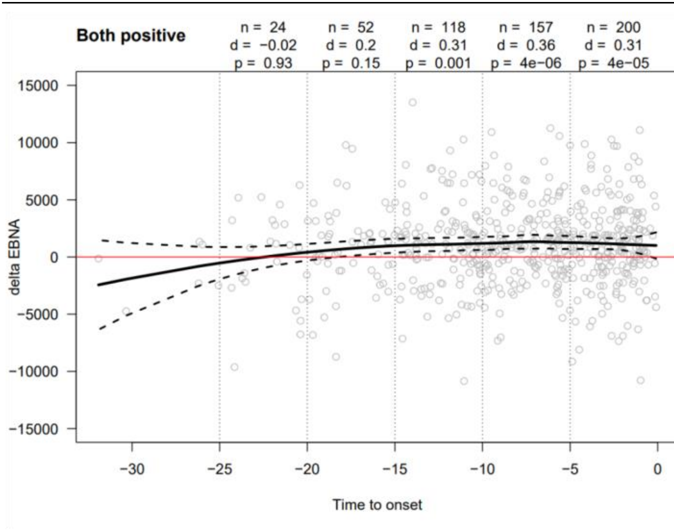

Figure 14. Delta EBNA1 values (difference case – healthy control) for EBV-positive samples plotted against time to MS onset (years). P values above graph represents results from t-tests within each age stratum. Pre-MS group has significantly higher EBNA1 values than controls for groups 10–15, 5–10, and 0–5 years to clinical MS onset. EBNA=Epstein Barr viral antigen 1, d = effect size (Cohen's d; i.e., mean/SD).

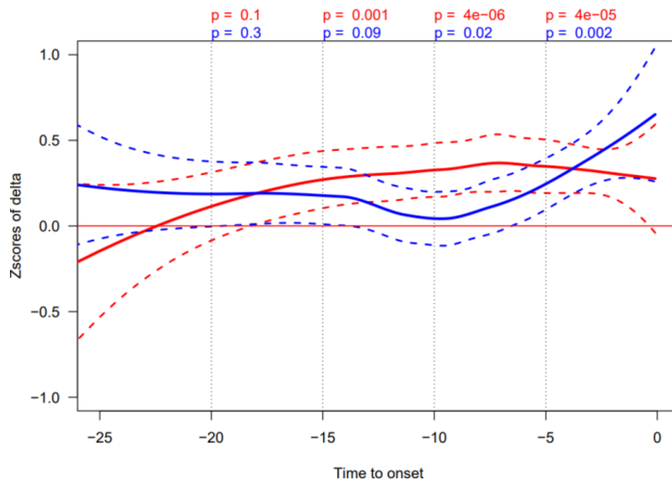

Figure 15. Z scores of delta values for pre-symptomatic sNfL (blue) and EBNA1 (red). This visualises that a significant increase in EBNA1 values is detectable before a significant increase in sNfL is detectable. P values above graph are from paired t-tests within each age stratum. Dotted lines show 95% confidence intervals.

6.5.2 EBV NEGATIVE SAMPLES – NEUROFILAMENT LIGHT

Seven percent (n=39) of the samples in the pre-MS group with sNfL measurement available were EBV negative (anti-EBNA1 and anti-VCAp18 negative). The highest sNfL in this group was 13.9%.

To compare the EBV-negative group to the EBV-positive group in pre-MS, we can no longer utilize the case–control design of the original study and instead try to correct for this by adjusting for age, since sNfL is age dependent, and sample size, since the EBV-positive group is larger than the EBV-negative group.

After age adjustment, based on the distribution of sNfL in the control group, 8% of the samples in the EBV-positive pre-MS group had an sNfL higher than 13.9. The expected number of sNfL values higher than 13.9 if the groups had the same size, calculated from the distribution of the EBV-negative group, was 2%. In other words, a higher percentage of the EBV-positive group had an increased sNfL than expected, when compared to the EBV-negative pre-MS group ($p = 0.038$).

When comparing the EBV-negative group with controls, no increase in sNfL was seen. For the final 10-year period prior to MS onset, the group was small (n=7). The ratio for sNfL in this group compared to controls was 1.02 (95% CI 0.52–1.99, $p = 0.95$). For the large group of EBV-positive pre-MS samples, the corresponding ratio was 1.18 (95% CI 1.09–1.28, $p = 8 \cdot 10^{-5}$).

7 DISCUSSION

Contemporary clinical research provides evidence for a relationship between EBV infection and MS. Herein we investigate the EBV–MS connection, both from a prospective examination of individuals with previous IM and by a retrograde examination of samples acquired prior to MS onset.

7.1 A POSSIBLE PATHWAY FROM INFECTIOUS MONONUCLEOSIS TO MS OR AN MS ENDOPHENOTYPE

The rationale for Studies **II** and **III** is the well-established connection between IM and subsequent MS (53, 54) and the concept of an endophenotype (6) or pre-symptomatic trait that in some individuals precedes disease development. In epidemiological studies, the IM–MS relationship was determined at the group level; however, no individual follow-up studies have investigated whether IM, rather than an asymptomatic EBV primary infection, may continue as a latent inflammation into MS or an MS endophenotype. An MS endophenotype is expected as an intermediate stage, because the prevalence of IM is two orders of magnitude higher than that of MS (110). In selecting a reasonable parameter to show persistent immunological activity after IM, serum anti-EBV immunoreactivity is a crucial finding present in the pre-symptomatic phase of MS. However, it might represent immunity rather than a chronic inflammatory process. A sustained intrathecal increase in cyto- or chemokine levels in the post-IM samples would indicate inflammatory activity, which may have the potential to be compared with known characteristics of MS and endophenotypes. In Study **II**, we examined pre-determined inflammatory chemokines and cytokines in the CSF of individuals 10 years after IM. The study was initially powered to include 70 individuals with previous IM and 70 controls with no known IM. However, practical limitations prevented us from reaching this target, so we accepted the evaluation of a pilot study using 22 samples. We did not demonstrate any increase of intrathecally enriched oligoclonal IgG in the post-IM group, which would have been the strongest indication of an endophenotype. Cyto- and chemokine levels showed an increasing trend in the post-IM group for all seven cyto- and chemokines (CCL2 expected to show an inverse trend), and there was an overall, albeit non-significant, trend towards higher cyto-chemokine levels in the post-IM group. A renewed power analysis showed that, provided this positive trend remained, a significantly increased level of intrathecal cyto- and chemokines could be

reached with an extension of the post-IM and control material to 50 individuals in each group.

In Study **III**, we found indications that IM elicits an increased long-term serological response to the Epstein-Barr virus, here detectable by an increase in sera anti-EBVgp350, compared to individuals with less symptomatic primary infections. It has previously been shown that levels of anti-gp350 antibodies directly correlate with higher EBV DNA levels at IM (107). So, one possible interpretation of the persistently increased EBVgp350 reactivity is that a higher viral load at the time of primary infection (IM) leads to a persistently elevated serological response to gp350. However, we did not find any long-term alterations of the EBNA1 response, which is the main alteration in MS patients. One could speculate that a higher viral load at IM and this consistent serological response indicate a higher number of EBV-infected B cells in post-IM. Given the increased risk of MS after IM, this could in such a case be said to formulate a dose–response relationship for the IM–MS connection. Of note, a previous study with a long-term follow-up of the EBV serological response after natural infection showed no significant decrease over time (estimated half-life: 11,552 years), and the EBV humoral response is likely maintained for life (139). Therefore, the time from primary EBV infection to follow-up, which differs for the post-IM group and controls, should not influence our recorded antibody reactivity.

The anti-viral MRZ index is an established feature of MS CSF. Therefore, one research question in Study **III** was whether IM induces an increase in the serological response to measles Ncore and varicella gE antigens. It is well known that IM elicits a polyclonal antibody response (104), and a few previous studies have suggested that increased IgG antibodies occur against several viruses in IM (105). The increased anti-measles and varicella antibodies in the present study may be part of a poly-specific reaction that has intriguing similarities with the MRZ index. Rubella was not included in this study, since we opted for recombinant single-proteins antigens and lacked access to such a method for rubella. Furthermore, the heightened anti-measles reactivity in the IM group had declined at follow-up but remained at the limit of significance. The serum findings were not mirrored in the CSF, and no differences regarding antibody reactivity in the CSF were detected.

7.2 NO DEVIATION IN EARLY B-CELL LINEAGES FROM MS PATIENTS

As stated above, the bone marrow study was originally motivated by a search for paramyxo- or unknown virus in MS bone marrow (67), a project that, after influential reports supporting an EBV-MS connection, was later limited to the current flow cytometry study. In this pilot study, we found no evidence for deviations in early B cell lineage in MS patients. A borderline significant reduction of the NKT cells was found in the bone marrow of MS patients, as previously reported in blood and CSF (140). The study is small, and our results must be interpreted with caution. Of interest is that a reduction in NKT cells also has been reported as a risk factor for EBV infection (141).

7.3 EBV INFECTION PRECEDES THE FIRST RECOGNIZABLE SIGNS OF MS

7.3.1 NEUROAXONAL DAMAGE STARTS APPROXIMATELY 10 YEARS BEFORE CLINICAL ONSET

One previous study of sNfL in pre-symptomatic MS has been undertaken (86). It showed that in samples acquired a median of 6 years (range, 4–10 years) before MS onset, sNfL was increased, compared to matched controls. The present Study **IV** adds some information. Firstly, this study examines samples from up to 32 years before MS onset, enabling us to show the development of the risk of pre-symptomatic axonal injury (i.e., when some of the pre-MS individuals starts to develop increased neurofilament disintegration). Secondly, this is a larger sample with a more representative sex ratio.

We found that sNfL starts to increase approximately 10 years before the clinical onset of MS. This strongly argues that CNS axonal damage starts gradually in some individuals up to 10 years before MS onset, defined as the first noticeable neurological symptoms of the disease. The 10-year time span was not exact and many individuals in the pre-MS group had a normal sNfL.

Several studies have investigated a clinical MS prodrome existing at least 5 years before MS onset. This prodrome includes the increased use of healthcare instances in individuals who later develop MS, for several issues,

including fatigue, depression, sleep disorder and pain (74, 76, 142). In the present study, we do not have any further information on these pre-MS individuals at the time of sampling and therefore have no way of knowing if pre-MS individuals with relatively increased sNfL also experience nonspecific symptoms in line with such a prodrome. However, the period of increasing risk of axonal damage observed here encompasses the hitherto reported period of the MS prodrome.

The difference in sNfL concentration between pre-MS individuals and matched controls increased with decreasing time to clinical onset. For the final 10 years before onset, there was a significant association between the sNfL ratio (pre-MS/control) and time to onset but not to biological age. We interpret this to mean that the explanation for the increase in sNfL is that pathological demyelinating and secondary axon injuring process has begun in these individuals. A tenfold increase in sNfL was associated with an increased risk of MS (OR= 4.1). Since we wanted to capture all increases in sNfL, including those within the normal range, sNfL has been used as a numeric predictor in the logistic model. The reason we express it as tenfold is because we use $\log^{10}$ sNfL as predictor in the model and one unit increase thus corresponds to a tenfold increase.

While not the aim of the study, one interesting question is whether sNfL testing could be used for screening and predicting future MS. However, there is a substantial overlap here in sNfL concentrations between pre-symptomatic MS individuals and controls. In this nested case–control study, individuals in the pre-MS group are at different time spans from clinical MS, and many individuals in the pre-MS group have normal sNfL values. Including this group of individuals before the start of pre-clinical pathology will lower the sensitivity of the complete sample using sNfL for possible prediction of future MS. Evaluating prediction from this material, the sensitivity will be low, and the positive predictive value, calculated using Bayes formula, will be just above the prior risk. However, for a particular risk group such as individuals with RIS, the association to sNfL could be different and imply a possibility of screening, especially if an increase was detected in subsequent samples from the same individual.

sNfL seems to be a very robust marker, a previous study reported a correlation of r=0.99 between two analyses of the same sample 11 years apart (143). Samples used in the present studies were drawn for either diagnostic or screening purposes at different times and possibly stored in different ways (see methods). It is known that NfL may be increased in various infections (87), but this random factor did not obscure the present findings.

7.3.2 EBV SERO-REACTIVITY IN PRE-SYMPTOMATIC MS

In Study **V**, we show that EBV sero-reactivity is significantly increased in pre-symptomatic MS patients from 10–15 years before MS onset. The increase in EBNA1 reactivity between pre-MS and controls in our material starts at 15–20 years before onset and reaches significance in the following 5-year period. The difference between case-controls then stays at approximately the same level and does not show any added increase in individuals who are sampled closer to MS onset. Of the antibodies we studied here, anti-EBNA1 was dominant. Anti-EBVgp350 shows a similar pattern, although rising slightly later, while the pre-symptomatic increase in VCAp18 was marginal. This is consistent with other reports of increased anti-EBNA1, but not anti-VCA reactivity, in pre-symptomatic MS (109) and multiple sclerosis (144). Some studies have reported common epitopes between EBNA1 and proteins in the CNS in relatively small proportions of MS patients: recently, anoctamin 2 and glial cell adhesion molecule (GlialCAM) (16, 62). Distinct autoreactivity has, to the best of our knowledge, not been reported in the majority of MS patients.

EBNA1 is an intranuclear antigen and expressed during virus latency I (96), and EBVgp350 is an immune-dominant surface lytic antigen of the virus. In the present study, reactivity to both EBNA1 and gp350 antigens were increased in pre-MS compared to controls. Unexpectedly, we saw no correlation between this reactivity to these two antigens in EBV-positive pre-MS individuals. One previous study that examined antibodies to one epitope of gp350 (145) showed absent correlation between EBNA1 and gp350 antibody reactivity in manifest MS, we confirm that the non-correlation origins in the pre-symptomatic phase. EBVgp350 antibody response was increased in post-IM, where it may represent a higher viral load during primary infection or possibly a higher number of viral reactivations. We propose that the increased gp350 antibody in pre-symptomatic MS also may represent a higher viral load.

7.3.3 INCREASE IN EBV SERO-REACTIVITY OCCURS BEFORE NEUROFILAMENT INCREASE IN PRE-SYMPTOMATIC MS

Our findings show that the increase in EBNA1 and EBVgp350 sero-reactivity in pre-symptomatic MS is present before the increase in sNfL. This suggests EBV infection as a primary event in multiple sclerosis pathophysiology. It

does not, however, rule out a possible confounder for the relationship (e.g., activity of T cells that was reported to regulate EBV activity [NK, NKT and CD8+ cells]) (141).

7.3.4 INDIVIDUALS WITH HIGH NEUROFILAMENT ACCUMULATE IN THE EBV POSITIVE PRE-SYMPTOMATIC MS GROUP

We demonstrate that the samples in the pre-symptomatic MS group that have a relatively increased sNfL are concentrated to the EBV-positive group. If we were to find samples with increased sNfL in the EBV-negative pre-symptomatic group, this would strongly argue against EBV infection as a prerequisite for future MS. We contend that we did not. The fact that whether the pre-clinical sample is EBV positive or not influences the risk of axonal damage in pre-MS strengthens the argument that EBV infection is necessary for developing a large percentage of MS. However, it is not statistically possible from our material to say that pre-clinical disease has not begun in 100% of the EBV-negative group.

The EBV-negative pre-symptomatic group is small, especially for the final 10-year period previous to MS onset (n=7). This is to be expected, since one of the main arguments for an EBV infection as a risk factor for MS is that virtually 100% of MS patients are EBV positive (48). The EBV-negative group did not show any elevation of sNfL compared to matched controls.

We here confirm and expand results from a previous repository study with a large sample that had the advantage of longitudinal samples (57). They showed that a group of pre-symptomatic MS patients who were EBV negative a median of 10 years before MS onset sero-converted before sNfL increased in that group. The lack of longitudinal samples is a weakness of our study. Even though a late EBV infection (such as IM) is a risk factor for future MS (112), most MS patients experience an earlier primary infection. The delay from EBV-positive sample to MS onset varied by decades in our material.

7.3.5 NO CORRELATION BETWEEN SNFL AND EBV SEROLOGIES IN EBV POSITIVE SAMPLES

For EBV-positive samples in the pre-symptomatic MS group, we found no correlation between the amount of antibody reactivity and sNfL concentrations, neither in the whole material nor in the final five-year period before onset. Our view is that this observation is not compatible with lytic

EBV infection during the final 5-year period before MS onset, but could be consistent with a model wherein EBV acts as a trigger for subsequent disease. Alternatively, a chronic effect from the expression of non-coding RNA during EBV latency is another possibility (108).

8 CONCLUSION

The findings in this thesis, primarily the positive results from Studies **IV** and **V**, add to the substantial body of evidence linking EBV infection to MS pathogenesis. We show that neuroaxonal damage is detectable 10 years before the onset of clinical MS but is still preceded by an increase in levels of EBV serological response (EBNA1 and EBVgp350). We also see indications that the neuroaxonal damage has not started in the small group of EBV-negative samples acquired before MS. This strengthens the connection between a previous EBV infection and the start of neuroaxonal damage in pre-symptomatic MS.

The lack of correlation found here between EBV serologies and sNfL in pre-symptomatic MS does not support a lytic EBV infection but could be consistent with a model wherein EBV acts as a trigger for subsequent disease or an unexplored chronic effect during EBV latency.

We found an increased EBV serological response (Study **III**) and some indications of an inflammatory reaction in the CSF after IM (Study **II**). However, our efforts to find a post-IM state as an endophenotype for MS must await better-powered investigations.

9 FUTURE PERSPECTIVES

Possibilities to further study the EBV–MS connection include the following:

Studies of high-risk populations for acquiring future MS. As an example, one possibility would be to start with individuals with a combination of IM and genetic risks and perform MRI examinations of a large number of healthy individuals with the hypothesis of discovering a number of individuals with RIS. This kind of study would be very interesting to combine with sNfL assays to determine whether this could be used to screen for the early development of neuroaxonal damage and possibly even be used as an indication for treatment. New definitions of what constitutes early signs of MS and what is an early prodrome would be necessary.

Studies with historically collected biomaterial in biobanks. These studies of individuals that we know now have definite MS could be expanded to include several risk factors, preferably including longitudinal samples.

Anti-viral treatment of EBV in MS patients. Previous small studies of anti-viral treatment in MS have used Acyclovir and Valacyclovir (146-148), but none show statistically significant effects on disease activity, but still trends in subgroup analysis. Neither Acyclovir nor Valacyclovir affect latent EBV infection; in fact, there are no currently available drugs with an effect on latent EBV infection. One ongoing trial is evaluating the effect of T cell immunotherapy that targets EBV-infected B cells, and the published phase 1 trial using this method in 10 patients showed a possible effect with clinical improvement (149, 150). Although very small, our findings in Study **V**, with a lack of correlation between EBV antibodies and sNfL increase, seem to raise a small objection against this approach, with the caveat that EBV infection in latency 0 and 1 is not necessarily non-pathogenic.

This raises the question of whether an EBV vaccine could reduce MS incidence. Should prophylaxis turn out to be efficacious in preventing MS, it would in principle be proof of EBV involvement in MS pathogenesis. Stringent follow-up would be needed for several years at a sufficient scale (e.g., in national registers) to determine such preventive effects. However, no EBV vaccination is currently in clinical use. Gp350 has been utilized in vaccine trials, where small series have suggested a clinical effect limited to increased seroconversion to gp350 and the reduced risk of IM (151). Recently, the development of an mRNA vaccine based on the major EBV glycoproteins was announced (152).

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11 REFERENCES

1. Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: Insights from the Atlas of MS, third edition. Mult Scler. 2020;26(14):1816-21.

2. Zalc B. One hundred and fifty years ago Charcot reported multiple sclerosis as a new neurological disease. Brain. 2018;141(12):3482-8.

3. Murray TJ. The history of multiple sclerosis: the changing frame of the disease over the centuries. J Neurol Sci. 2009;277 Suppl 1:S3-8.

4. Kabat EA, Freedman DA, et al. A study of the crystalline albumin, gamma globulin and total protein in the cerebrospinal fluid of 100 cases of multiple sclerosis and in other diseases. Am J Med Sci. 1950;219(1):55-64.

5. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. I. Clinical results of a multicenter, randomized, double-blind, placebo-controlled trial. The IFNB Multiple Sclerosis Study Group. Neurology. 1993;43(4):655-61.

6. Ramagopalan SV, Dobson R, Meier UC, Giovannoni G. Multiple sclerosis: risk factors, prodromes, and potential causal pathways. Lancet Neurol. 2010;9(7):727-39.

7. Jons D, Sundstrom P, Andersen O. Targeting Epstein-Barr virus infection as an intervention against multiple sclerosis. Acta Neurol Scand. 2015;131(2):69-79.

8. Compston A, Coles A. Multiple sclerosis. Lancet. 2008;372(9648):1502-17.

9. Dobson R, Giovannoni G. Multiple sclerosis - a review. Eur J Neurol. 2019;26(1):27-40.

10. Kappos L, Wolinsky JS, Giovannoni G, Arnold DL, Wang Q, Bernasconi C, et al. Contribution of Relapse-Independent Progression vs Relapse-Associated Worsening to Overall Confirmed Disability Accumulation in Typical Relapsing Multiple Sclerosis in a Pooled Analysis of 2 Randomized Clinical Trials. JAMA Neurol. 2020;77(9):1132-40.

11. Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology. 1996;46(4):907-11.

12. Okuda DT, Mowry EM, Beheshtian A, Waubant E, Baranzini SE, Goodin DS, et al. Incidental MRI anomalies suggestive of multiple sclerosis: the radiologically isolated syndrome. Neurology. 2009;72(9):800-5.

13. Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann Neurol. 1983;13(3):227-31.

14. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. 2018;17(2):162-73.

15. Wootla B, Eriguchi M, Rodriguez M. Is multiple sclerosis an autoimmune disease? Autoimmune Dis. 2012;2012:969657.

16. Lanz TV, Brewer RC, Ho PP, Moon JS, Jude KM, Fernandez D, et al. Clonally expanded B cells in multiple sclerosis bind EBV EBNA1 and GlialCAM. Nature. 2022;603(7900):321-7.

17. Filippi M, Bar-Or A, Piehl F, Preziosa P, Solari A, Vukusic S, et al. Multiple sclerosis. Nat Rev Dis Primers. 2018;4(1):43.

18. Broman T. Blood-Brain Barrier Damage in Multiple Sclerosis Supravital Test-Observations. Acta Neurol Scand Suppl. 1964;40:SUPPL 10:21-4.

19. Frischer JM, Weigand SD, Guo Y, Kale N, Parisi JE, Pirko I, et al. Clinical and pathological insights into the dynamic nature of the white matter multiple sclerosis plaque. Ann Neurol. 2015;78(5):710-21.

20. Klaver R, De Vries HE, Schenk GJ, Geurts JJ. Grey matter damage in multiple sclerosis: a pathology perspective. Prion. 2013;7(1):66-75.

21. Magliozzi R, Howell O, Vora A, Serafini B, Nicholas R, Puopolo M, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. Brain. 2007;130(Pt 4):1089-104.

22. Frischer JM, Bramow S, Dal-Bianco A, Lucchinetti CF, Rauschka H, Schmidbauer M, et al. The relation between inflammation and neurodegeneration in multiple sclerosis brains. Brain. 2009;132(Pt 5):1175-89.

23. Reiber H, Ungefehr S, Jacobi C. The intrathecal, polyspecific and oligoclonal immune response in multiple sclerosis. Mult Scler. 1998;4(3):111-7.

24. Jarius S, Eichhorn P, Franciotta D, Petereit HF, Akman-Demir G, Wick M, et al. The MRZ reaction as a highly specific marker of multiple sclerosis: re-evaluation and structured review of the literature. J Neurol. 2017;264(3):453-66.

25. Hottenrott T, Dersch R, Berger B, Rauer S, Huzly D, Stich O. The MRZ reaction in primary progressive multiple sclerosis. Fluids Barriers CNS. 2017;14(1):2.

26. Ruprecht K, Wildemann B, Jarius S. Low intrathecal antibody production despite high seroprevalence of Epstein-Barr virus in multiple sclerosis: a review of the literature. J Neurol. 2018;265(2):239-52.

27. Li R, Patterson KR, Bar-Or A. Reassessing B cell contributions in multiple sclerosis. Nat Immunol. 2018;19(7):696-707.

28. Cencioni MT, Mattoscio M, Magliozzi R, Bar-Or A, Muraro PA. B cells in multiple sclerosis - from targeted depletion to immune reconstitution therapies. Nat Rev Neurol. 2021;17(7):399-414.

29. Ahlgren C, Oden A, Lycke J. High nationwide incidence of multiple sclerosis in Sweden. PLoS One. 2014;9(9):e108599.

30. Koch-Henriksen N, Sorensen PS. The changing demographic pattern of multiple sclerosis epidemiology. Lancet Neurol. 2010;9(5):520-32.

31. Kurtzke JF. Geographic distribution of multiple sclerosis: An update with special reference to Europe and the Mediterranean region. Acta Neurol Scand. 1980;62(2):65-80.

32. Simpson S, Jr., Wang W, Otahal P, Blizzard L, van der Mei IAF, Taylor BV. Latitude continues to be significantly associated with the prevalence of multiple sclerosis: an updated meta-analysis. J Neurol Neurosurg Psychiatry. 2019;90(11):1193-200.

33. Dean G. Annual incidence, prevalence, and mortality of multiple sclerosis in white South-African-born and in white immigrants to South Africa. Br Med J. 1967;2(5554):724-30.

34. Gale CR, Martyn CN. Migrant studies in multiple sclerosis. Prog Neurobiol. 1995;47(4-5):425-48.

35. Bager P, Nielsen NM, Bihrmann K, Frisch M, Hjalgrim H, Wohlfart J, et al. Childhood infections and risk of multiple sclerosis. Brain. 2004;127(Pt 11):2491-7.

36. Sadovnick AD, Baird PA. The familial nature of multiple sclerosis: age-corrected empiric recurrence risks for children and siblings of patients. Neurology. 1988;38(6):990-1.

37. Willer CJ, Dyment DA, Risch NJ, Sadovnick AD, Ebers GC, Canadian Collaborative Study G. Twin concordance and sibling recurrence rates in multiple sclerosis. Proc Natl Acad Sci U S A. 2003;100(22):12877-82.

38. Fagnani C, Neale MC, Nistico L, Stazi MA, Ricigliano VA, Buscarinu MC, et al. Twin studies in multiple sclerosis: A meta-estimation of heritability and environmentality. Mult Scler. 2015;21(11):1404-13.

39. Westerlind H, Ramanujam R, Uvehag D, Kuja-Halkola R, Boman M, Bottai M, et al. Modest familial risks for multiple sclerosis: a registry-based study of the population of Sweden. Brain. 2014;137(Pt 3):770-8.

40. Parnell GP, Booth DR. The Multiple Sclerosis (MS) Genetic Risk Factors Indicate both Acquired and Innate Immune Cell Subsets Contribute to MS Pathogenesis and Identify Novel Therapeutic Opportunities. Front Immunol. 2017;8:425.

41. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: A comprehensive review. J Autoimmun. 2015;64:13-25.

42. Afrasiabi A, Parnell GP, Fewings N, Schibeci SD, Basuki MA, Chandramohan R, et al. Evidence from genome wide association studies implicates reduced control of Epstein-Barr virus infection in multiple sclerosis susceptibility. Genome Med. 2019;11(1):26.

43. Olsson T, Barcellos LF, Alfredsson L. Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. Nat Rev Neurol. 2017;13(1):25-36.

44. Nielsen TR, Rostgaard K, Nielsen NM, Koch-Henriksen N, Haahr S, Sorensen PS, et al. Multiple sclerosis after infectious mononucleosis. Arch Neurol. 2007;64(1):72-5.

45. Sundqvist E, Sundstrom P, Linden M, Hedstrom AK, Aloisi F, Hillert J, et al. Epstein-Barr virus and multiple sclerosis: interaction with HLA. Genes Immun. 2012;13(1):14-20.

46. Virtanen JO, Jacobson S. Viruses and multiple sclerosis. CNS Neurol Disord Drug Targets. 2012;11(5):528-44.

47. Ahlgren C, Oden A, Toren K, Andersen O. Multiple sclerosis incidence in the era of measles-mumps-rubella mass vaccinations. Acta Neurol Scand. 2009;119(5):313-20.

48. Abrahamyan S, Eberspacher B, Hoshi MM, Aly L, Luessi F, Groppa S, et al. Complete Epstein-Barr virus seropositivity in a large cohort of patients with early multiple sclerosis. J Neurol Neurosurg Psychiatry. 2020;91(7):681-6.

49. Dobson R, Kuhle J, Middeldorp J, Giovannoni G. Epstein-Barr-negative MS: a true phenomenon? Neurol Neuroimmunol Neuroinflamm. 2017;4(2):e318.

50. Sundstrom P, Juto P, Wadell G, Hallmans G, Svenningsson A, Nystrom L, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology. 2004;62(12):2277-82.

51. Ascherio A, Munger KL, Lennette ET, Spiegelman D, Hernan MA, Olek MJ, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. JAMA. 2001;286(24):3083-8.

52. Levin LI, Munger KL, O'Reilly EJ, Falk KI, Ascherio A. Primary infection with the Epstein-Barr virus and risk of multiple sclerosis. Ann Neurol. 2010;67(6):824-30.

53. Handel AE, Williamson AJ, Disanto G, Handunnetthi L, Giovannoni G, Ramagopalan SV. An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. PLoS One. 2010;5(9).

54. Lindberg C, Andersen O, Vahlne A, Dalton M, Runmarker B. Epidemiological investigation of the association between infectious mononucleosis and multiple sclerosis. Neuroepidemiology. 1991;10(2):62-5.

55. Ramagopalan SV, Hoang U, Seagroatt V, Handel A, Ebers GC, Giovannoni G, et al. Geography of hospital admissions for multiple sclerosis in England and comparison with the geography of hospital admissions for infectious mononucleosis: a descriptive study. J Neurol Neurosurg Psychiatry. 2011;82(6):682-7.

56. Warner HB, Carp RI. Multiple sclerosis and Epstein-Barr virus. Lancet. 1981;2(8258):1290.

57. Bjornevik K, Cortese M, Healy BC, Kuhle J, Mina MJ, Leng Y, et al. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. Science. 2022;375(6578):296-301.

58. Aloisi F, Serafini B, Magliozzi R, Howell OW, Reynolds R. Detection of Epstein-Barr virus and B-cell follicles in the multiple sclerosis brain: what you find depends on how and where you look. Brain. 2010;133(Pt 12):e157.

59. Sargsyan SA, Shearer AJ, Ritchie AM, Burgoon MP, Anderson S, Hemmer B, et al. Absence of Epstein-Barr virus in the brain and CSF of patients with multiple sclerosis. Neurology. 2010;74(14):1127-35.

60. Willis SN, Stadelmann C, Rodig SJ, Caron T, Gattenloehner S, Mallozzi SS, et al. Epstein-Barr virus infection is not a characteristic feature of multiple sclerosis brain. Brain. 2009;132(Pt 12):3318-28.

61. Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. J Exp Med. 2007;204(12):2899-912.

62. Tengvall K, Huang J, Hellstrom C, Kammer P, Bistrom M, Ayoglu B, et al. Molecular mimicry between Anoctamin 2 and Epstein-Barr virus nuclear antigen 1 associates with multiple sclerosis risk. Proc Natl Acad Sci U S A. 2019;116(34):16955-60.

63. Pender MP. Infection of autoreactive B lymphocytes with EBV, causing chronic autoimmune diseases. Trends Immunol. 2003;24(11):584-8.

64. Laurence M, Benito-Leon J. Epstein-Barr virus and multiple sclerosis: Updating Pender's hypothesis. Mult Scler Relat Disord. 2017;16:8-14.

65. Serafini B, Rosicarelli B, Veroni C, Mazzola GA, Aloisi F. Epstein-Barr Virus-Specific CD8 T Cells Selectively Infiltrate the Brain in Multiple Sclerosis and Interact Locally with Virus-Infected Cells: Clue for a Virus-Driven Immunopathological Mechanism. J Virol. 2019;93(24).

66. Miyauchi E, Shimokawa C, Steimle A, Desai MS, Ohno H. The impact of the gut microbiome on extra-intestinal autoimmune diseases. Nat Rev Immunol. 2022.

67. Goswami KK, Cameron KR, Russell WC, Lange LS, Mitchell DN. Evidence for the persistence of paramyxoviruses in human bone marrows. J Gen Virol. 1984;65 (Pt 11):1881-8.

68. Mitchell DN, Porterfield JS, Micheletti R, Lange LS, Goswami KK, Taylor P, et al. Isolation of an infectious agent from bone-marrows of patients with multiple sclerosis. Lancet. 1978;2(8086):387-91.

69. Carrai V, Donnini I, Mazzanti B, Alterini R, Amato MP, Barilaro A, et al. Immunohistochemistry analysis of bone marrow biopsies in multiple sclerosis patients undergoing autologous haematopoietic stem cells transplantation. Clin Neurol Neurosurg. 2013;115(7):1044-8.

70. Shi K, Li H, Chang T, He W, Kong Y, Qi C, et al. Bone marrow hematopoiesis drives multiple sclerosis progression. Cell. 2022;185(13):2234-47 e17.

71. Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. Nat Rev Microbiol. 2007;5(6):405-17.

72. O'Keeffe J, Podbielska M, Hogan EL. Invariant natural killer T cells and their ligands: focus on multiple sclerosis. Immunology. 2015;145(4):468-75.

73. Chung BK, Tsai K, Allan LL, Zheng DJ, Nie JC, Biggs CM, et al. Innate immune control of EBV-infected B cells by invariant natural killer T cells. Blood. 2013;122(15):2600-8.

74. Tremlett H, Marrie RA. The multiple sclerosis prodrome: Emerging evidence, challenges, and opportunities. Mult Scler. 2021;27(1):6-12.

75. Makhani N, Tremlett H. The multiple sclerosis prodrome. Nat Rev Neurol. 2021;17(8):515-21.

76. Yusuf F, Wijnands JM, Kingwell E, Zhu F, Evans C, Fisk JD, et al. Fatigue, sleep disorders, anaemia and pain in the multiple sclerosis prodrome. Mult Scler. 2021;27(2):290-302.

77. Haghighi S, Andersen O, Rosengren L, Bergstrom T, Wahlstrom J, Nilsson S. Incidence of CSF abnormalities in siblings of multiple sclerosis patients and unrelated controls. J Neurol. 2000;247(8):616-22.

78. De Stefano N, Cocco E, Lai M, Battaglini M, Spissu A, Marchi P, et al. Imaging brain damage in first-degree relatives of sporadic and familial multiple sclerosis. Ann Neurol. 2006;59(4):634-9.

79. Xia Z, Steele SU, Bakshi A, Clarkson SR, White CC, Schindler MK, et al. Assessment of Early Evidence of Multiple Sclerosis in a Prospective Study of Asymptomatic High-Risk Family Members. JAMA Neurol. 2017;74(3):293-300.

80. Draborg AH, Duus K, Houen G. Epstein-Barr virus in systemic autoimmune diseases. Clin Dev Immunol. 2013;2013:535738.

81. Lebrun C. The radiologically isolated syndrome. Rev Neurol (Paris). 2015;171(10):698-706.

82. Lebrun-Frenay C, Kantarci O, Siva A, Sormani MP, Pelletier D, Okuda DT, et al. Radiologically Isolated Syndrome: 10-Year Risk Estimate of a Clinical Event. Ann Neurol. 2020;88(2):407-17.

83. Matute-Blanch C, Villar LM, Alvarez-Cermeno JC, Rejdak K, Evdoshenko E, Makshakov G, et al. Neurofilament light chain and oligoclonal bands are prognostic biomarkers in radiologically isolated syndrome. Brain. 2018;141(4):1085-93.

84. Rossi S, Motta C, Studer V, Macchiarulo G, Germani G, Finardi A, et al. Subclinical central inflammation is risk for RIS and CIS conversion to MS. Mult Scler. 2015;21(11):1443-52.

85. Pawlitzki M, Sweeney-Reed CM, Bittner D, Lux A, Vielhaber S, Schreiber S, et al. CSF-Progranulin and Neurofilament Light Chain Levels in Patients With Radiologically Isolated Syndrome-Sign of Inflammation. Front Neurol. 2018;9:1075.

86. Bjornevik K, Munger KL, Cortese M, Barro C, Healy BC, Niebuhr DW, et al. Serum Neurofilament Light Chain Levels in Patients With Presymptomatic Multiple Sclerosis. JAMA Neurol. 2020;77(1):58-64.

87. Gaetani L, Blennow K, Calabresi P, Di Filippo M, Parnetti L, Zetterberg H. Neurofilament light chain as a biomarker in neurological disorders. J Neurol Neurosurg Psychiatry. 2019;90(8):870-81.

88. Disanto G, Barro C, Benkert P, Naegelin Y, Schadelin S, Giardiello A, et al. Serum Neurofilament light: A biomarker of neuronal damage in multiple sclerosis. Ann Neurol. 2017;81(6):857-70.

89. Jakimovski D, Kuhle J, Ramanathan M, Barro C, Tomic D, Hagemeier J, et al. Serum neurofilament light chain levels associations with gray matter pathology: a 5-year longitudinal study. Ann Clin Transl Neurol. 2019;6(9):1757-70.

90. Kuhle J, Plavina T, Barro C, Disanto G, Sangurdekar D, Singh CM, et al. Neurofilament light levels are associated with long-term outcomes in multiple sclerosis. Mult Scler. 2020;26(13):1691-9.

91. Epstein MA, Achong BG, Barr YM. Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. Lancet. 1964;1(7335):702-3.

92. Amon W, Farrell PJ. Reactivation of Epstein-Barr virus from latency. Rev Med Virol. 2005;15(3):149-56.

93. Jensen TZT, Niemann J, Iversen KH, Fotakis AK, Gopalakrishnan S, Vagene AJ, et al. A 5700 year-old human genome and oral microbiome from chewed birch pitch. Nat Commun. 2019;10(1):5520.

94. Ehlers B, Spiess K, Leendertz F, Peeters M, Boesch C, Gatherer D, et al. Lymphocryptovirus phylogeny and the origins of Epstein-Barr virus. J Gen Virol. 2010;91(Pt 3):630-42.

95. Münz C. Epstein barr virus volume 1: One herpes virus: Many diseases: Springer; 2015.

96. Thorley-Lawson DA. EBV Persistence--Introducing the Virus. Curr Top Microbiol Immunol. 2015;390(Pt 1):151-209.

97. Hjalgrim H, Friborg J, Melbye M. The epidemiology of EBV and its association with malignant disease. In: Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, Whitley R, et al., editors. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge2007.

98. Luzuriaga K, Sullivan JL. Infectious mononucleosis. N Engl J Med. 2010;362(21):1993-2000.

99. Crawford DH, Macsween KF, Higgins CD, Thomas R, McAulay K, Williams H, et al. A cohort study among university students: identification of risk factors for Epstein-Barr virus seroconversion and infectious mononucleosis. Clin Infect Dis. 2006;43(3):276-82.

100. Niederman JC, Evans AS, Subrahmanyan L, McCollum RW. Prevalence, incidence and persistence of EB virus antibody in young adults. N Engl J Med. 1970;282(7):361-5.

101. Axelrod P, Finestone AJ. Infectious mononucleosis in older adults. Am Fam Physician. 1990;42(6):1599-606.

102. Hochberg D, Souza T, Catalina M, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Acute infection with Epstein-Barr virus targets and overwhelms the peripheral memory B-cell compartment with resting, latently infected cells. J Virol. 2004;78(10):5194-204.

103. Balfour HH, Jr., Holman CJ, Hokanson KM, Lelonek MM, Giesbrecht JE, White DR, et al. A prospective clinical study of Epstein-Barr virus and host interactions during acute infectious mononucleosis. J Infect Dis. 2005;192(9):1505-12.

104. Golaszewska E, Kurowska E, Duk M, Koscielak J. Paul-Bunnell antigen and a possible mechanism of formation of heterophile antibodies in patients with infectious mononucleosis. Acta Biochim Pol. 2003;50(4):1205-11.

105. Haukenes G, Viggen B, Boye B, Kalvenes MB, Flo R, Kalland KH. Viral antibodies in infectious mononucleosis. FEMS Immunol Med Microbiol. 1994;8(3):219-24.

106. De Paschale M, Clerici P. Serological diagnosis of Epstein-Barr virus infection: Problems and solutions. World J Virol. 2012;1(1):31-43.

107. Weiss ER, Alter G, Ogembo JG, Henderson JL, Tabak B, Bakis Y, et al. High Epstein-Barr Virus Load and Genomic Diversity Are Associated with Generation of gp350-Specific Neutralizing Antibodies following Acute Infectious Mononucleosis. J Virol. 2017;91(1).

108. Münz C. Epstein barr virus volume 2: One herpes virus: Many diseases: Springer; 2015.

109. Levin LI, Munger KL, Rubertone MV, Peck CA, Lennette ET, Spiegelman D, et al. Temporal relationship between elevation of epstein-barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA. 2005;293(20):2496-500.

110. Rostgaard K, Balfour HH, Jr., Jarrett R, Erikstrup C, Pedersen O, Ullum H, et al. Primary Epstein-Barr virus infection with and without infectious mononucleosis. PLoS One. 2019;14(12):e0226436.

111. Bistrom M, Alonso-Magdalena L, Andersen O, Jons D, Gunnarsson M, Vrethem M, et al. High serum concentration of vitamin D may protect against multiple sclerosis. Mult Scler J Exp Transl Clin. 2019;5(4):2055217319892291.

112. Bistrom M, Jons D, Engdahl E, Gustafsson R, Huang J, Brenner N, et al. Epstein-Barr virus infection after adolescence and human herpesvirus 6A as risk factors for multiple sclerosis. Eur J Neurol. 2021;28(2):579-86.

113. Engdahl E, Gustafsson R, Huang J, Bistrom M, Lima Bomfim I, Stridh P, et al. Increased Serological Response Against Human Herpesvirus 6A Is Associated With Risk for Multiple Sclerosis. Front Immunol. 2019;10:2715.

114. Hillert J, Stawiarz L. The Swedish MS registry - clinical support tool and scientific resource. Acta Neurol Scand. 2015;132(199):11-9.

115. Biström M. Environmental risk factors for the occurrence of multiple sclerosis: Umeå university; 2020.

116. Liao X, Makris M, Luo XM. Fluorescence-activated Cell Sorting for Purification of Plasmacytoid Dendritic Cells from the Mouse Bone Marrow. J Vis Exp. 2016(117).

117. Chantepie SP, Cornet E, Salaun V, Reman O. Hematogones: an overview. Leuk Res. 2013;37(11):1404-11.

118. Persson Berg L, Thomsson E, Hasi G, Backstrom M, Bergstrom T. Recombinant Epstein-Barr virus glycoprotein 350 as a serological antigen. J Virol Methods. 2020;284:113927.

119. Persson L, Longhi S, Enarsson J, Andersen O, Haghigi S, Nilsson S, et al. Elevated antibody reactivity to measles virus NCORE protein among patients with multiple sclerosis and their healthy siblings with intrathecal oligoclonal immunoglobulin G production. J Clin Virol. 2014;61(1):107-12.

120. Thomsson E, Persson L, Grahn A, Snall J, Ekblad M, Brunhage E, et al. Recombinant glycoprotein E produced in mammalian cells in large-scale as an antigen for varicella-zoster-virus serology. J Virol Methods. 2011;175(1):53-9.

121. Measles virus nomenclature update: 2012. Wkly Epidemiol Rec. 2012;87(9):73-81.

122. Brenner N, Mentzer AJ, Butt J, Michel A, Prager K, Brozy J, et al. Validation of Multiplex Serology detecting human herpesviruses 1-5. PLoS One. 2018;13(12):e0209379.

123. Malmestrom C, Andersson BA, Haghighi S, Lycke J. IL-6 and CCL2 levels in CSF are associated with the clinical course of MS: implications for their possible immunopathogenic roles. J Neuroimmunol. 2006;175(1-2):176-82.

124. Bonneh-Barkay D, Wang G, Starkey A, Hamilton RL, Wiley CA. In vivo CHI3L1 (YKL-40) expression in astrocytes in acute and chronic neurological diseases. J Neuroinflammation. 2010;7:34.

125. Comabella M, Fernandez M, Martin R, Rivera-Vallve S, Borras E, Chiva C, et al. Cerebrospinal fluid chitinase 3-like 1 levels are associated with conversion to multiple sclerosis. Brain. 2010;133(Pt 4):1082-93.

126. Canto E, Tintore M, Villar LM, Costa C, Nurtdinov R, Alvarez-Cermeno JC, et al. Chitinase 3-like 1: prognostic biomarker in clinically isolated syndromes. Brain. 2015;138(Pt 4):918-31.

127. Scarpini E, Galimberti D, Baron P, Clerici R, Ronzoni M, Conti G, et al. IP-10 and MCP-1 levels in CSF and serum from multiple sclerosis patients with different clinical subtypes of the disease. J Neurol Sci. 2002;195(1):41-6.

128. Hakansson I, Tisell A, Cassel P, Blennow K, Zetterberg H, Lundberg P, et al. Neurofilament levels, disease activity and brain volume during follow-up in multiple sclerosis. J Neuroinflammation. 2018;15(1):209.

129. Hu WT, Howell JC, Ozturk T, Gangishetti U, Kollhoff AL, Hatcher-Martin JM, et al. CSF Cytokines in Aging, Multiple Sclerosis, and Dementia. Front Immunol. 2019;10:480.

130. Novakova L, Axelsson M, Khademi M, Zetterberg H, Blennow K, Malmestrom C, et al. Cerebrospinal fluid biomarkers as a measure of disease activity and treatment efficacy in relapsing-remitting multiple sclerosis. J Neurochem. 2017;141(2):296-304.

131. Gaudreault E, Fiola S, Olivier M, Gosselin J. Epstein-Barr virus induces MCP-1 secretion by human monocytes via TLR2. J Virol. 2007;81(15):8016-24.

132. Stein J, Xu Q, Jackson KC, Romm E, Wuest SC, Kosa P, et al. Intrathecal B Cells in MS Have Significantly Greater Lymphangiogenic Potential Compared to B Cells Derived From Non-MS Subjects. Front Neurol. 2018;9:554.

133. Ribeiro CM, Oliveira SR, Alfieri DF, Flauzino T, Kaimen-Maciel DR, Simao ANC, et al. Tumor necrosis factor alpha (TNF-alpha) and its soluble receptors are associated with disability, disability progression and clinical forms of multiple sclerosis. Inflamm Res. 2019;68(12):1049-59.

134. Rossi S, Motta C, Studer V, Macchiarulo G, Volpe E, Barbieri F, et al. Interleukin-1beta causes excitotoxic neurodegeneration and multiple sclerosis disease progression by activating the apoptotic protein p53. Mol Neurodegener. 2014;9:56.

135. Sisay S, Lopez-Lozano L, Mickunas M, Quiroga-Fernandez A, Palace J, Warnes G, et al. Untreated relapsing remitting multiple sclerosis

patients show antibody production against latent Epstein Barr Virus (EBV) antigens mainly in the periphery and innate immune IL-8 responses preferentially in the CNS. J Neuroimmunol. 2017;306:40-5.

136. Gaetani L, Hoglund K, Parnetti L, Pujol-Calderon F, Becker B, Eusebi P, et al. A new enzyme-linked immunosorbent assay for neurofilament light in cerebrospinal fluid: analytical validation and clinical evaluation. Alzheimers Res Ther. 2018;10(1):8.

137. Rissin DM, Kan CW, Campbell TG, Howes SC, Fournier DR, Song L, et al. Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations. Nat Biotechnol. 2010;28(6):595-9.

138. Thulin M. Two-sample tests and one-way MANOVA for multivariate biomarker data with nondetects. Stat Med. 2016;35(20):3623-44.

139. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med. 2007;357(19):1903-15.

140. Svenningsson A, Andersen O, Hansson GK, Stemme S. Reduced frequency of memory CD8+ T lymphocytes in cerebrospinal fluid and blood of patients with multiple sclerosis. Autoimmunity. 1995;21(4):231-9.

141. Munz C. Co-Stimulatory Molecules during Immune Control of Epstein Barr Virus Infection. Biomolecules. 2021;12(1).

142. Cortese M, Riise T, Bjornevik K, Bhan A, Farbu E, Grytten N, et al. Preclinical disease activity in multiple sclerosis: A prospective study of cognitive performance prior to first symptom. Ann Neurol. 2016;80(4):616-24.

143. Axelsson M, Sjogren M, Andersen O, Blennow K, Zetterberg H, Lycke J. Neurofilament light protein levels in cerebrospinal fluid predict long-term disability of Guillain-Barre syndrome: A pilot study. Acta Neurol Scand. 2018;138(2):143-50.

144. Ruprecht K, Wunderlich B, Giess R, Meyer P, Loebel M, Lenz K, et al. Multiple sclerosis: the elevated antibody response to Epstein-Barr virus primarily targets, but is not confined to, the glycine-alanine repeat of Epstein-Barr nuclear antigen-1. J Neuroimmunol. 2014;272(1-2):56-61.

145. Lindsey JW, Hatfield LM, Vu T. Epstein-Barr virus neutralizing and early antigen antibodies in multiple sclerosis. Eur J Neurol. 2010;17(10):1263-9.

146. Bech E, Lycke J, Gadeberg P, Hansen HJ, Malmestrom C, Andersen O, et al. A randomized, double-blind, placebo-controlled MRI study of anti-herpes virus therapy in MS. Neurology. 2002;58(1):31-6.

147. Lycke J, Svennerholm B, Hjelmquist E, Frisen L, Badr G, Andersson M, et al. Acyclovir treatment of relapsing-remitting multiple sclerosis. A randomized, placebo-controlled, double-blind study. J Neurol. 1996;243(3):214-24.

148. Friedman JE, Zabriskie JB, Plank C, Ablashi D, Whitman J, Shahan B, et al. A randomized clinical trial of valacyclovir in multiple sclerosis. Mult Scler. 2005;11(3):286-95.

149. Ioannides ZA, Csurhes PA, Douglas NL, Mackenroth G, Swayne A, Thompson KM, et al. Sustained Clinical Improvement in a Subset of Patients With Progressive Multiple Sclerosis Treated With Epstein-Barr Virus-Specific T Cell Therapy. Front Neurol. 2021;12:652811.

150. Pender MP, Csurhes PA, Smith C, Douglas NL, Neller MA, Matthews KK, et al. Epstein-Barr virus-specific T cell therapy for progressive multiple sclerosis. JCI Insight. 2020;5(20).

151. Sokal EM, Hoppenbrouwers K, Vandermeulen C, Moutschen M, Leonard P, Moreels A, et al. Recombinant gp350 vaccine for infectious mononucleosis: a phase 2, randomized, double-blind, placebo-controlled trial to evaluate the safety, immunogenicity, and efficacy of an Epstein-Barr virus vaccine in healthy young adults. J Infect Dis. 2007;196(12):1749-53.

152. Sun C, Chen XC, Kang YF, Zeng MS. The Status and Prospects of Epstein-Barr Virus Prophylactic Vaccine Development. Front Immunol. 2021;12:677027.

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12 APPENDIX

Survey filled out by study participants for study **II** and **III**

Forskningsprojekt kring körtelfeber och inflammatorisk sjukdom

(enkät och blodprov)

Studie ID

Frågeformulär

Har Du någon sjukdom? Ja Nej

Om ja, vilka?____

Tar Du några mediciner? Ja Nej

Om ja, vilka?____

Har Du haft körtelfeber (mononukleos)? Ja Nej Vet ej

Finns någon av följande sjukdomar i Din släkt?

multipel skleros Ja Nej

Om ja, ange släktskap:________________________________

ledgångsreumatism Ja Nej

Om ja, ange släktskap:________________________________

SLE Ja Nej

Om ja, ange släktskap:________________________________

Röker Du eller har Du tidigare rökt? Ja Nej

Om ja, mellan vilka år har Du rökt? __________________

Vad har Du rökt/röker Du? Cigaretter Pipa

Hur många cigaretter/paket tobak har Du i genomsnitt rökt/dag? __________

Snusar Du eller har Du tidigare snusat? Ja Nej

Om ja, mellan vilka år har Du snusat? __________________

Hur många snusdosor har Du i genomsnitt förbrukat/vecka? __________