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Multiple Sclerosis and the impact of Epstein-Barr virus and other Neurotrophic Viruses

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Preface

This Master Thesis project and the work presented herein were performed at the department of Autoimmunology at Statens Serum Institut (SSI). The 60 ECTS project was initiated in February 2017 with the aim of obtaining a Master of Science (MSc) degree in Medical Biology and Molecular Biology from Roskilde University.

The external supervisor was Professor Gunnar Houen, from the department of Autoimmunology and Biomarkers at SSI, and the internal supervisor on the project was Jesper Troelsen, professor at Department of Science and Environment at Roskilde University.

Firstly, I would like to thank Professor Gunnar Houen for the opportunity to work with this project and for great guidance during the process. A special thanks to Professor Jette Frederiksen, Glostrup Hospital for her engagement during this project, and for providing me with patient samples.

Furthermore, I would also like to thank everyone who have been involved is this project, and all the employees and the other students at the Department of Autoimmunology and Biomarkers at SSI for great discussions throughout the entire project and for sharing their knowledge with me whenever I have a question. It has been a pleasure to come to work every day and I am very grateful to have been given this opportunity to do my thesis at SSI.

Finally, I would like to thank my family and friends for their support and encouragement during this process.

I hope you will enjoy reading this thesis.

Julie Heiden Nielsen

Copenhagen, January 2018

Abstract

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease causing degeneration of the myelin sheaths of the central nervous system. The etiology of MS is still unknown, but a combination of a genetic predisposition and environmental triggers as Epstein-Barr virus (EBV) is the most reasonable theory. EBV is the causative agent of infectious mononucleosis (IM), and the virus infects primarily B cells. Previous studies have revealed a connection between human herpes viruses (HHVs), especially EBV, and MS, and many research groups have also investigated the impact of different neurotropic viruses like measles, mumps and rubella. The most important tools today in the diagnosis of MS are MRI scans and the investigation of intrathecal production of antibodies, by the presence of oligoclonal bands (OCBs) and an elevated IgG-index.

In this project, the immune response towards EBV, other HHVs and the neurotropic viruses measles, mumps and rubella are conducted by ELISA technique in serum and CSF samples from MS patients, compared to patients diagnosed with optic neuritis (ON) which can be a precursor of MS, and various control groups. An antigen panel called the EZMMR panel, consisting of EBV (EBNA-1), VZV, measles, mumps and rubella virus antigens was invented, and the results showed a significant elevated level of serum and CSF IgG antibodies directed against EBNA-1, VZV, measles and mumps in relapsing-remitting MS (RRMS) patients, but no difference regarding rubella antibodies. The specific antibody index (AI) was calculated, to determine if the specific antibodies were intrathecal synthesized. The sensitivity of the EZMMR panel regarding RRMS patients was approximately 50% if three positive AIs were required, and approximately 85% if only two positive AIs were required. Compared with the MRZ panel where only 50% of the RRMS patients had two positive AIs, the EZMMR panel revealed improved sensitivity. Unfortunately, it was not possible to determine the specificity.

In conclusion, the results obtained in this study support the theory of the impact of viruses according to MS etiology, and especially EBV. It is still unknown if the viruses are the primary pathogenic agents, and how they are related according to development of MS, or whether the increased prevalence of antibodies just is a consequence of the disease.

Resumé (Danish summary)

Multipel sclerose (MS) er en kronisk inflammatorisk autoimmun sygdom, der forårsager degenerering af myelinskederne i centralnervesystemet. MS ætiologien er stadig ukendt, men en kombination af genetisk prædisposition og miljømæssige faktorer som Epstein-Barr-virus (EBV) er den mest fornuftige teori. EBV er den forårsagende virus til infektiøs mononukleose (IM), og EBV inficerer primært B-celler. Tidligere undersøgelser har afsløret forbindelse mellem humane herpesvirus (HHV), i særdeleshed EBV, og MS. Andre forskningsgrupper har undersøgt indflydelsen af forskellige neurotrope vira som giver mæslinger, fåresyge og rubella (røde hunde). De vigtigste diagnostiske midler til at stille diagnosen MS i dag, er MRI scanninger og undersøgelsen af intratekal produktion af antistoffer ved tilstedeværelsen af oligoklonale bånd (OCB) og et forhøjet IgG-indeks.

I dette projekt undersøges immunresponset mod EBV, andre HHV og de neurotrope vira, mæslinger, fåresyge og rubella ved hjælp af ELISA teknik i serum og CSF prøver fra MS patienter, sammenlignet med patienter med synsnervebetændelse (ON) som kan være en form for forstadie til MS, og forskellige kontrolgrupper. Et antigenpanel, der kaldes EZMMR-panelet, bestående af EBV (EBNA-1), VZV, mæslinger, fåresyge og rubella virus antigener blev opfundet, og resultaterne viste et signifikant forhøjet niveau af serum- og CSF IgG antistoffer rettet mod EBNA-1, VZV, mæslinger og fåresyge i patienter med relapsing-remitting MS (RRMS), men ingen forskel i rubella antistoffer. Det specifikke antistof index (AI) var udregnet for at bestemme om de specifikke antistoffer var syntetiseret intratekalt. EZMMR panelets følsomhed vedrørende RRMS patienter var ca. 50%, hvis der kræves tre positive AI og ca. 85%, hvis der kun kræves to positive AI. Sammenlignet med MRZ panelet, hvor kun 50% af RRMS-patienterne havde to positive AI, viste EZMMR-panelet bedre følsomhed. Det var desværre ikke muligt at bestemme specificiteten.

Som konklusion, understøtter de opnåede resultater i dette studie teorien om at forskellige vira spiller en stor rolle i MS ætiologien og især EBV. Det er stadig ukendt om vira er den primære årsag til MS, eller den øgede forekomst af antistoffer blot er en konsekvens af sygdommen.

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List of Abbreviations

AD	Alzheimer's disease
Alb	Albumin
AI	Antibody index
AP	Alkaline phosphatase
APC	Antigen presenting cell
AP	Alkaline phosphatase
BBB	Blood brain barrier
BCR	B cell receptor
BMVEC	Brain microvascular endothelial cell
CIS	Clinical isolated syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EBV-EA/D	Epstein-Barr virus diffuse early antigen
EBV-VCA	Epstein-Barr virus viral capsid antigen
ELISA	Enzyme-linked immunosorbent assay
EZMMR	EBNA-1-Zoster-Measles-Mumps-Rubella
Fab	Fragment antigen-binding
Fc	Fragment crysallisable region
gG	Glycoprotein G
H-chain	Heavy polypeptide chain
HC	Healthy control
HCV	Hepatitis C virus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPC	High-positive control
HPV	Human papillomavirus
HSV	Herpes simplex virus
HTLV	Human T-lymphotropic virus
Ig	Immunoglobulin
IM	Infectious mononucleosis
JCV	JC virus (John Cunningham)
kDa	Kilodalton
L-chain	Light polypeptide chain

LPC	Low positive control
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MMR	Measles-Mumps-Rubella
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MRZ	Measles-Rubella-Zoster
MS	Multiple Sclerosis
NK	Natural killer cells
OCB	Oligoclonal band
ON	Optic neuritis
p-NPP	*p(para)*-nitrophenylphosphat
p-NP	*p(para)*-nitrophenol
PAMP	Pathogen-associated molecular pattern
PLP	Proteolipid protein
PNS	Peripheral nervous system
PPMS	Primary progressive multiple sclerosis
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
SLE	Systemic lupus erythematosus
SPMS	Secondary progressive multiple sclerosis
SZ	Schizophrenia
TCR	T cell receptor
TTN	Tris-Tween-NaCl
Q	Quotient
VZV	Varicella zoster virus

Introduction

Multiple sclerosis (MS) is a chronic neurological disease characterised by inflammation, demyelination and axonal degeneration of the central nervous system (CNS). In MS the immune system causes attacks on the myelin sheaths surrounding the nerves of the CNS, and so far the disease is considered an autoimmune disease involving B- and T-cells.[1]

The Immune System

The immune system is a network of cells, tissues and organs that work together to defend the body against attacks by pathogenic microbes from infection-causing organisms such as bacteria, viruses, fungi and parasites. First line of defence is the physical barriers like the skin and mucosa, the cilia in the respiratory tract, coughing and sneezing, and the mucus secreted by the epithelial cells of the gut. The immune system is build up by the innate immune system (non-specific), and the adaptive immune system (specific) which function as cooperative systems. [2-4]

Innate immunity

If a pathogen invades the physical barriers, the innate immune response will be activated and provide immediate host defence. The system is called “innate” or “natural” because it is present before infection, although the amount of some components may increase following infection. The innate immune response is unspecific, and cannot distinguish between different pathogens, but it is able to discriminate foreign molecules from self. The innate immune system consists of phagocytic cells such as macrophages, dendritic cells, and neutrophils, and other leukocytes like eosinophils, basophils, mast cells and natural killer cells (NK cells) that eliminate foreign molecules and cells, and is involved in recruitment and activation of cells from the adaptive immune system by production of cytokines. It is important that the immune system only targets foreign objects and not healthy tissue, and the way the innate immune system distinguishes foreign molecules from self, is because the cells of the innate immune system has pattern recognition receptors (PRRs) on the surface. The PRRs recognize the foreign molecules by their pathogen-associated molecular patterns (PAMPs). If the innate immune response is unable to prevent the infection, the adaptive immune system or “acquired” immune system is activated. [2-4]

Adaptive immunity

The adaptive immunity creates immunological memory after an initial response to a specific pathogen, and leads to an enhanced response to subsequent encounters with the same pathogen. The cells of the adaptive immune system are lymphocytes; the two most important types are B cells and T cells, where B cells produce antibodies when activated. The adaptive immune response can be divided into cell-mediated and humoral responses. T cells are involved in the cell-mediated immune response, and B cells are involved in the humoral immune response.

T cells are produced in the bone marrow, and they are derived from the multipotent hematopoietic stem cells. The maturation of T cells takes place in the thymus. T cells can be divided into two major classes after maturation; the T helper cells that express CD4 molecules on the surface, and T cytotoxic cells that express CD8 molecules on the surface. T cells are involved in the cell-mediated immune response, which means that the T cell receptors (TCR) recognise antigens presented by major histocompatibility molecules (MHC) on antigen-presenting cells (APCs). Antigen-presenting cells include macrophages, dendritic cells and B cells. This recognition of antigen by the T-cell receptor is different for CD4+ and CD8+ T cells. CD4+ T cells only recognise antigen presented by MHC class II, and CD8+ T cells only by MHC class I. The MHC molecule that is used to present an antigen will therefore determine the type of effector response. The activated cytotoxic T cells (CD8+) will kill any cell that expresses the antigen on their MHC class I, whereas activation of T helper cells (CD4+) leads to production of cytokines, which in turn activate a wide range of cells around them, all with the purpose of eliminating the infection. [2-4]

B cells are produced and matured in the bone marrow, and like T cells they are derived from the multipotent hematopoietic stem cells. B cells are involved in the humoral immune response, and produce antigen-specific antibodies that serve to neutralise toxins, prevent organisms adhering to mucosal surfaces, activate complement, opsonise bacteria for phagocytosis, and sensitise tumour and infected cells for antibody dependant cytotoxic attack by killer cells. Thus, antibodies act to enhance elements of the innate immune system. B cells usually recognize free antigens by the membrane-bound B cell receptor

(BCR), which is an antibody molecule, and the antigen is then internalised, processed, and re-expressed on the MHC class II molecule of the B cell. This can present the antigen to a specific T cell that will produce cytokines leading to B-cell division and maturation. Most of the B cells will differentiate into antibody-secreting cells (plasma cells) that are short-lived (2-3 days), and approximately 10 % will differentiate into long-lived memory B cells. [2-4]

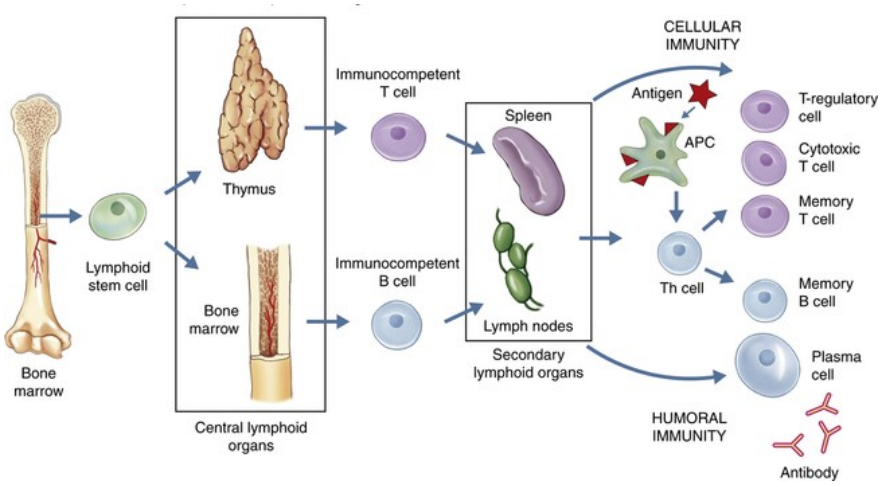

Figure 1: Overview of the adaptive immune system. Lymphoid stem cells from the bone marrow migrate to the central lymphoid organs, where they undergo a series of cellular division and differentiation stages resulting in either immunocompetent T cells or immunocompetent B cells. The immunocompetent cells enter the circulation and migrate to the secondary lymphoid organs (e.g., spleen and lymph nodes) where the cells undergo a second stage of cellular proliferation. Link: https://basicmedicalkey.com/adaptive-immunity/

The innate and adaptive immune systems act together, with the innate response representing the first line of host defence, and with the adaptive response becoming prominent after several days, as antigen-specific T and B cells have undergone clonal expansion. Components of the innate system contribute to activation of the antigen-specific cells. Additionally, the antigen-specific cells amplify their responses by recruiting innate effector mechanisms to bring about the complete control of invading microbes. Thus, while the innate and adaptive immune responses are fundamentally different in their mechanisms of action, synergy between them is essential for an intact, fully effective immune response. [2]

Antibodies

Antibodies, also known as immunoglobulins (Igs) are produced by plasma cells that differentiate from antigen-specific B cells. The basic immunoglobulin molecule is a 150 kDa protein depicted as a Y-shaped structure, and is composed of two identical heavy polypeptide chains (H-chain) and two identical light polypeptide chains (L-chains), held together by interchain disulfide bonds. The N-terminal domains are responsible for binding antigen, and are called the antigen-binding fragments (Fab region), and are located in the variable domains of the H- and L-chains. The heavy C-terminal domains form the constant region, and are called the fragment crystallisable region (Fc region). The Fc region interacts with cell surface receptors called Fc receptors on phagocytes and some proteins of the complement system. In this way, it mediates different physiological effects of antibodies. [2,5,6]

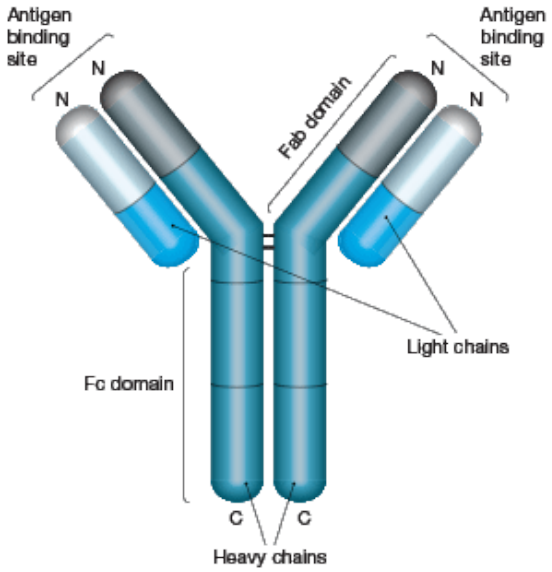

Figure 2: The classical representation of an antibody. The antibody is formed as a Y-shaped molecule composed of four subunits with two identical heavy- and light chains. The N-terminus of each heavy chain associates with one of the light chains to create two antigen-binding domains. They are termed fragment antigen binding (Fab) domains. The C-terminal of the two heavy chains form the fragment crystallization (Fc) domain. The Fc domain is important for the interaction with effector cells. The four polypeptide chains are held together by covalent disulfide bridges and non-covalent bonds.
Link: https://www.bio-rad-antibodies.com/immunoglobulin-antibody.html

There are five classes of human immunoglobulins; IgG, IgM, IgA, IgD and IgE, and some of them have subclasses. There are four subclasses of IgG and two subclasses of IgA. Each class of antibodies has different heavy chains; γ (IgG), μ(IgM), α(IgA), δ (IgD) and ε (IgE). Each class or subclass of the antibody has a unique set of functions determined

by the class of heavy chain at the Fc region of the molecule. The specificity of the antibody is related to the shape of the Fab region, meaning different classes of antibodies can have the same antigen specificity. IgM is always the first class of antibody made by a developing B cell, but eventually many B cells switch to making other classes of antibodies, with a majority of IgG-producing B cells. The immediate precursor of a B cell, called a pre-B cell, initially makes μ chains (IgM) and insert it into the plasma membrane. At this point, the cell is called an immature naïve B cell. After leaving the bone marrow, the cell starts to produce cell-surface IgD as well, with the same antigen-binding site as the IgM molecules. With both IgM and IgD on the surface, functioning as B cell receptors (BCRs), the B cell is called a mature naïve B cell, and the B cell is now ready to respond to foreign antigen. [5,7]

B cell activation

Engagement of the B cell receptor (BCR) initiates two interdependent processes, signalling and receptor internalization. When two or more receptor molecules on the naïve B cell are cross-linked by antigens, intracellular signalling is initiated. The cross-linking by antigen alone is not sufficient to activate a B cell completely; it also requires stimulation from T helper cells. The B cell antigen receptor takes up the antigen, which is processed and presented as antigenic peptides on surface MHC class II molecules. The peptide presented by the MHC II molecule can be recognised by antigen-specific T helper cells, stimulating them to synthesize cytokines that, in turn, cause the B cell to proliferate and differentiate into antibody-secreting plasma cells. Some of the B cells differentiate into IgM secreting plasma cells, but the majority of the B cells undergo class switch to IgG-secreting plasma cells. The secreted immunoglobulins are involved in the elimination of pathogens by working as an opsonin, and activation of the complement system, which itself produces opsonizing and chemotactic factors. The IgM- and IgG-secreting plasma cells are the main source of antibodies in a primary response and IgM reach their peak after 4 days, and IgG after 2 weeks of exposure to an antigen. After 4-7 days, some of the B cells and T helper cells migrate to primary follicles, where germinal centres will form. Germinal centres are specialised structures that form within lymphoid tissue following an encounter with antigen, and four main events occur in the germinal centres; antibody class switching, affinity maturation of antibody, differentiation of B

cells into plasma cells, and differentiation of B cells into memory cells. The purpose of the germinal centre is to enhance later parts of the primary immune response by production of antigen-specific plasma cells that secrete high affinity antigen-specific immunoglobulins, and generate memory cells to counteract any subsequent infection with the same antigen. Germinal centre B cells that differentiate into plasma cells are destined to become nondividing and terminally differentiated cells. Memory B cells are long-lived cells, surviving sometimes for up to many years. These cells divide very slowly, if at all, and they express surface immunoglobulins, but they do not produce antibodies. [2,5,8]

Autoimmunity

There are more than 80 identified autoimmune diseases, and MS is one of them. Autoimmunity occurs when the immune system recognizes and attacks host tissue. Environmental triggers as viruses, bacteria and other infectious diseases are thought to play a major role in the development of an autoimmune disease. [9] There are multiple mechanisms by which host infection by a pathogen can lead to autoimmunity, one of them is molecular mimicry. Molecular mimicry is a mechanism by which infectious pathogens can break immunological tolerance, and it implicates that the pathogen expresses a stretch of proteins that share antigenic structures with the host tissue. If the pathogen-encoded epitope is presented by the MHC, it may activate potentially self-reactive T cells, and as a consequence the tolerance to autoantigen breaks down a and pathogen-specific immune response cross-reacts with host structures to cause tissue damage and disease. [10] Another possible mechanism that can induce autoimmunity is bystander activation, which is a non-specific mechanism occurring within the inflammatory context generated by virus infection. A functional consequence of bystander activation and local tissue damage is the phenomenon known as epitope spreading. This often occurs in the setting of persistent infection where a prolonged anti-pathogen specific immune response leads to tissue destruction resulting in release of endogenous or cryptic self-epitopes that are presented by APCs, and activate autoreactive T cells. Chronic viral infection and the accompanying inflammation poses a significant risk for inducing autoimmunity. [11] In MS, myelin epitope-specific CD4+ T cells are assumed to infiltrate the CNS and attack the protective myelin sheaths, and maybe the cause is cross-reactivity, bystander activation or epitope spreading caused by chronic viral infection in the central nervous system.

The central nervous system (CNS)

The CNS is composed of the brain and the spinal cord. The CNS consists of approximately 100 billion neurons and about 100 billion supporting glia cells that make a complex network with approximately 10^{14} synapses for signal transmission, with a speed of up to 400 km/h. The most important supporting glia cells are microglia, oligodendrocytes, and astrocytes.

Microglia are macrophage-like cells that strive to retain CNS homeostasis. Microglia promote neuroinflammatory and neurodegenerative events in MS by releasing inflammatory mediators and stimulating leukocyte activity and infiltration into the CNS, but microglia also assist in CNS repair through the production of neurotrophic factors and clearance of inhibitory myelin debris. [12]

The primary function of oligodendrocytes is the formation and maintenance of myelin sheaths. The myelin sheaths are wrapped around the axons, and form a compact and insulating lipid layer that secures a rapid signalling between neurons. The main protein of the myelin sheaths is Myelin Basic Protein (MBP) and Proteolipid Protein (PLP), and the myelin sheaths also contain Myelin Oligodendrocyte Glycoprotein (MOG), and Myelin-Associated Glycoprotein (MAG). [13]

Astrocytes are the most abundant cells in the CNS and constitute approximately 90 % of the human brain, and one of the major functions of astrocytes is the support of neural transmission. Astrocytes provide functional support to neurons by maintaining local ion and pH homeostasis, storing CNS glycogen, and clearing neuronal waste. Another major function of astrocytes is maintenance of the blood brain barrier (BBB). Astrocytes can contribute to the pathogenesis of MS by inhibiting remyelination, modulate the permeability of the BBB and promote inflammation.[14]

Blood-brain barrier (BBB)

With the crucial functions of CNS, protection from infections and other injuries is extremely important. The CNS is protected by the cranium and vertebral column, and from the inside, the CNS is protected from the periphery by the blood-brain barrier

(BBB). The BBB is a cellular metabolic filter that regulates the exchange of materials between the blood and brain. The primary function of the BBB is to protect the brain microenvironment from influx of plasma components and harmful substances, which may disturb the neuronal function. The BBB is mainly composed of endothelial cells, pericytes, astrocytes and the basement membrane, and is largely impervious to hydrophilic substances, with the exception of certain substances such as glucose. Several membrane transporters maintain the influx and efflux of essential molecules such as nucleosides, amino acids, electrolytes and peptides, as well as protection against toxic waste products /environmental toxins. This isolation and strictly regulated blood supply are very important for the proper function of the brain. The BBB is disrupted in many pathological conditions, such as HIV, encephalitis, Alzheimer's disease, age-related dementia, stroke and Multiple Sclerosis. The disruption of the BBB refers to a reduction of the barrier tightness and an increase in leakiness, and as a result of these changes, several blood proteins deposit in the brain parenchyma. The deposits are often observed very early in a disease, suggesting that BBB disruption may participate in disease onset and progression. [15,16]

The blood-cerebrospinal fluid barrier serves the same purpose as the blood-brain barrier, but facilitates the transport of different substances into the brain due to distinct structural characteristics between the two barrier systems. The barrier between the blood and the cerebrospinal fluid mainly consist of the epithelial layer of the plexus choroideus, and here the ultrafiltration of serum takes place to form cerebrospinal fluid (CSF). [17]

The cerebrospinal fluid (CSF)

The CNS is surrounded by cerebrospinal fluid (CSF), with an average volume of 150 mL in adults. CSF originates from choroid plexus in the ventricles, flows through cisternae and subarachnoid space and finally drains through the arachnoid villi into venous blood. Blood proteins enter additionally into CSF along its way between ventricles and lumbar subarachnoid space, inducing a 2.5-fold increase in total protein concentration between ventricular and lumbar CSF. Protein transfer from the brain into CSF and from blood into CSF follows the laws of diffusion as function of molecular size. The CSF/serum quotients are larger for smaller molecules. Albumin equilibrates faster between blood and CSF than

the larger immunoglobulin molecules. $Q_{Alb} > Q_{IgG} > Q_{IgA} > Q_{IgM}$. [18] Serum is the source of 80% of all CSF proteins, and the protein concentration in CSF have an average concentration of 0,4-0,5 g/l, and consists mostly of albumin (0,2-0,4 g/l) and IgG (up to 0,4 g/l). The plexus restricts the protein- and immunoglobulin-flow into the CSF, but smaller molecules diffuse more easily into the CSF. The total Ig concentration is about 500 times lower of IgG, and about 5000 times lower of IgM than in blood. The CSF-albumin/serum-albumin quotient (Q_{alb} = Alb_{CSF}/Alb_{serum}) assesses blood-CSF barrier dysfunction, because albumin is only synthesized in the liver.[17]

BBB dysfunction plays a major role in a wide range of neurological disease, including MS. The significant consequence of BBB disruption is the increased permeability. Under normal conditions, T- and B-cells are not able to enter the CNS, but when the permeability increases larger molecules as T- and B-cells can get access to CNS and cause immune responses.

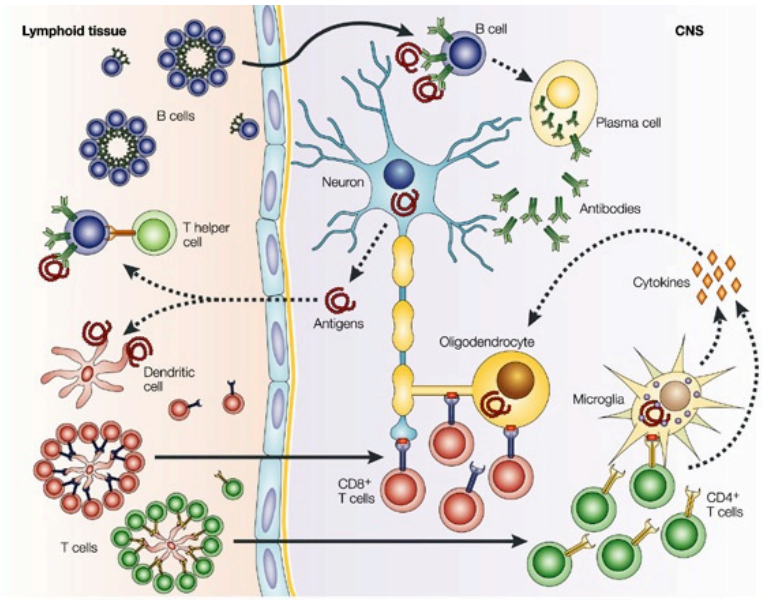

Figure 3: Hypothetical view of the immune response in CNS during acute MS. MS is associated with the infiltration of $CD4^+$ and $CD8^+$ T-cells and B-cells within the acute inflammatory lesions or the areas of demyelination. The presence of these immune cells at these locations, indicate alterations in BBB structure, which allowed their crossing into the central nervous system (CNS). Figure from nature review[19]

Virus infection in the nervous system

Virus infections usually begin in peripheral tissue, often at epithelial or endothelial cell surfaces and can, in some cases, invade the nervous system. The virus can spread into the peripheral nervous system (PNS) and more rarely the central nervous system (CNS).

Viruses that are capable of entering and affecting the nervous system are called neurotropic viruses. The CNS is normally protected from virus infections by effective immune responses and the BBB, but some viruses gain access to the CNS because of diminished host defence that fails to limit peripheral infections such as Epstein-Barr virus (EBV), cytomegalovirus (CMV) and other herpesviruses. PNS is relatively accessible to peripheral infections because nerves are in direct contact with all types of tissues, while CNS has several layers of protection. The BBB limits the spread of infection from the blood to the CSF in CNS.[15] Virus infections that leave the peripheral tissue and enter the PNS or CNS do so either by direct infection of nerve endings in the tissue, or by infecting cells of the circulatory system that carry the infection through the BBB into the CNS. Most alfa herpesviruses such as herpes simplex-1 (HSV-1), herpes simplex-2 (HSV-2) and varicella zoster (VZV) enter PNS by membrane fusion with sensory nerve endings, and engage dynein motors for retrograde transport to the neuronal cell body or soma. The viruses thereby establish a life-long persistent infection, by instilling the viral DNA in the nucleus. Despite the direct connection between PNS neurons and CNS, spreading of alfa herpesviruses into the CNS is rare.[15] Other viruses gain access to the nervous system by infection of leukocytes. Leukocytes circulate in the blood, and may infiltrate in the brain parenchyma by passing the BBB, carrying the virus into the CNS. Virus particles from EBV, CMV, hepatitis C virus (HCV), human T-lymphotropic virus 1 (HTLV-1) and JC virus in the circulatory system can infect brain microvascular endothelial cells (BMVECs), a major constituent of the BBB. Infection of BMVEC cells often leads to disruption of the BBB integrity, and cause uncontrolled migration of immune cells into the CNS. Prevalent human DNA viruses like EBV and JC virus can damage the CNS by infecting BMVECs, and establishing life-long latency. EBV establishes life-long latency in memory B-cells, and can infect BMVECs. Reactivation of EBV in these cells increases expression of inflammatory cytokines and chemokines, and affecting the integrity of the BBB, which might lead to progression of the inflammatory neurological disease multiple sclerosis. Other members of neurotropic viruses are measles virus, mumps virus and rubella virus. [15,20] These examples demonstrate that, while virus replication itself may cause neuropathology, the immune system also contributes to the neuronal damage in an effort to eradicate the infection.

Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease affecting the central nervous system (CNS), in which inflammation, demyelination and axonal loss occur from the very early stages of the disease. MS mainly affects young people between 20 and 40 years, with a female predominance. [21] The disease is the most common cause of disability in the young adult population, and is an on-going clinical problem. The prevalence of MS has increased 10-fold over the last 50 years in some regions, and nearly 2.5 million individuals are affected by MS globally. The majority of MS patients reside in Europe and North America, and the disease incurs an annual cost of 15 billion US dollars (100 billion DKK).[22-25] There is still no cure for MS, and the treatment today relies on managing symptoms, accelerating recovery from relapse, and reducing the number and severity of relapses.

Patients with MS have variable clinical courses and symptoms, and today there are three MS phenotypes: Relapsing-remitting (RRMS), more than 85% of MS patients are classified as having relapsing-remitting MS (RRMS), where full recovery can occur in the beginning of the illness, but eventually the disease will cause permanent damage of the nerves. These patients are predominantly female and typically 20-30 years old at the presentation of initial symptoms. The remaining 10-15% of MS patients are classified as having primary progressive MS (PPMS), which is characterised by continuous worsening of symptoms from the onset. The patients in this group are typically between 30-50 years old, and men and women are affected with similar frequency. The last phenotype is and secondary progressive MS (SPMS), which in the beginning follows the relapsing-remitting course, and then becomes progressive. Studies have demonstrated that after 20 years, about 80 % of the patients transit to a progressive phase. Clinically Isolated Syndrome (CIS) can also be defined as a phenotype, and is a term that refers to the first clinical manifestation of the disease that by definition is isolated in time or not preceded by any neurologic event.[25,26]

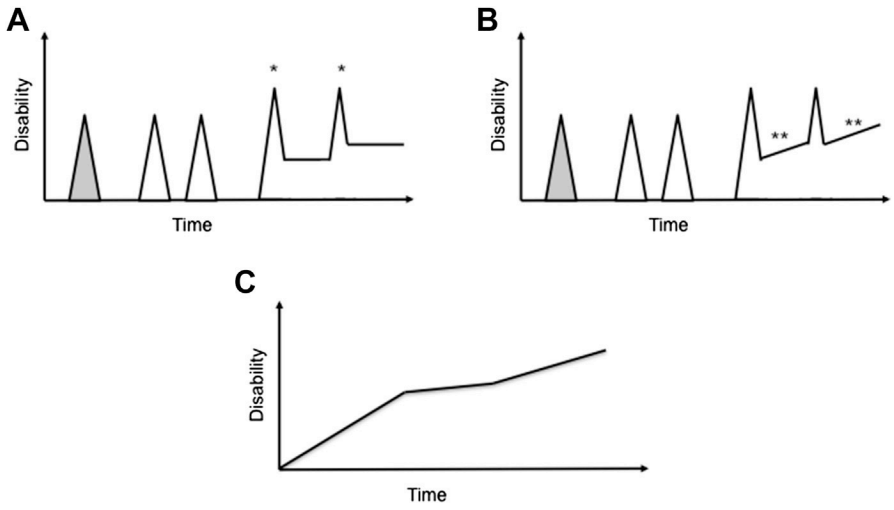

Figure 4: Multiple sclerosis phenotypes. A) Relapsing-remitting MS: after the first event or clinical isolated syndrome (light grey), new relapses will occur and the recovery of these relapses may be complete or partial. After this initial period, some patients enter a more progressive phase where the recovery is incomplete. B) Secondary-progressive MS: After the initial period with relapses and recovery, the disease will be progressive. C) Primary-progressive: Patients present a slow progressive neurologic disability since onset, and no recovery occur. Figure from [25]

Pathogenesis and symptoms

MS affects the central nervous system, including the brain, spinal cord and optic nerves. The immune system attacks the protective myelin sheaths that covers nerve fibres, and multifocal zones of inflammation due to focal T-lymphocyte and macrophage infiltration, and oligodendrocyte death is the primary cause of myelin sheath destruction.[21,27] According to the function of myelin sheaths to facilitate electric signals between the neurons quickly and efficiently, destruction of the myelin sheaths will cause communication problems between the brain and rest of the body, because the signal impulse from the brain cannot be transmitted to the muscles. Remyelination can occur in the beginning of the illness, but eventually, the disease will cause permanent damage of the nerves.[14] Symptoms will vary between patients, depending on where the lesions in the CNS occur, and which subtype of MS the patient is suffering from. In RRMS the lesions can generate symptoms such as visual impairment, tingling and numbness, episodic bouts of fatigue, intestinal and urinary system disorders, spasticity, and learning and memory impairment. Patients diagnosed with PPMS tend to have fewer brain lesions, and the disease largely affects the nerves of the spinal cord. Induced symptoms include problems with walking, weakness, stiffness, and trouble with balance. [21]

Demyelination of the neurons leave scars, multiple sclerosis means "scar tissue in multiple areas.", and the areas where there are either not enough myelin or no myelin are called plaques or lesions. Plaques are randomly distributed. They have a predilection for the periventricular white matter, optic nerves and spinal cord, but they can occur everywhere in the CNS. About 90 % of MS patients have detectable oligoclonal IgG bands in their cerebrospinal fluid (CSF). Given that these antibodies are directed towards antigenic targets in the CSF and usually only are present in infectious CNS disorders, it suggests that one or more infectious agents are implicated in MS. Although myelin is the main biochemical target in MS, the distribution of lesions in the brain is patchy, and many myelinated areas remain unaffected, suggesting an etiology with specific tropism.[24]

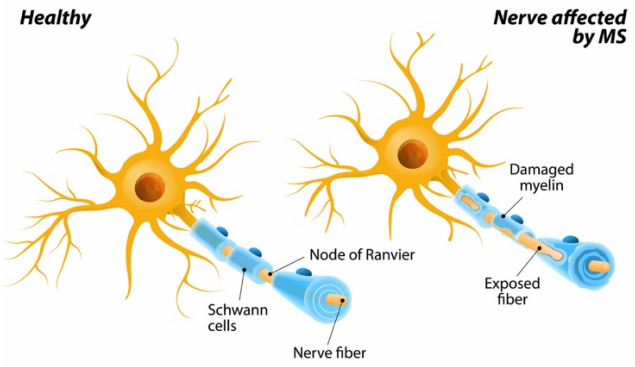

Figure 5: Healthy neuron vs. neuron affected by MS. Myelin sheaths are damaged and the nerve fibre is exposed. Link:https://anova-irm-stemcell-center.com/en/treatments/stem-cell-autoimmune-diseases/multiple-sclerosis-ms

Etiology

MS has been known for more than 160 years, and no common etiologic factor has been identified. The more we understand the mechanism of MS, the clearer it has become that MS is truly a complex disease with environmental, genetic and immunological components.

Environmental factors

The infectious etiology of MS has been suspected for well over one hundred years, and several viruses and other infectious agents have been associated with MS during this time. Several studies show a strong association between MS and different viruses, notably Epstein-Barr virus (EBV), and in particular infectious mononucleosis (IM) caused by EBV. Also other viruses as Human herpes virus 6 (HHV-6), Cytomegalovirus (CMV),

Varicella zoster virus (VZV), Measles virus and Influenza virus have been suggested as contributors to the risk of MS.[24] Other studies suggesting MRZ reaction, which is the presence of antibodies in CSF against Measles, Rubella and VZV, as a causative agent.[28] The heterogeneity of the disease might also suggest that MS is not caused or triggered by only one virus, but rather a more complex set of viral infections could act in genetically susceptible individuals.

Vaccines

Vaccinations have also been investigated as a trigger of MS. Different vaccines as hepatitis B, human papillomavirus (HPV), seasonal influence, measles-mumps-rubella (MMR) tetanus, polio or diphtheria has been investigated, and no evidence in risk of developing MS has been found.[29]

Smoking, vitamin D sufficiency and obesity

Smoking and low vitamin D levels caused by insufficient sun exposure and/or dietary intake have also been presented as factors that can influence the pathogenesis of MS. More recently also obesity during adolescence is suggested to have an influence on developing MS. [30]

Genetic susceptibility

Previous studies of the heritability of MS have estimated that a sibling of an individual with MS has almost 17-fold increased risk of developing MS. [31] However, recent studies have estimated a siblings' relative risk to be increased by 7-fold, indicating a much lower importance of genetic predisposition.[30] Genes within the human leukocyte antigen (HLA) complex are the strongest genetic risk factor, and especially HLA class II and I genes are relevant modifiers of disease risk. Variants of class II genes encode products that present antigens to CD4+ T cells, and class I products present antigens to CD8+ T cells. The class II variant HLA-DRB1*10:01 has strong association with an increased risk of developing MS.[30]

Diagnosing MS

Clinical definite MS (CDMS) is diagnosed by a combination of neurological investigations and severity of symptoms over time according to the revised 2010

McDonald criteria.[32,33] The most important tools in diagnosis of MS are MRI scans, presence of oligoclonal bands (OCBs) and an elevated IgG-index.

OCBs and IgG index

The most sensitive and most widely used laboratory method to detect a significant intrathecal IgG synthesis is isoelectric focusing, where distinct bands of serum- and CSF-IgG are visually compared on a gel. The test is positive if there are 2-4 distinctive CSF bands that are not present in serum. Quantitative measurements calculated from IgG and albumin in serum and CSF can also demonstrate intrathecal synthesis of immunoglobulins, known as IgG index.[17] Under normal physiological conditions there are no antibody-producing B cells in the CNS. Patients with multiple sclerosis typically have an intrathecal synthesis of immunoglobulin G. The intrathecal production of IgG is maintained by B cells that have entered the CNS. It is still unclear when and why these cells invade the CNS, but infection with EBV is suspected as a causative agent. In MS patients, intrathecal production of IgG is found in more than 90%.[34] The B lineage cells responsible for intrathecal production of antibodies must migrate into the CNS from the systemic circulation. At the time an antiviral antibody-producing cell enters the CNS, the specific B cell has to be present in the systemic circulation. Therefore, the patient must have undergone infection with the respective virus, or vaccination against it, to generate either B memory cells, or antibody-producing B cells against the specific virus.[34]

Magnetic Resonance Imaging (MRI)

MRI scanning of the brain is one of the most important tools in diagnosing MS, and is also used to track progression of the disease. It is the most non-invasive and sensitive method of imaging the brain, spinal cord or other part of the body. MRI uses a strong magnetic field and computer-generated radio waves to measure the relative water content in the tissue. It creates detailed images of areas to reveal nerve damage. Myelin is fatty and repels water, and demyelinated nerves retain more water. Therefore, demyelinated areas will appear as white spots or plaques in the MRI scan. If a patient has 2 separate plaques, it supports the MS diagnose.[35]

Optic Neuritis and MS

Optic neuritis (ON) is an acute inflammatory condition affecting the optic nerve and causing retro-orbital pain and visual loss. ON is highly associated with MS, and 50 % of patients diagnosed with ON develop MS after 15 years.[36] The optic nerve is considered a part of the CNS, and it shares many histological characteristics, i.e. myelination of the optic nerves is produced by oligodendrocytes, and it has a blood brain barrier (BBB). It is found that patients with ON that develop MS within 2 years from ON onset, have a significantly higher permeability of the BBB in some parts of the brain.[37]

Treatment of Multiple Sclerosis

There is still no cure for MS, but an increasing number of drugs are being developed for the treatment of MS. Few licenced treatments are available to slow progressive MS, whereas numerous disease-modifying treatments are available to reduce the frequency of relapses in relapsing-remitting MS (RRMS).[38] The disease-modifying medicines are the standard treatment for patients with MS, and current drugs such as Prednisolone, Beclofen, Interferon-beta, Glatiramer and Natalizumab are administered to slow down progression of MS and reduce its symptoms. The drugs have different mechanisms of action, but they have in common that they supress the immune response and inhibit the inflammatory processes.[39]

Stem-cell-based therapies have recently emerged as a promising medication for treatment of MS. Various stem cell sources such as mesenchymal, embryonic and neural have been identified, and because of their self-renewal and differentiation capacity into various cell types, they can contribute to the regeneration of the demyelinated areas and preventing disease progression effectively. The research is still ongoing, and the stem-cell-based therapeutics are still in an experimental stage. [40]

Viruses and vaccines

Epstein-Barr virus (EBV) and other human herpes viruses (HHVs)

Epstein-Barr virus (EBV) is one of the eight known viruses in the human herpes virus (HHV) family, and affects more than 90% of the world's adult population. People are often infected with EBV during childhood, and in most cases, infection with EBV is asymptomatic or is indistinguishable from other mild illnesses of childhood. When infection with EBV occurs in the late teenage years or during adolescence, it causes infectious mononucleosis (IM) in 35 to 70% of the cases, where up to 20% of B-cells are infected. EBV is a double-stranded DNA (dsDNA) virus enclosed by a capsid, which is surrounded by the tegument and a host cell membrane-derived envelope embedded with glycoproteins.[41,42]

EBV is transmitted in saliva and initially infects epithelial cells in the oropharynx and nasopharynx, and subsequently EBV enters the underlying tissue and infects B-cells. B cells are the primary targets of EBV, and after primary lytic infection EBV persists in immortalized resting memory B cells for the rest of the host's life, where it can shift between an active lytic cycle and a latent stage. To promote viral persistence in the memory B cells, EBV has evolved a variety of strategies to modulate the host immune response; including inhibition of immune cell function, blunting of apoptotic pathways, and interfering with antigen processing and presentation pathways.[43-45]

During the latent stage, EBV nuclear antigens (EBNAs) are the only genes that are expressed, and in particular EBNA-1 which is the only protein required for maintenance of the viral genome serving as a replication factor. When B-cells are latently infected for longer periods, EBV will only express EBNA-1. The exact trigger for lytic cycle reactivation is unknown, but the process is a dynamic interaction between the host's immune response to EBV and the infection state. The lytic genes are divided into immediate early, early and late genes, according to their expression in the lytic cycle. Early antigen diffuse (EA/D), is an early gene as the name indicate, and is one of the six early proteins that form the initiation complex to facilitate replication of the virus. The viral capsid antigen (VCA) p23, is expressed in the acute phase, and the expression peaks at 2-4 weeks after onset.[42,46]

EBV is associated with a lot of severe cancer diseases, such as Hodgin´s lymphoma, Burkitt´s lymphoma, nasopharyngeal carcinoma and gastric cancer.[47] There is also evidence that infection with EBV is associated with a higher risk of other certain autoimmune diseases, especially dermatomyositis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS).[48]

Other members of the HHV family is herpes simplex virus 1 (HSV-1), herpes simplex virus-2 (HSV-2), varicella zoster virus (VZV), cytomegalovirus (CMV), human herpes virus 6A and 6B (HHV-6), HHV-7 and HHV-8. All herpesviruses can establish latent infection within specific tissues of the host, which are characteristic for each virus. HSV-1 and HSV-2 are the infectious agents causing herpes. HSV-1 approximately affects 70 % of the population, and the transmission often occurs during childhood. HSV-2 is primarily transmitted through sexual contact, and approximately 30 % of the population are infected. The HSV-1 and 2 replicates initially in epithelial cells, and establish latency in the dorsal root ganglia, where it during reactivation can spread the virus distally and initiate new cutaneous lesions.[49] VZV causes varicella (chickenpox) in the first time of infection, and more than 90 % of the adult population in Denmark are infected. Infection with chickenpox often occurs in early childhood, and reactivation of VZV often in adolescence will cause herpes zoster (shingles). VZV is usually transmitted by droplets, and replicates initially in the nasopharynx. Latency is established in dorsal root ganglia, like HSV-1 and 2. VZV is highly contagious and about 95% of adults are seropositive of the virus. CMV can cause a variety of symptoms, and sometimes the virus is asymptomatic. CMV infection can cause hepatosplenomegaly, retinitis, rash and CNS involvement. In about 10 % of older children or adults, primary CMV infection causes a mononucleosis syndrome. CMV replicates mainly in salivary glands and kidneys, and is shed in saliva and urine. The virus induces characteristic giant cells with intranuclear inclusions. CMV is affecting approximately 50% of the adult population.[49]

Measles-Mumps-Rubella (MMR)

The health authorities in almost all countries recommend vaccination of children against severe diseases that can cause permanent injury and in worst case be fatal. In Denmark, children are vaccinated against:

3, 5 and 12 months: Diphtheria, Tetanus, Whooping cough (pertussis), Polio, Meningitis and epiglottis caused by *Haemophilus influenzae* type b, Meningitis and other serious diseases caused by pneumococci.
5 years: Diphtheria, Tetanus, Whooping cough (pertussis), Polio revaccination
15 months and 4 years: Measles, Mumps, Rubella (MMR)
12 years: HPV

Link: Sundhedsstyrelsen.https://www.sst.dk/en/disease-and-treatment/~/media/B74655FEA6DF4771998A6BDEA96A374A.ashx

The multivalent MMR vaccine became available in 1971, and the vaccine is currently provided to children by the age of 15 months and 4 years, and sometimes by the age of 12 years, if the child only has been given one vaccination earlier. The vaccine is a mixture of live attenuated viruses, and is administered by subcutaneous injection.[50]

The MMR vaccination includes vaccination against measles, mumps and rubella virus. Measles virus can cause high fever, coughing, common cold, eye irritation, skin rash and possible serious complications such as pneumonia or encephalitis. Mumps are characterised by swollen salivary glands and a slight fever, and possibly complicated by meningitis or orchitis with permanent damage. Rubella is a virus causing skin rash, swollen lymph glands and a slight fever. Infection during pregnancy can result in miscarriage or congenital malformation of the baby. The three viruses are members of the neurotropic viruses, and they are capable of infecting the nervous system.[20,50,51]

Method Theory

Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a simple technique used to detect the amount of either antigen or antibody in a liquid sample, as for instance sera or CSF. ELISA is based on the principle of antibody-antigen interactions. A number of different ELISA methods have been developed, and the different ELISA assays detect either antigen or antibodies. An indirect ELISA method is used to quantify the amount of primary antibody.[52] A specific antigen is adsorbed to the wells of a microtitre plate, which binds both hydrophilic and hydrophobic domains. After incubation with the antigen, the plate is washed and blocked to prevent further bindings to the wells, followed by incubation with the liquid solution containing antibodies. In this case sera and CSF samples. An enzyme-conjugated secondary antibody is added, in this case an alkaline-phosphatase(AP)-conjugated antibody that binds to the primary antibody. The interaction between antigen and antibody is then visualised by adding a substrate for the enzyme, in this case para(*p)*-nitrophenylphosphate (*p*NPP) that is converted to the yellow water-soluble *p*-nitrophenol (*p*NP) by alkaline-phosphatase. The intensity of the colour reaction is proportional to the amount of antibody-antigen interactions, and is measured by a spectrophotometer. [53]

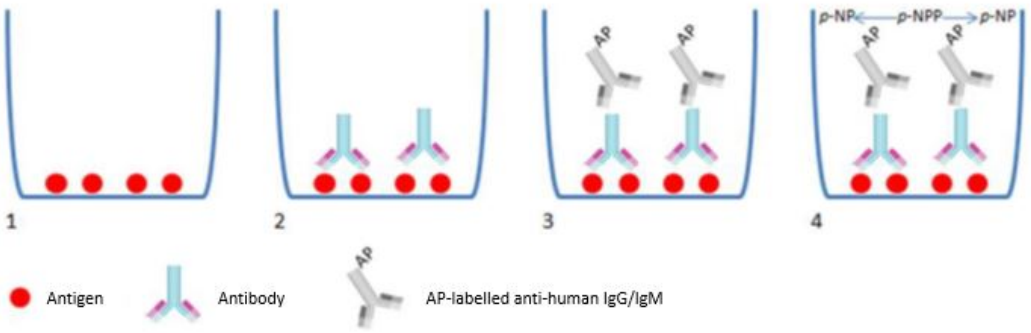

Figure 6: Indirect ELISA method for quantification of antibodies. 1. Recombinant antigen is coated in microtiter wells. **2.** Antibodies against the coated antigen from serum or CSF will bind. **3.** Alkaline-phosphatase (AP)-conjugated anti-human IgG/M is added to the well. **4.** *ρ*-nitrophenylphosphate (*ρ*-NPP) is added and converted to theyellow water-soluble *ρ*-nitrophenol (*ρ*-NP) by alkaline-phosphatase. The color formation is measured by spectrophotometry. (Modified figure from Anette Holck Draborg)

IgG index

Patients with multiple sclerosis (MS) typically have an intrathecal synthesis of IgG. Concentrations of total IgG and albumin are measured quantitatively in both serum and CSF, using nephelometry (BN ProSpec System, Siemens). The principle is to measure turbidity in a liquid sample by passing light through the sample, and the turbidity is proportional with the amount of antibodies in the sample, because of use of an antibody reagent. The IgG index is the best index of local IgG production, adjusted both for leakage of IgG due to BBB dysfunction and for albumin and IgG concentrations in serum. This index is increased in more than 80% of cases of MS. It is not only specific for MS, it can be increased in any condition where there is a local CNS IgG synthesis.

Antibody Index (AI)

Specific antibody synthesis in the CNS is used to determine if a specific antibody production occur within the CNS or not, and the index could be calculated by the ratio between the CSF/serum quotients for specific antibodies. $AI = Q_{IgG[spec]}/ Q_{IgG[total]}$ $Q_{IgG[spec]} = CSF_{IgG[spec]}/Serum_{IgG[spec]}$ and $Q_{IgG[total]} = CSF_{IgG[total]}/Serum_{IgG[total]}$.
AI values above 1,5 indicates a specific intrathecal production of antibodies. [54]

Isoelectric focusing

Isoelectric focusing is used to detect oligoclonal bands (OCB) in cerebrospinal fluid (CSF), which indicates intrathecal synthesis of IgG. The principle behind isoelectric focusing is that proteins are separated by their isoelectric point in an agarose or PAGE gel with a pH gradient. This allows easy separation of proteins without denaturing them. In this study, isoelectric focusing was performed on the semi-automatic Hydrasys (Sebia) applying the hydrogel 9 CSF isofocusing kit. The patient's serum and CSF samples are analysed in parallel, in order to compare the IgG distribution. If the bands are visible at both serum and CSF or not visible at all, the test is negative for intrathecal synthesis of antibodies. If the bands are visible only at CSF, the test is positive for OCB, and indicating an intrathecal production of antibodies. [55]

Aim of study

Multiple sclerosis (MS) is an autoimmune disease presumably linked to Epstein-Barr virus (EBV). Infectious mononucleosis, caused by EBV, in the late teens or in adulthood, confers a more than twofold higher risk to develop MS.[56] Conversely, the risk is extremely low in rare individuals who are seronegative for EBV. [57] The hypothesis is that EBV-infected B cells enter the CNS predominantly at the time of and triggered by acute primary EBV infection. Infectious mononucleosis increases the amount of B cells invading the CNS, and the different kinds of B cells that are circulating in the peripheral system entering the CNS - producing different virus antibodies.

Today the diagnosis of multiple sclerosis relies on clinical features and magnetic resonance imaging (MRI), only a few biomarkers are known (e.g. IgG index, oligoclonal bands in CSF). The aim of this project is to get closer to the etiology of MS and to identify potential biomarkers of MS. ELISA screening for antibodies against different human herpes viruses (including EBV), as well as screening for antibodies against other neurotrophic viruses. ELISA methods will be applied using recombinant proteins, and sera and cerebrospinal fluid from MS patients, healthy donors and various disease controls will be tested.

Materials and Methods

The project can be divided into two phases:

1) A screening phase where serum and CSF pools were tested against a broad panel of antigens, to determine a composition of antigens for further investigation.
2) A more thorough investigation of serum and CSF samples, against selected antigens.

The studies from the second phase will be the main focus in this thesis.

Materials

Buffers and reagents

- Tris Buffer 20 mM (0.5 M Tris (Tris-HCl, Trisma-base) pH 7.5)
- TTN buffer (0.05 M Tris, 1% Tween 20, 0.3 M NaCl, pH 7.5) (SSI diagnostica, Copenhagen, Denmark)
- Alkaline Phosphatase (AP) substrate buffer (1 M diethanolamine, 0.5 mM MgCl2, pH 9.8) (SSI diagnostica, Copenhagen, Denmark)
- *p*-nitrophenylphosphate (*p*NPP) (Sigma-Aldrich, St. Louis, MO, USA)

Antibodies

- AP-conjugated goat anti-human IgG (Sigma-Aldrich, St. Louis, MO, USA)
- AP-conjugated goat anti-human IgM (Sigma-Aldrich, St. Louis, MO, USA)
- Human purified IgG (IVIG) (SSI diagnostica, Copenhagen, Denamrk)

Recombinent proteins

- EBV EBNA-1 (Rec. protein, Prospec-Tany TechnoGene Ltd., Israel)
- EBV VCA p23 (Rec. protein, Prospec-Tany TechnoGene Ltd., Israel)
- CMV pp52 (Rec. protein, Prospec-Tany TechnoGene Ltd., Israel)
- HSV-1 gG (Rec. protein, Bio-Rad Laboratories Inc., Denmark)
- VZV (cell lysate antigen, Department of virology SSI, Denmark)
- Measles virus (Rec. protein, Baltymas, Lithuania)
- Mumps virus (Rec. protein, Baltymas, Lithuania)
- Rubella virus (Capsid protein, Baltymas, Lithuania)

Patient material and antigens in the screening phase

In the beginning of the project, pools were made of sera and cerebrospinal fluid (CSF), positive and negative for oligoclonal bands. All four pools consist of approximately 100 individual patient samples. Pools were tested for oligoclonal bands, and IgG index. Pools were also tested against a broad panel of antigens using an indirect ELISA technique. The Department of Virology, SSI, performed some of the tests in the screening process. They tested the pools for antibodies against CMV, HSV, VZV, rubella, measles, mumps, pertussis, pneumococci, salmonella and campylobacter. Furthermore, two pools with sera and CSF from patients diagnosed with MS and ON were made. As controls, a pool of healthy controls was used as serum control, and because of complications getting CSF from healthy controls, CSF tested for Alzheimer's Disease (AD) were used; one pool with low amyloid-β and high Tau (assumed Alzheimer's disease), and one pool with high amyloid-β and low Tau (possibly some other dementia). The serum and CSF pools were screened against a panel of antigens to determine which antigens are important, and which ones are relevant to test against the patient cohorts. The methods used in the screening phase are primarily ELISA, and antibodies of subtype IgG, IgM and IgA were examined. Western blot and line blots were also performed during the screening phase. For more detail about these techniques se appendix.

Table 1: Overview of the screening phase

Serum pools	CSF pools
Multiple Sclerosis	Multiple Sclerosis
Optic Neuritis	Optic Neuritis
OCB positive	OCB positive
OCB negative	OCB negative
Healthy control (control_1)	AD (↓Amyloid-β, ↑Tau) (control_1)
Control Donor (control_2)	AD (↑Amyloid-β, ↓Tau) (control_2)

EBNA-1	Morbilli virus
EBNA-2	Rubella virus
EA/D	Parotitis virus
EBV VCA p23	HPV-6
CMV pp52	Parainfluenza (hPIV1)
HHV-6 p41	Respiratory syncytial virus (RSV)
VZV	Influenza A (IAV)
JC virus	Influenza B (IBV)

Antibodies in MS, ON and control patients

Followed by the screening phase, a new cohort of patients was examined. The new cohort was thoroughly investigated, first mixed as pools, and then as single samples. According to the results obtained in the screening phase, a panel of eight antigens was selected. The reason for the selection of antigens was based on the results from the screening phase, and the theory about EBV and other herpes viruses influence on MS, and the Danish Childhood Vaccine program.

Antigen panel

Patient pools of sera and CSF are tested against eight antigens, and both IgG and IgM antibodies were analysed. The antigen panel consisted of five kinds of human herpes viruses, and three viruses, children in Denmark are vaccinated against, Measles, Mumps and Rubella (MMR).

Table 2: Antigen panel tested on pools

EBNA-1	EBV VCA p23	CMV	HSV-1	VZV	Measles	Mumps	Rubella
IgG IgM	IgG IgM	IgG IgM	IgG IgM	IgG IgM	IgG IgM	IgG IgM	IgG IgM

After testing the pools, the five most interesting antigens are selected for further investigation. The patient cohorts, both sera and CSF samples were tested against the antigens, and only for IgG antibodies. The antigen panel consisted of two human herpes viruses, and three viruses, children in Denmark are vaccinated against (MMR).

Table 3: Antigen panel tested on the patient cohort

EBNA-1	VZV	Measles	Mumps	Rubella
IgG	IgG	IgG	IgG	IgG

Patient material

Patient sera and CSF were supplied from the Sclerosis Clinic, Department of Neurology, Glostrup Hospital, where they were diagnosed with MS or ON. Control sera and CSF were already at the Department of Autoimmunology and Biomarkers, SSI, where the project was performed. Controls have been used for other research projects, or investigated for other diseases than MS. The new cohorts of sera and CSF were prepared as pools, and then tested individually.

Table 4: Pools tested against the antigen panel in table 2.

Diagnosis	No. of patients in the pool
RRMS	25
ON_OCB+	10
ON_OCB-	14
SZ	17
Serum/CSF control 1: HC/AD (↓Amyloid-β, ↑Tau)	UN/20
Serum/CSF control 2: SLE/AD (↑Amyloid-β, ↓Tau)	30/20

Abbreviations in table 4 and 5
RRMS: Relapsing-Remitting Multiple Sclerosis
ON: Optic Neuritis
OCB: Oligoclonal Bands
SZ: Schizophrenia
HC: Healthy controls
AD: Alzheimer's Disease
SLE: Systemic Lupus Erythematosus
UN: Unknown

Overview of the individual patient samples, and the number of patients tested against the antigen panel in table 5. Serum and CSF samples were divided into 3 groups each, with the same MS and ON patients, and a different control group.

Table 5: Patient cohorts, single samples tested against the antigen panel in table 3.

Serum samples	No. of patients	CSF samples	No. of patients
RRMS	29	RRMS	29
ON	24	ON	24
Serum controls (HC)	21	CSF controls (SZ)	17

Methods

Enzyme-linked Immunosorbent Assay (ELISA)

Maxisorp microtitre plates (Nunc, Roskilde Denmark) were used for all the ELISA measurements. TTN buffer was used for washing steps, blocking and dilution of patient samples and conjugate. Recombinant antigens were diluted to a concentration of 1µg/ml in TRIS buffer. 100 µl was used for incubation of wells with diluted antigens, diluted samples, secondary antibodies (conjugate), and enzyme substrate. Antigens diluted in TRIS buffer was applied to all wells and incubated over night at 4°C. TRIS buffer without antigen was applied as control. After incubation, the wells were washed 3 x 1 min with 200 µl TTN buffer. Two different standard curves were included on every plate. The first one coated with human-IgG in known concentrations, diluted 2-fold and in duplicates. The highest standard 0,025 µg/ml for serum and 0,01 µg/ml for CSF. 2) The other one coated with the respective antigen, and then a serum or CSF pool in appropriate dilution, and diluted 2-fold and in duplicates as well. The standard curve with serum or CSF is also tested in uncoated wells to subtract the background noise. The serum and CSF samples were normalized to both standard curves. Serum samples were diluted 1:800 (EBNA-1), 1:1000 (Measles), 1:400 (VZV, Mumps and Rubella), and CSF samles 1:40 (EBNA-1 and Measles), 1:20 (VZV, Mumps and Rubella), and 100 µl of diluted samples was applied in each well. 100 µl TTN in the conjugate control wells. The plate was incubated for 1 hour at room temperature (RT), on a shaking table. The wells were washed 3 x 1 min with 200 µl TTN, and incubated with 1:2000 dilution of alkaline-phosphatase(AP)-conjugated goat anti-human IgG (or IgM for the pools) for 1 hour at RT on a shaking table. The plate was washed 3 x 1 min with 200 µl TTN, and developed by adding AP-substrate (ρ-NPP) diluted in AP-substrate buffer to a concentration of 1 mg/ml. Absorbance was measured at an excitation wavelenght of 405 nm, and a reference wavelenght of 650 nm using the ELISA reader sunrise™, Tecan, and the Magellan data analysis software. The plates were read after an appropriate time. Serum samples were read after 15 min and 30 min, and CSF samples were read after 30 min and 60 min. The absorbance values of the conjugate control wells were substracted from all the values on the plate, and the absorbance value from the uncoated wells were subtracted from the respective coated wells with the same patient sample. All the samples were measured in duplicates.

Inter-assay and intra-assay variation in ELISA

The intra-assay and inter-assay variation test determines the reproducibility of an assay. Intra-variation is defined as the CV percent (CV%) of a sample run in a double determination within the same plate. Inter-variation is defined as the CV percent of a sample run in different plates and in different moments. The intra-assay variation was measured in at least 10 double determinations per antigen in the same plate, and the test was carried out for both serum and CSF. The inter-assay variation is calculated according to a low and a high positive control (LPC and HPC) included in each plate for every antigen. The intra-variation is accepted if CV% is less than 10%, while inter-assay variation of maximum 15% is accepted.

Isoelectric focusing

One of the experienced technicians kindly performed isoelectric focusing. The patient cohort has already been tested for OCB by isoelectric focusing, and the IgG index has been calculated. Therefore, the majority of the results is looked up in the LIMS database. Isoelectric focusing was performed on the semi-automatic Hydrasys (Sebia), according to the instruction manual. Prior to performing the test, it is mandatory to quantify the IgG concentrations in both serum and CSF, in order to adjust the IgG concentration at the same level. The samples were loaded onto an applicator 10 µl/well. The agarose gel was placed in the migration chamber of the Hydrasys, and buffer strips soaked in anodic and cathodic solutions were placed on the holders so they were in contact with the top and the buttom of the gel. After protein separation, the gel was immunofixed with peroxidase-labelled anti-IgG. After incubation with anti-IgG the gel was washed to remove unbound proteins, before enzymatic reaction. [55]

IgG index

The nephelometry method (BN ProSpec System, Siemens) was used to measure the IgG and albumin concentrations in serum and CSF, and the index is calculated using the formula below:

$$\text{CSF IgG index} = \frac{\text{CSF IgG x serum albumin}}{\text{CSF albumin x serum IgG}}$$

AI index

Specific antibody synthesis in CNS was calculated by the ratio between the CSF/serum quotients for specific antibodies.

$$AI = Q_{IgG[spec]} / Q_{IgG[total]}$$

$$Q_{IgG[spec]} = CSF_{IgG[spec]}/Serum_{IgG[spec]} \text{ and } Q_{IgG[total]} = CSF_{IgG[total]}/Serum_{IgG[total]}$$

Specific antibody concentrations are measured using ELISA technique, and the absorbance measurements are normalized to the standard curve coated with human IgG in known concentrations. The total antibody concentrations in serum and CSF are determined using nephelometry as described in IgG index measurements.

Statistics

Statistical analysis was carried out using Graph Pad Prism 7.0 software. Comparison of antibody concentrations between groups (RRMS, ON and control group) was performed using the nonparametric unpaired two-tailed Mann Whitney U test. A $p<0,05$ was considered significant.

Results

The project was initiated by a screening phase, and the screening phase led to a defined antigen panel that was tested against a set of different patient pools, and then a cohort of individual patients diagnosed with multiple sclerosis (MS), optic neuritis (ON) and various control groups.

Screening phase

In the screening phase, different pools of MS, ON, OCB positive, OCB negative, and healthy controls were tested against a lot of different antigens. The results from the screening phase are not shown in the results section, but selected results are shown in appendix 1. The OCB positive and OCB negative pools were examined for oligoclonal bands (OCB) by isoelectric focusing, and the IgG index was calculated. The pool with OCB positive samples consists of almost 100 different patients. Each of them positive for OCB, but when they were pooled, they turned out negative for OCB. That indicates the specificity of the IgG's varies between patients.

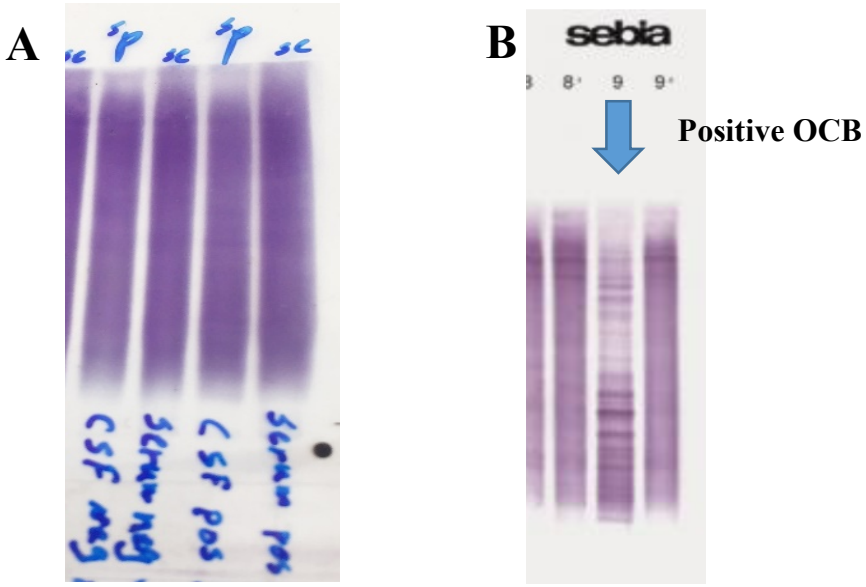

Figure 7: Oligoclonal bands in pools. A) Serum and CSF pools are shown in parallels. Serum and CSF pos means serum from patients with a positive test for OCB, and serum and CSF neg means patients with negative OCB. Picture obtained by isoelectric focusing method, used to detect OCBs. B) Example of a test with positive OCBs.

The IgG index of the pools was calculated, and the OCB positive pool has an IgG-index of 0,90 which indicates a positive intrathecal synthesis of IgG, and the OCB negative pool has an IgG index of 0,53 which indicate no intrathecal synthesis of IgG.
(Reference for IgG-index < 0.7)

After the screening phase, a panel of antigens for further investigation was selected, and the panel was reduced to eight different antigens.

Antibodies in serum and CSF pools in MS and ON patients

In order to determine which antibodies that are dominant in MS and ON patients, pools were tested against the panel of eight antigens. A pool consisting of patients all diagnosed with relapsing-remitting multiple sclerosis (RRMS), and all with a positive test of oligoclonal bands (OCBs), a pool containing patients diagnosed with optic neuritis (ON), and with a positive test of OCB, and a pool containing patients with ON and a negative test of OCB. Besides the MS and ON pools, control pools were included. In the serum setup a pool of healthy controls (HCs) a pool of schizophrenia (SZ) patients, and a pool of systemic lupus erythematosus (SLE) patients was included. For the CSF setup a pool with CSF of SZ patients, a pool possibly positive for alzheimer's disease (AD), with low amyloid-β and high Tau, and a pool with high amyloid-β and low Tau from patients possibly suffering from some kind of dementia. Pools can be used as an efficient and rapid method to determine a set of proteins (e.g. antibodies), which the patients within the pool have in common, and thereby deny or confirm a hypothesis.[58] All pools were tested against eight antigens, and both IgG and IgM antibodies were measured. The antigen panel consisted of five human herpes viruses (HHVs); EBNA-1, EBV VCA p23, CMV, HSV-1 and VZV, and three viruses in the MMR panel, Measles, Mumps and Rubella. The content of antibodies in the serum pools was determined by ELISA technique. Absorbance measurements (405/650 nm) were normalized to a standard curve, and arbitrary units (U) were defined. Serum pools were tested in appropriate dilutions defined in the pre-assay (IgG: EBNA-1 and measles 1:800, VCA p23, HSV-1, VZV, mumps and rubella 1:400) (IgM: all 1:100).

Table 6: Overview of the pools used in the measurements of antibodies directed against EBV and other human herpes viruses (HHVs), and the MMR panel.

Serum pools	No. of patients
HC	UN
RRMS	25
ON_OCB+	10
ON_OCB-	14
SZ	17
SLE	30

CSF pools	No. of patients
RRMS	25
ON_OCB+	10
ON_OCB-	14
SZ	17
AD (↓Amyloid-β, ↑Tau)	20
AD (↑Amyloid-β, ↓Tau)	20

Antibodies directed against EBV and other HHVs in the serum pools

Serum pools were examined for IgG and IgM antibodies against EBNA-1, which is the only EBV protein expressed in both latent and lytic modes of EBV infection, and the only protein expressed in the deepest latency state[59], and for VCA p23, which is a viral capsid protein expressed in the acute phase.[41]

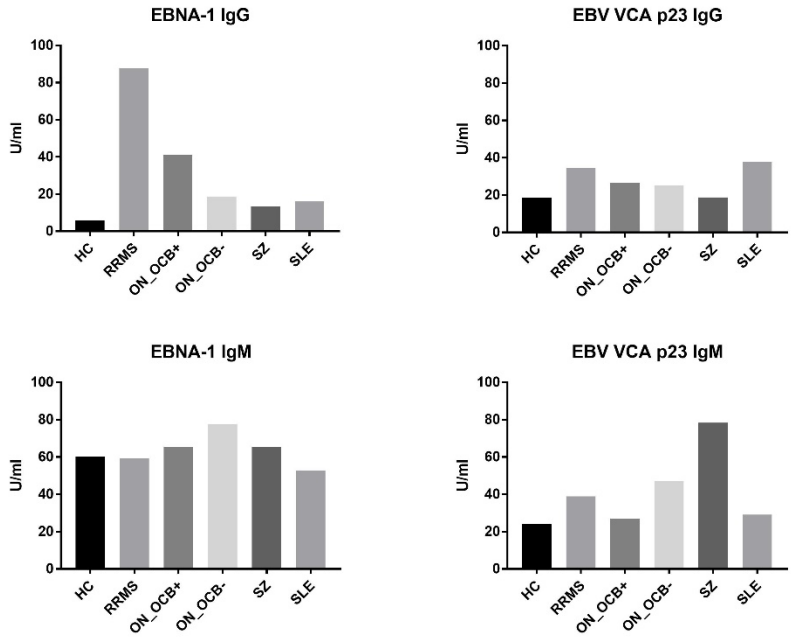

Figure 8: Prevalence of serum antibodies directed against EBNA-1 and EBV VCA p23. IgG and IgM antibodies were analyzed using ELISA technique, and U/mL was defined by the absorbance measurements (405/650 nm) normalized to a standard curve.

The results showed an elevated EBNA-1 IgG amount in the RRMS pool compared to the healthy control pool, and a minor elevation in the ON-OCB positive pool. The EBV VCA p23 IgG quantity is almost equal in the pools, only with a small increase in the RRMS pool and the SLE control pool. The quantity of EBNA-1 IgM is almost equal between the pools, whereas the amount of EBV VCA p23 IgM is raised in the SZ control pool.

Antibodies directed against three other HHVs were examined. CMV pp52 is an early lytic gene essential for lytic replication. The presence of antibodies directed against early antigens usually indicate an ongoing or recent lytic infection.[43] HSV-1 glycoprotein G (gG) is a virus envelope glycoprotein, and the presence of antibodies directed against it indicates a previous exposure of the virus.[60] The VZV antigen is kindly supplied from the Department of Virology at SSI, and is a cell lysate antigen.

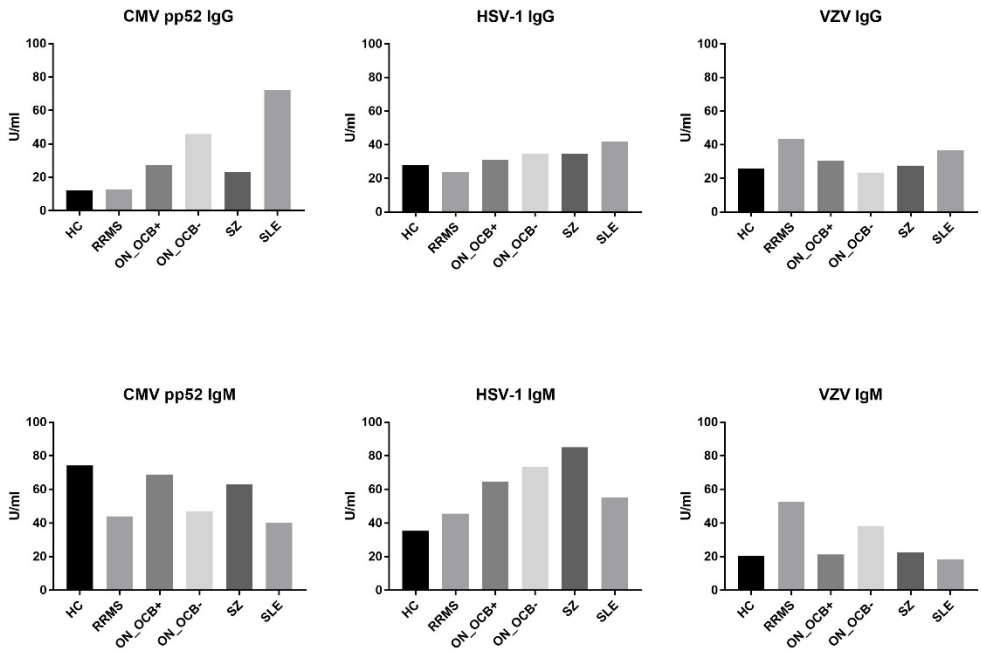

Figure 9: Quantity of serum antibodies directed against other herpes viruses, CMV, HSV-1 and VZV. IgG and IgM measurements were performed to detect antibodies in the serum pools. ELISA method was used and the absorbance measurements were normalized to a standard curve, and units were determined.

The quantity of antibodies directed against the three different human herpes viruses, showed an elevated amount of CMV pp52 IgG-directed antibodies in SLE patients, but no difference between RRMS patients and healthy controls. The amount of HSV-1 IgG antibodies were almost equal between the pools, and the VZV-directed IgG antibodies

were slightly increased in the RRMS pool compared to the healthy controls and the other pools. IgM antibodies directed against CMV and HSV-1 showed the highest titre in the healthy controls, and a low titre in the RRMS pools. VZV-directed IgM antibodies have the highest titre in the RRMS pool.

Antibodies directed against the MMR panel in serum pools

The MMR panel consists of three viruses, children in Denmark are vaccinated against. Measles virus, which causes erythematous rash known as measles. Mumps virus, which is the most common viral cause of parotitis, and rubella virus, which is the pathogenic agent of the disease rubella (also known as German measles).[51]

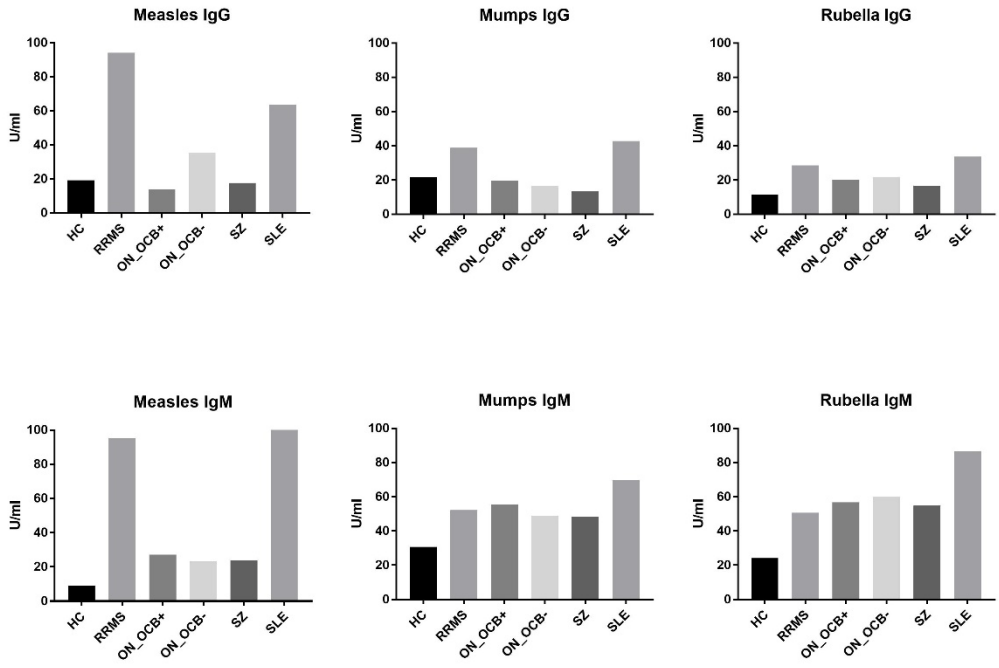

Figure 10: The amount of serum antibodies directed against the MMR panel. Serum pools were tested for IgG and IgM antibodies directed against the MMR panel, consisting of measles, mumps and rubella virus. ELISA method was used and the absorbance measurements were normalized to a standard curve, and units were determined.

The IgG and IgM antibody titres against measles are very high in the RRMS pool, and the titre is high in the SLE pool as well. The calculated units is almost 100 U/mL in the RRMS pool, compared to the healthy control pool, where units were below 20 U/mL. Antibodies directed against measles are definitely increased in RRMS patients. The quantity of antibodies directed against mumps is in general lower than the quantity of

antibodies against measles, but the pattern in the different pools is the same as for antibodies against measles. The amount of IgG antibodies against mumps have the highest titre in the RRMS pool and the SLE pool. Regarding IgM antibodies directed against mumps, the amount of antibodies are more equal between the pools, but the titre in the healthy control pool was the lowest. The quantity of antibodies directed against rubella shows the same pattern as the amount of mumps antibodies. The healthy control pool contains the lowest amount of antibodies directed against rubella, whereas the RRMS and SLE pools have the highest titres. Especially regarding the IgM antibodies against rubella, the SLE pool contains a high amount of antibodies.

Fractions of antibodies in the RRMS and control serum pools are shown in the figure below, and are used to get an overview of the composition of IgG antibodies in the pools. Comparison of the amount of specific antibodies in the RRMS pool, compared with the healthy control (HC) pool.

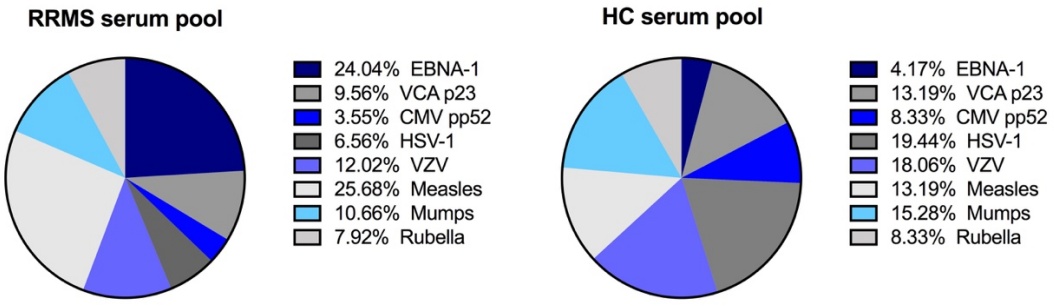

Figure 11: Fractions of the eight tested antibodies in the RRMS and control serum pool. The figure illustrates a big difference in the RRMS and healthy control pools regarding the fraction of IgG antibodies directed against EBNA-1 and measles.

In the RRMS pool, the most distinctive difference is that EBNA-1 and Measles are the dominant antibodies, and they comprise approximately 25% of the fraction each. Compared to the control pool, where EBNA-1 and measles antibodies only comprise 4% and 13%, respectively. Notably, the fraction scheme is made of the eight antibodies that are tested for, and not the total fraction of IgG antibodies.

Antibodies directed against EBV and other HHVs in the CSF pools

The quantity of IgG antibodies in CSF is in general about 500 times lower than in serum, and the quantity of IgM antibodies is about 5000 times lower.[17] The arbitrary units are

calculated using a serum standard curve in a concentration of 1:1000, and the CSF pools were tested in a 1:10 concentration. The IgM antibody titre was tested in the pools as well, but the absorbance measurements in both standard curves and in the pools, were below the detection limit. The results from the IgM antibodies are not shown, and it is assumed that the amount of IgM antibodies in CSF is close to zero.

An increased amount of antibodies within the CNS can be a consequence of a leaky blood-brain barrier (BBB), or it can be a result of intrathecal production maintained by B-cells in the CNS. Activation of a memory B-cells will cause the B cells to differentiate into antibody-producing plasma cells.[8]

EBV IgG antibodies against EBNA-1 and VCA p23 were measured in the CSF pools. The quantity of EBNA-1 directed antibodies was highest in the ON pool with positive OCB, and the amount in the RRMS pool is also higher, compared with the ON pool with negative OCB, and the three control pools. The level of VCA p23-directed antibodies is equal between all pools.

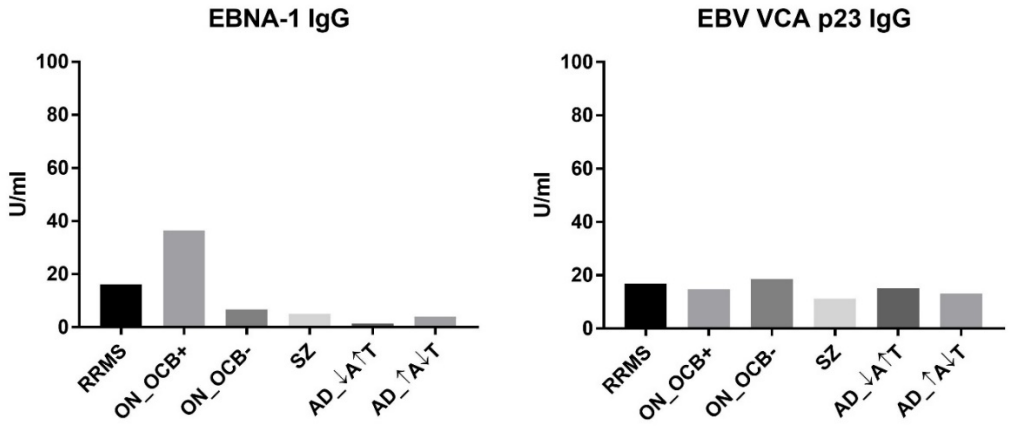

Figure 12: EBV directed antibodies in the CSF pools. EBNA-1 and VCA p23 IgG antibodies were analysed using ELISA technique, and U/mL was defined by the absorbance measurements (405/650 nm) normalized to a standard curve.

The CSF pools were also tested against CMV, HSV-1 and VZV (figure 13). The amount of CMV-directed antibodies have the highest titre in the ON OCB negative pool, and the amount is also slightly higher in the RRMS pool compared to the control pools. HSV-1-directed antibodies have in general a low titre, and the RRMS pool actually has the lowest

titre. Regarding the VZV-directed antibodies, it shows a very big difference of antibodies between the pools, and the RRMS pool has a very high titre. The calculated amount is above 100 U/ml, calculated by the formula:

$$U = \frac{\text{Absorbance(pool)}}{\text{Absorbance(highestStd.)}}\ x\ 100$$

The amount of VZV antibodies in the ON OCB positive pool is also increased compared to the control pools.

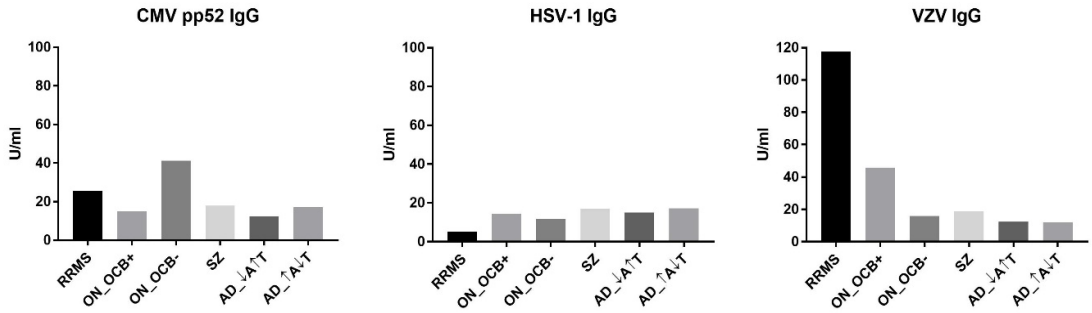

Figure 13: Quantity of CSF antibodies directed against other herpes viruses, CMV, HSV-1 and VZV. ELISA measurements were performed to identify antibodies in the CSF pools. The CSF pools were diluted 1:10, and normalized to a serum standard curve diluted 1:1000.

Antibodies directed against the MMR panel in CSF pools

Quantification of antibodies directed against the MMR panel in the CSF pools showed interesting results. The same pattern is revealed regarding antibodies directed against both measles, mumps and rubella. The RRMS pool has a highly increased amount of IgG antibodies against all three viruses in the MMR panel.

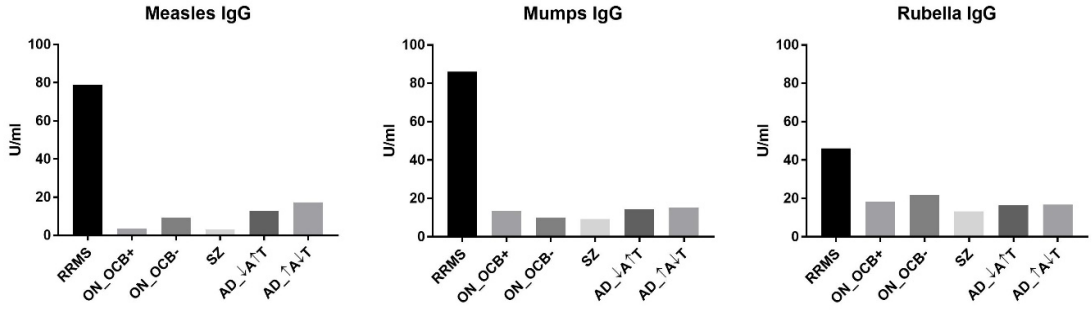

Figure 14: The amount of antibodies directed against the MMR panel in CSF pools. CSF pools tested for IgG antibodies directed against the MMR panel, consisting of measles, mumps and rubella virus. ELISA measurements were performed to identify antibodies in the CSF pools. The CSF pools were diluted 1:10, and normalized to a serum standard curve diluted 1:1000.

Like the comparison of antibody fractions in the serum pool, the CSF antibody fraction between the eight tested antibodies is used to compare the difference of antibody composition in the RRMS pool and a control pool (SZ).

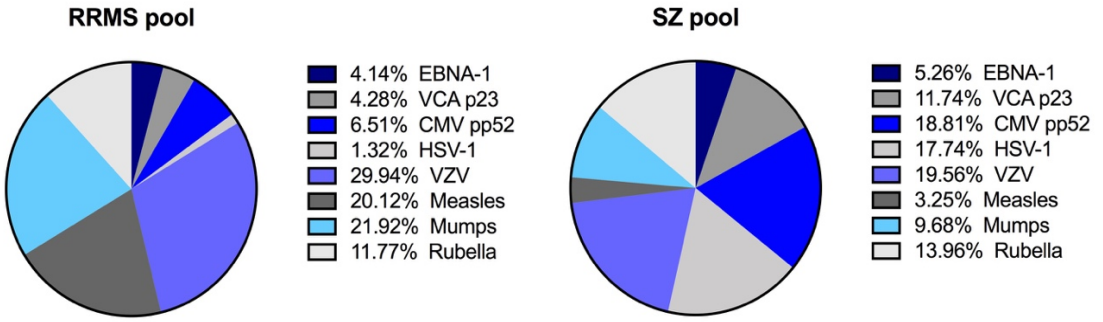

Figure 15: Fractions of the eight tested antibodies in the RRMS and control CSF pool (SZ). The RRMS CSF pool has a higher fraction of VZV, measles, mumps and rubella compared to the control pool (SZ), which have the highest fraction of CMV, HSV-1 and VZV antibodies.

The majority of antibodies in the RRMS CSF pool are directed against VZV, measles, mumps and rubella, compared to the control pool (SZ) where the antibodies directed against CMV, HSV-1 and VZV have the highest fraction. The fraction of EBNA-1 is relatively low in both pools, compared to the fraction of the other antibodies.

Total amount of IgG and IgM in serum and CSF pools

To quantify the amount of total IgG and IgM in the pools, an ELISA setup was performed. A kind of direct ELISA technique, where the microtiter wells were coated with the serum and CSF pools. The antibodies within the pools will adsorb to the surface of the wells in the microtiter plate, and by adding of secondary antibody (IgG and IgM) labelled with AP, detection of the total amount of IgG and IgM antibodies is possible. The absorbance measurements were normalized to a standard curve coated directly with human IgG or human IgM in known concentrations, and the immunoglobulin concentration in the pools was determined.

In order to compare the quantification of the total amount of IgG and IgM calculated by the ELISA setup, the pools were run in the nephelometry analysis on the ProSpec device, and the total amount of IgG and IgM antibodies was measured. The amounts of antibodies measured by the nephelometry analysis, were far from the results obtained in the performed ELISA setup. Only the serum pools were measured for comparison, due to lack of CSF material.

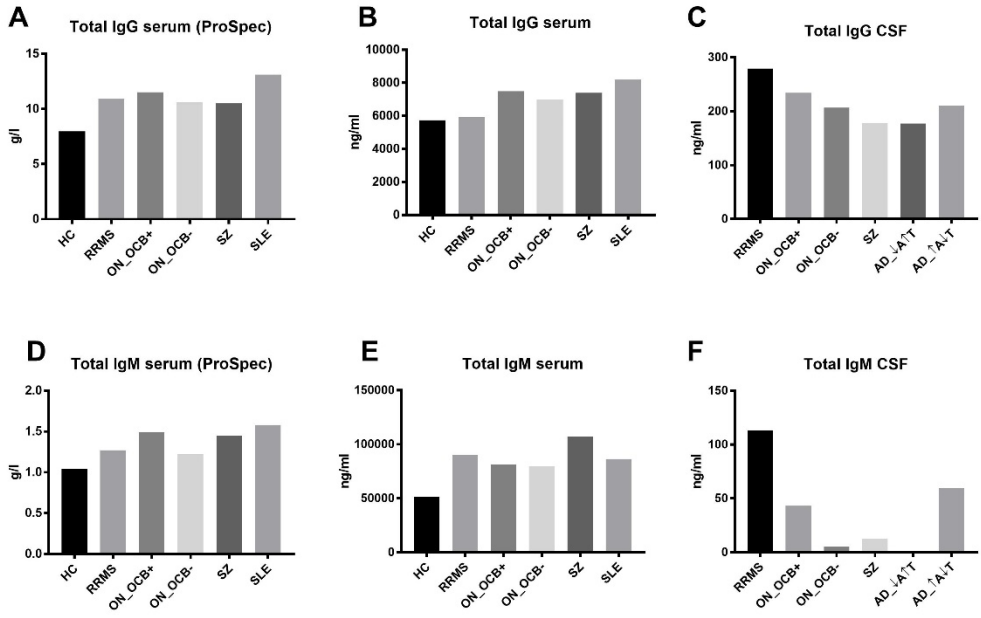

Figure 16: The total amount of IgG and IgM in serum and CSF pools. The quantity of IgG and IgM antibodies are measured by nephelometry at the ProSpec device (A and D), and determined by an ELISA setup where pools were coated directly in the wells, and the secondary anti-human AP-labelled antibody detects the amount of antibodies (B, C, E and F). The calculated concentrations are dissimilar, but the proportion between the pools are almost similar.

The amount of total IgG antibodies in the serum pools was very high in all the pools, as expected, no significant difference between the pools were seen. The amount of measured total IgG and IgM by the two methods varies significantly. The concentration of total IgG in the serum RRMS pool was determined to 5,9 mg/l by the ELISA method, and 10,9 g/l by the nephelometry analysis at the ProSpec device. Regarding IgM, the RRMS pool contains 90 mg/l (0,09 g/l) according to the ELISA assay, and the measured amount by ProSpec was 1,27 g/l. Despite the big difference in concentrations, the proportion of antibodies between the pools are almost similar. Comparison of figure 16A and 16B show the same patterns, with the lowest concentration of IgG in the healthy controls, and the highest concentration of IgG in the SLE pool. The proportion between the pools seen in figure 16D and 16E is almost equal as well.

In the CSF pools, the amount of total IgG antibodies was only measured by the experimental ELISA setup because of lack of material. The amount was highest in the RRMS pool, followed by the ON OCB positive pool. The amount of IgM antibodies in the serum pools was low in the healthy control pool compared to the pools of RRMS, ON, SZ and SLE. The quantity of total IgM in the CSF pools was in general very low, but the concentration was highest in the RRMS pool.

Antibodies directed against EBV, VZV and the MMR panel

Single samples were subsequently examined against a reduced panel of antigens, and only for IgG antibodies, according to the results from the measurements of the pools where IgM antibodies in CSF were undetectable using the ELISA assay that was set up. Sera and CSF from patients diagnosed with MS or ON were tested in pairs, and the samples were tested against IgG antibodies to EBNA-1, VZV, Measles virus, Mumps virus and Rubella virus – an extended MRZ panel[28] called EZMMR panel (by author).

Serum samples tested against the EZMMR panel

An indirect ELISA technique was applied for examination of the antibody concentration in the patient samples. To secure the best and most valid results, optimization tests were run to find the right concentration of standard curves, patient samples and control samples. Different serum samples were tested in different concentrations against all the antigens in the EZMMR panel. The best candidates to use as low positive and high positive controls (LPC and HPC) were found, and the dilution of the serum samples was chosen (EBNA-1 1:800, VZV 1:400, Measles 1:1000, Mumps 1:400 and Rubella 1:400).

Table 7: Serum samples tested against the EZMMR panel

Diagnosis	No. of patients	Average age	Gender	IgG-index	OCB status
RRMS	29	42 (23-58)	♀19/♂10	20 pos >0,7 9 neg <0,7 Avg. 0,98	26 OCB pos 3 OCB neg
ON	24	38 (23-56)	♀14/♂10	6 pos >0,7 18 neg <0,7 Avg. 0,71	10 OCB pos 14 OCB neg
Healthy controls (HC)	21	39 (22-61)	♀19/♂2	-	-

The serum patient samples were divided into three groups, comprising patients diagnosed with either relapsing-remitting MS (RRMS), Optic neuritis (ON) and healthy controls (HCs). The three groups have almost the same average age (38-42 years), and all with an overload of women.

Antibodies in serum samples directed against EBNA-1 and VZV

The three groups in the serum setup was tested against the two HHV antigens included in the EZMMR panel, EBNA-1 and VZV. Figure 17 illustrates the results with all measurements normalized to a standard curve, and arbitrary units were determined. The results revealed significantly higher titres of EBNA-1 IgG antibodies in RRMS patients compared to HCs (p=0,021). No significant difference was revealed in antibody titre of EBNA-1 between RRMS and ON patients, and between ON and HCs (p=0,280 and p=0,085, respectively). The amount of VZV-directed IgG antibodies revealed significant difference between RRMS patients and HCs (p=0,039), and no significant difference between RRMS and ON patients, and between ON and HCs (p=0,084 and p=0,731, respectively).

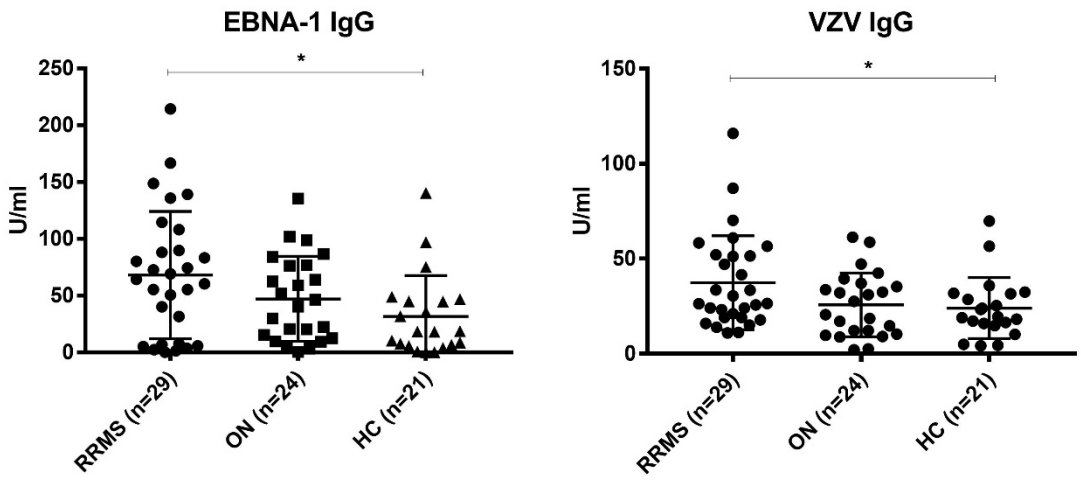

Figure 17: Quantity of antibodies directed against EBNA-1 and VZV in serum samples. ELISA was applied for testing antibodies directed against EBNA-1 and VZV in RRMS patients (n=29), ON patients (n=24) and heathy controls (n=21). The antibody titres are presented as units generated from a normalization to a standard curve. Middle horizontal bars represent the standard deviation (SD). Significant difference between the RRMS patients and the healthy controls are shown for both EBNA-1 (p=0,021) and VZV (p=0,039), using Mann-Whitney U test.

Antibodies in serum directed against the MMR panel

Antibodies directed against the viruses comprised in the MMR vaccine, are tested against the serum samples in the three groups. The quantity of antibodies directed against measles revealed significant difference between the RRMS and the ON patients (p=0,016), and no

significant differences between the RRMS and HC, or the ON and HC (p=0,052 and p=0,382, respectively). Antibodies directed against mumps showed a significant difference between the RRMS and ON samples as well (p=0,009), and no difference between RRMS and HC (p=0,072), despite visualization of the figure 18 regarding mumps, indicates that there is a difference between RRMS and HC samples. No significant difference between ON and HC samples was found (p=0,334). The amount of antibodies against rubella indicated no significant difference between the RRMS and ON, RRMS and HC or ON and HC samples (p=0,515, p=0,744 and p=0,546, respectively).

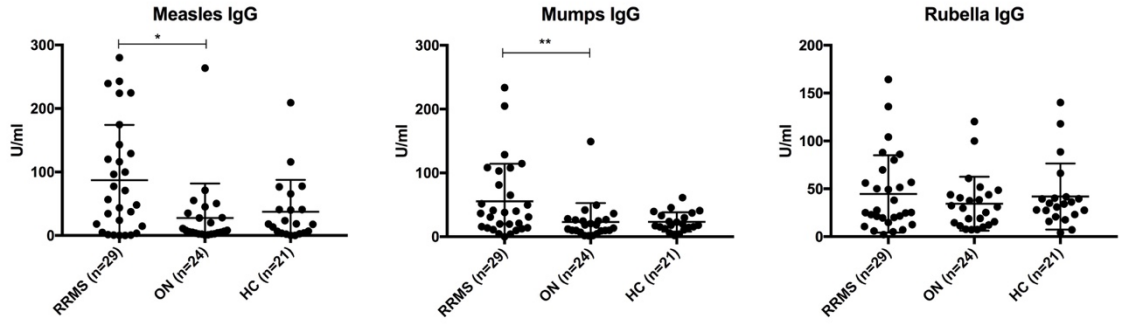

Figure 18: Quantity of antibodies directed against the MMR panel in serum samples. ELISA was applied for testing antibodies directed against measles, mumps and rubella in RRMS patients (n=29), ON patients (n=24) and heathy controls (n=21). The antibody titres are presented as units generated from normalization to a standard curve. Middle horizontal bars represent the standard deviation (SD). Significant difference between the RRMS patients and the ON patients are shown for both measles (p=0,016) and mumps (p=0,009), and no significant difference was found for antibodies directed against rubella. Mann-Whitney U test is used.

Inter-assay and intra-assay variation serum samples

Inter- and intra-assay variation test determine the robustness and reproducibility of the assay, and a low variation indicates that the assay can provide consistent data. The inter-assay variation reflects the variation of results obtained from repeated experiments, in this case a low positive control (LPC) and a high positive control (HPC) were included in six plates pr. antigen, and a CV% <15% is acceptable. The intra-assay variation describes the variation of results in the same experiment, in this case a patient sample repeated 10 times within the same plate. The acceptable CV% <10%.

Table 8: The inter-assay and intra-assay variation in serum measurements.

Inter-assay variation	EBNA-1	VZV	Measles	Mumps	Rubella
LPC CV%	11,4	8,5	6,9	18,6	8,9
HPC CV%	7,0	7,8	17,1	11,2	6,2
Mean CV% (LPC and HPC)	9,2	8,2	12,0	14,9	7,6
Intra-assay variation					
Patient sample CV%	11,1	8,3	8,8	10,1	11,1

The HPC in the measles assay is slightly above the 15%, but the calculated units were above 100, and it gives an inaccuracy of the measurement and the calculated CV%. The LPC in the mumps assay was also slightly above 15%. The assays were considered acceptable if the mean CV% of the LPC and the HPC was below 15%. The intra-assay variation showed CV% close to, and slightly above the 10%.

CSF samples tested against the EZMMR panel

To examine antibodies in the CSF samples, ELISA assays were performed. To secure the best and most valid results, optimization tests were run to find the right concentration of standard curves, patient samples and control samples. Different serum and CSF samples were tested in different concentrations against all the antigens in the EZMMR panel. The best candidates to use as low positive and high positive controls (LPC and HPC) were found, and the dilution of the CSF samples was chosen (EBNA-1 and measles 1:40, VZV, Mumps and Rubella 1:20). The units are calculated using normalization to a serum standard curve in a concentration of 1:2000 (EBNA-1) or 1:1000 (mumps, rubella and VZV), and because of the high amount of antibodies directed against measles in the serum pool, a new setup was performed, using a CSF pool containing RRMS patients as standard curve in a 1:20 concentration.

Table 9: CSF samples tested against the EZMMR panel

Diagnosis	No. of patients	Average age	Gender	IgG-index	OCB status
RRMS	29	42 (23-58)	♀19/♂10	20 pos >0,7 9 neg <0,7 Avg. 0,98	26 OCB pos 3 OCB neg
ON	24	38 (23-56)	♀14/♂10	6 pos >0,7 18 neg <0,7 Avg. 0,71	10 OCB pos 14 OCB neg
CSF controls (SZ)	17	28 (21-48)	♀6/♂11	1 pos >0,7 16 neg <0,7 Avg. 0,52	1 OCB pos 16 OCB neg

The CSF patient samples were divided into three groups, comprising patients diagnosed with either relapsing-remitting MS (RRMS), Optic neuritis (ON) or schizophrenia (SZ). SZ CSF samples are used as control samples, due to the complications getting CSF from healthy donors. IgG index was calculated for all the samples, and in the RRMS group 20 patients were above the reference value for IgG index (>0,7), indicating intrathecal synthesis of IgG, and 9 patients were below 0,7. Oligoclonal bands (OCBs) were also tested, and 26 patients in the RRMS group appeared positive in the OCB test, and only 3 RRMS patients had a negative OCB test. In the ON group, only 6 patients showed an elevated IgG index, whereas 18 patients were below the limit 0,7. Regarding the OCB test of the ON group, 10 patients were positive for OCB, and 14 patients were negative for OCB. As expected, the majority of SZ patients showed no intrathecal production of IgG, and only 1 patient (SZ group n=17) revealed an elevated IgG index and a positive test of OCB.

Antibodies in CSF samples directed against EBNA-1 and VZV

The quantity of antibodies directed against EBNA-1 and VZV in the CSF samples was measured using the same method as for serum samples. The results are shown in figure 19, and show significant difference in antibody titre between the RRMS patients and the SZ patients for EBNA-1 (p=0,029), but no significant difference between RRMS and ON patients, or ON and SZ patients (p=0,427 and p=0,166, respectively).

The amount of antibodies directed against VZV shows also a significant difference between the RRMS and SZ patients (p=0,002), and also between RRMS and ON patients (p=0,002). No significant difference between ON and SZ patients was shown (p=0,729).

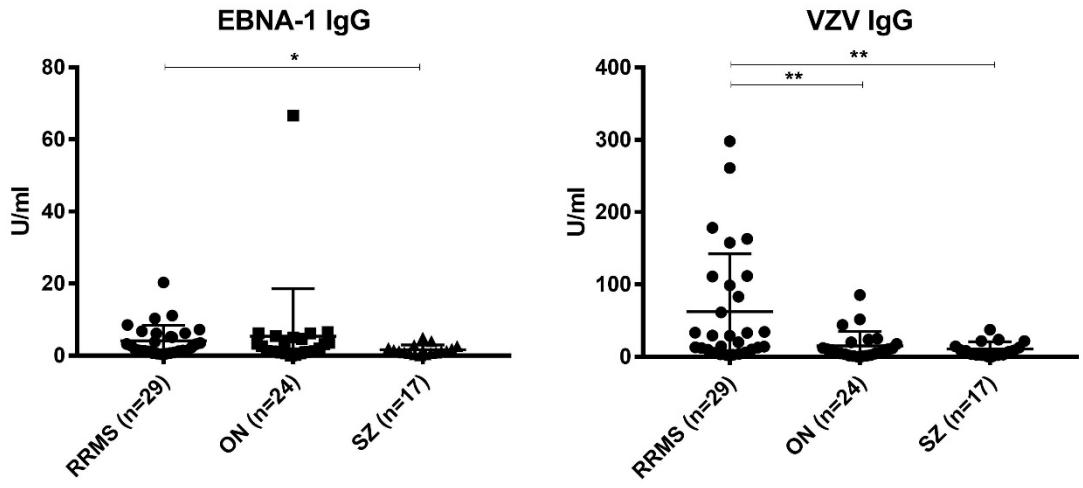

Figure 19: Quantity of antibodies directed against EBNA-1 and VZV in CSF samples. ELISA was applied for testing antibodies directed against EBNA-1 and VZV in RRMS patients (n=29), ON patients (n=24) and as controls, Schizophrenia (SZ) patients (n=17). The antibody titres are presented as units generated from normalization to a standard curve. Middle horizontal bars represent the standard deviation (SD). Significant differences between the RRMS patients and SZ patients are shown for both EBNA-1 (p=0,029) and VZV (p=0,002). The amount of VZV-directed antibodies reveal also significant difference between RRMS and ON patients (p=0,002). Mann-Whitney U test is used to determine the p-values.

Antibodies in CSF directed against the MMR panel

The quantity of antibodies against measles, mumps and rubella was measured in the CSF patient samples, and the results are shown in figure x. The amount of antibodies directed against measles are in general very high in the RRMS samples, and comparison of the three groups showed significant differences between both the RRMS and ON patients (p=0,001) and RRMS and SZ patients (p=0,002), but no difference between the ON and SZ patients (p=0,744). The antibody titre against mumps revealed the same pattern as for antibodies directed against measles, and the results showed a significant difference between the RRMS and ON patients, and RRMS and SZ patients (p=0,012 and p=0,023, respectively), and no difference between ON and SZ patients (p=0,964). Regarding antibodies directed against rubella, no significant difference was revealed between any of the groups, despite visualization of the figure of the amount of rubella antibodies indicate

a difference between RRMS patients and both the ON and SZ group (p=0,167 and p=0,101, respectively), and no difference between ON and SZ patients (p=0,699).

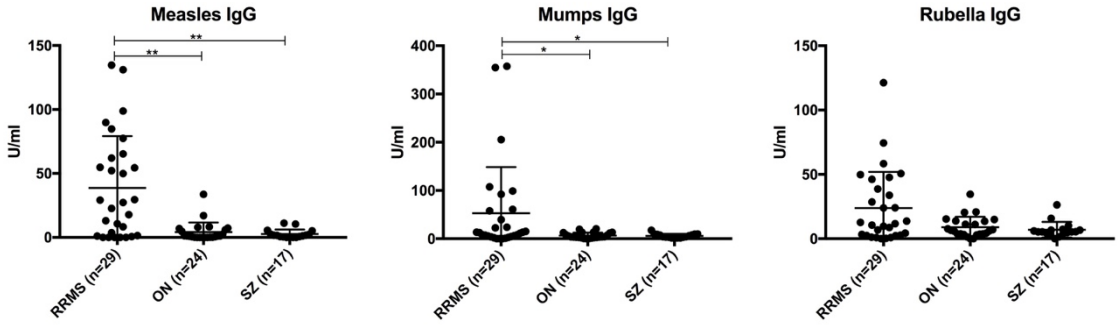

Figure 20: Quantity of antibodies directed against the MMR panel in CSF samples. ELISA was applied for testing antibodies directed against measles, mumps and rubella in RRMS patients (n=29), ON patients (n=24) and SZ (n=17). The antibody titres are presented as units generated from normalization to a standard curve. Middle horizontal bars represent the standard deviation (SD). Significant differences between the RRMS patients and the ON patients are shown for both measles (p=0,001) and mumps (p=0,012), and also significant difference between RRMS patients and SZ patients are shown for both measles (p=0,002) and mumps (p=0,023). No significant difference was found for antibodies directed against rubella. Mann-Whitney U test is used to determine the p-value.

Inter-assay and intra-assay variation CSF samples

As explained according to the serum assays, a low positive control (LPC) and a high positive control (HPC) were included in six plates pr. antigen, and a CV% <15% is acceptable of the inter-assay variation. The intra-assay variation is calculated using a patient sample, repeated approximately 10 times within the same plate. The acceptable CV% <10%.

Table 10: The inter-assay and intra-assay variation in CSF measurements.

Inter-assay variation	**EBNA-1**	**VZV**	**Measles**	**Mumps**	**Rubella**
LPC %CV	9,3	7,1	7,0	5,7	7,7
HPC %CV	11,8	4,2	6,2	4,1	8,7
Mean CV% (LPC and HPC)	10,6	5,7	6,6	4,9	8,2
Intra-assay variation					
Patient sample %CV	9,6	9,4	9,9	4,3	7,5

The calculation of inter-assay and intra-assay variation in the CSF setup shows impressive results. All the calculated CV% are below 15% for the inter-assay variation test, and below 10% for the intra-assay variation test.

Specific antibody index (AI)

The specific antibody index (AI) is calculated to the viruses that are included in the EZMMR panel (EBNA-1, VZV, measles, mumps and rubella). The formula for calculation of AI is:

$$\boldsymbol{AI} = \frac{\mathbf{QIgG[spec]}}{\mathbf{QIgG[total]}} \qquad \mathrm{QIgG[spec]} = \frac{\mathrm{CSFIgG[spec]}}{\mathrm{SerumIgG[spec]}} \qquad \mathrm{QIgG[total]} = \frac{\mathrm{CSFIgG[total]}}{\mathrm{SerumIgG[total]}}$$

The normal reference range for AI is between 0,7 and 1,3. Values of AI ≥1,5 indicates a local specific antibody synthesis in CNS, according to Reiber[61]. The AI index is calculated in 28 RRMS patients, and 23 ON patients.

Table 11: Positive antibody indices and the mean (range) AI for all five antigens

Positive AIs	RRMS (n=28)	Mean AI RRMS	ON (n=23)	Mean AI ON
EBNA-1	21 (75%)	6,64 (0,8-61,4)	22 (96%)	12,11 (0,7-95,9)
VZV	15 (54%)	3,16 (0,4-14,4)	7 (30%)	2,26 (0,2-10,9)
Measles	20 (71%)	3,10 (0,7-13,8)	20 (87%)	7,03 (0,1-76,7)
Mumps	9 (32%)	1,83 (0,0-14,0)	10 (43%)	2,29 (0,0-13,4)
Rubella	6 (21%)	1,20 (0,0-7,1)	5 (22%)	0,77 (0,0-2,5)

In accordance with the AI findings seen in table 11, the majority of RRMS patients have a positive AI for EBNA-1 (75%), and almost all ON patients are assumed having an intrathecal synthesis of EBNA-1 (96%). The AI findings for measles indicate a very high frequency of patients with an intrathecal production of IgG antibodies against measles (RRMS: 71%, ON:86%). The AI findings for all of the five antibodies in the EZMMR panel, indicate a minimum of 20% positive in each patient group. The mean values of the five AIs are also shown in table 11, and it reveals that the mean AIs for EBNA-1 is higher compared to the AI value for the other virus antibodies. The range of AI values for EBNA-1 varies a lot, and some of the values are extremely high, indicating a huge amount of intrathecal production of EBNA-1 antibodies.

Previous studies regarding the MRZ panel have varied in the conclusion of a positive or a negative MRZ reaction. Some studies have concluded the MRZ reaction positive with only one of the three AI positive, and other required two positive specific AIs.[54] In this study, the panel is extended to five parameters, and it is decided that a positive EZMMR reaction requires three or more positive AIs.

Table 12: Number of positive antibody indices in the patient samples

Patients with:	RRMS (n=28)	ON (n=23)
0 positive AI	1 (4%)	0 (0%)
1 positive AI	3 (11%)	4 (17%)
2 positive AI	11 (39%)	7 (30%)
3 positive AI	8 (29%)	5 (22%)
4 positive AI	4 (14%)	4 (17%)
5 positive AI	1 (4%)	3 (13%)

The results shown in table 12 illustrate the number of positive AIs in the patient samples, and it reveals that 13 of the RRMS patients (46%), and 11 of the ON patients (48%) are positive for the EZMMR reaction with 3 or more positive AIs. Additional, 24 of the RRMS patients (86%) and 19 of the ON patients (83%) are positive for the EZMMR reaction if only two positive AIs are required. A minor group of patients was only positive for one or zero AIs (RRMS: 14%, ON:17%). Statistical analysis is not performed due to lack of an appropriate control cohort.

MRZ reaction

The results obtained are tested for MRZ reaction as well. Out of the 28 RRMS patients, 3 patients were not positive for any AIs (11%), 11 patients have one positive AI (39%), 12 patients have two positive AIs (43%), and 2 patients were positive of all 3 AIs (7%). Specific intrathecal antibody production directed against measles is the most frequent in general. In the ON patient cohort, the MRZ reaction was tested on 23 patients, whereas 3 patients were not positive for any AIs (13%), 11 patients have one positive AI (48%), 6 patients have two positive AIs (26%), and 3 patients were positive of all 3 AIs (13%). If the requirement of a positive MRZ reaction is two specific positive AIs, 14 RMMS patients are positive (50%), and 9 ON patients are positive (39%).

Comparison of the two methods that are used to create standard curves

Two different standard curves were included in every plate during this study. The first method was the known approach where the wells in the microtiter plate are coated with the respective antigen, and then a serum or CSF pool in appropriate dilution is added. The other method is on an experimental basis, and the approach is that the wells are coated with human-IgG in known concentrations, diluted 2-fold. The stock concentration is known, and the highest standard is diluted to 0,025 µg/ml for serum samples and 0,01 µg/ml for CSF samples. The serum and CSF samples were normalized to both standard curves in every assay.

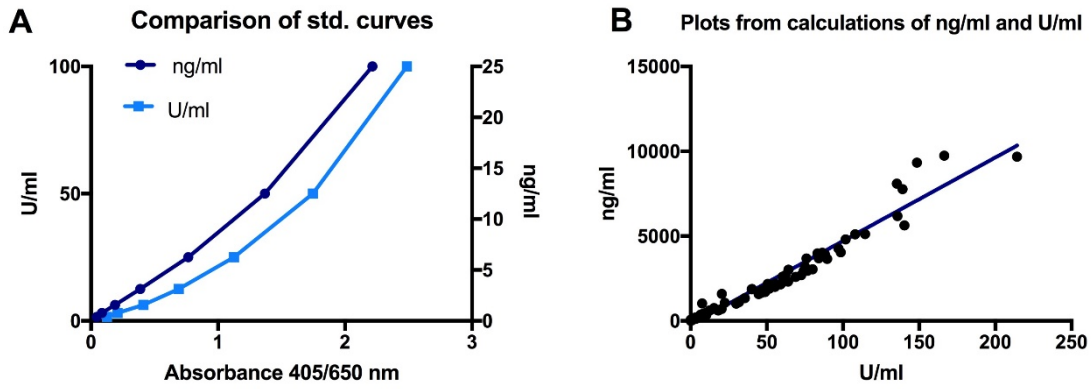

Figure 21: Comparison of the two approaches of making a standard curve. Representative figures from the result processing. A) Illustration of two standard curves, one was created by coating of antigen (measles in this case), and then addition of a serum standard pool, conjugate, and substrate, and then measurement of absorbance. The other standard curve was created using human IgG as coating in known concentrations, and then directly addition of conjugate before substrate addition and absorbance measurements. B) The absorbance measurements from a patient cohort (n=74) are normalized to both standard curves, and ng/ml and U/ml are determined and the values are plotted against each other in the B part of the figure.

The two standard curves go together, and demonstrate almost parallel slopes (figure 21A). In figure 21B, the calculated ng/ml and U/ml are plotted against each other, and the linear regression fits the plotted values well.

Discussion

In the beginning of this study, serum and CSF pools were screened against a broad panel of antigens, to determine which antibodies that are present, and which would be interesting to investigate further. The use of pools is a quick an efficient screening method to confirm or deny a hypothesis, and in this case, to determine which antibodies that different patient groups have in common. Sternbæk L. et al.[58] demonstrated the usefulness of pools, which enables an efficient and timesaving research method.

Screening phase

In the beginning of the screening phase, pools were tested for OCBs. One of the most interesting findings was that when approximately 100 OCB positive patient samples were pooled, the pool revealed no positive OCBs, indicating that the specificity of the OCBs varies a lot between patients. Despite the findings of positive OCBs in the CSF of approximately 90% of MS patients, the exact specificity of the antibodies within the OCBs remain undetermined. Many studies have investigated the OCB specificity in the past.[54,62-66] In the screening phase, different pools were tested for many antibodies, and the results led to a panel of antigens for further investigation. Selected results obtained in the screening phase are shown in appendix 1, and the results show raw absorbance measurements just to compare the difference between pools. The tested CSF pools in the screening phase showed in general a high amount of different IgG antibodies in the OCB positive pool (probably MS patients), against EBNA-1, VCA p23, measles, mumps and rubella, and against different respiratory viruses as well (different influenza subtypes). IgM antibodies in CSF indicated an interesting difference between MS patients and the various controls, and the amount of IgM antibodies in the pool with OCB positive patients showed an elevated level against EBV, CMV, HHV-6, measles, mumps, rubella, parainfluenza, HPV-6, MC virus and JC virus. Based on the results obtained in the screening phase, a panel of eight antigens was selected. The project was focused on different human herpes viruses (HHVs), and the measles, mumps, rubella viruses (MMR) that are included in the vaccine. The respiratory viruses, HPV-6, MC and JC viruses were deselected, out of necessity of limitation of the project.

Antibodies in serum and CSF pools

In order to determine the content of antibodies in the pools, ELISA assays were developed. In the serum setup, six pools were included, one containing patients diagnosed with RRMS, two different pools with patients diagnosed with ON, two pools with control diseases, and a pool with healthy controls (HCs). In the CSF setup six pools were included as well, but the biggest issue was that no pool with healthy controls was included, due to the complications getting CSF from healthy controls. It is easy to get blood samples from healthy controls, but a lumbar puncture is not under normal conditions performed on healthy individuals, given that it will induce more pain and physical discomfort. Because of that, CSF from other neurological diseases was used as control pools.

The investigation of antibodies in the different serum pools showed an elevated titre of EBNA-1 IgG, VZV IgG and IgM, measles IgG and IgM, and mumps IgG in the RRMS pool compared to the healthy control pool and the other disease controls. Regarding EBV antibodies, no big difference between pools was shown for EBNA-1 IgM, and only a slight difference was shown for EBV VCA p23 IgG, where the RRMS and SLE pools have the highest amount of antibodies. EBV VCA p23 IgM showed an elevated level in the control pool of serum from SZ patients. EBNA-1 antibodies indicate a latent state of EBV infection, whereas the presence of EBV VCA p23 antibodies indicate an active EBV virus infection. EBV is not supposed to have an impact on SZ patients.[67] Antibodies directed against the other three HHVs showed an elevated level of CMV pp52 IgG in the SLE patients, which indicated a recent CMV infection because CMV pp52 is expressed in the early lytic phase of infection. Other studies previously have demonstrated the connection between CMV and SLE.[43,68] Serum antibodies directed against the three viruses contained in the MMR vaccine were also investigated, and the results showed a huge amount of both IgG and IgM antibodies against measles in the RRMS and SLE pool, compared to the other pools. Mumps- and rubella-directed IgG antibodies also showed an elevated level in the RRMS and SLE pools, and regarding IgM antibodies also an elevated level in the SLE pool, but not in the RRMS pool. The SLE pool contains in general many antibodies against the tested viruses.

Measurements of IgG and IgM antibodies in the CSF pools were also performed, but the amount of IgM antibodies in the CSF pools was imponderable using the applied ELISA assay. When the measurements of background and unspecific binding from the uncoated wells were subtracted, the absorbance values of the CSF pools were negative, indicating very small amounts of IgM antibodies in the CSF pools directed against the tested viruses. The lack of being able to measure IgM is frustrating, because the search of IgM antibodies in the screening phase indicated a difference in IgM amount between MS patients and different controls. The quantity of IgG antibodies in the CSF pools were examined, and the results showed an extremely elevated amount of antibodies directed against VZV, measles, mumps and rubella in the RRMS pool compared to the other CSF pools. EBNA-1 IgG antibodies were also elevated in the RRMS pool, and even more in the ON OCB positive pool.

The amounts of total IgG and IgM were measured by two methods, and the amount of measured total IgG and IgM by the two methods varies significantly. The concentration of total IgG in the serum RRMS pool was determined to 5,9 mg/l by the ELISA method, and 10,9 g/l by the nephelometry analysis at the ProSpec device. Regarding IgM, the RRMS pool contains 90 mg/l according to the ELISA assay, and the measured amount by ProSpec was 1,27 g/l. It is an enormous difference, and raise doubts about the experimental ELISA setups usefulness. However, the big difference in the concentrations can probably be explained by 1) The coating methods, where serum pools were coated directly in the wells, and there is a big probability that only a limited amount of the total immunoglobulin in the serum sample has adhered to the bottom of the wells. 2) The absorbance measurements were normalized to the standard curve of directly coated human IgG in known concentration, and the same issue of the antibody adherence to the wells come into play. Both explanations will supplement each other in the explanation of a much lower IgG and IgM concentration measured by the ELISA setup. The difference in the calculated IgM concentrations was lesser compared to the IgG calculation, and can maybe be explained by the amount of IgM molecules, and the amount of IgG molecules in the sample. The IgM molecules are bigger than the IgG molecules, and there are fewer IgM molecules. Probably a bigger part of the IgM molecules is capable of adhering to the wells, and many IgG molecules are left within the liquid because of the huge amount, and

thereby washed away in the washing steps. Despite the big difference in concentration, the proportion of antibodies between the pools are almost similar, which indicates the method can be used as comparison of the Ig level between pools, but not the exact concentration.

Two standard curves were included in every experiment, and the measured absorbance values of the patient samples were normalized to both standard curves, determine either arbitrary units (U/ml), or a concentration (ng/ml). The calculated concentrations are probably way to low according to the measurements of the total IgG by the two methods, where the measured concentrations by the validated device ProSpec, were much higher than the calculated concentrations, using normalization to the human IgG coated standard curve. Like the determination of total IgG and IgM, the standard curve coated directly with human IgG is useful for comparison between samples, but not as an alternative to measuring the exact concentrations of immunoglobulins in a sample.

The most interesting results from the serum setup with pools, concerning RRMS patients, were IgG antibodies against EBNA-1, VZV, measles, mumps and rubella. Additionally, the results from the CSF setup showed exciting results of VZV, measles, mumps and rubella amounts in the RRMS pool. To confine the project, the most interesting antigens were selected for further investigation of individual samples, and the reduced panel of antigens consisted of EBNA-1, VZV, measles, mumps and rubella, named the EZMMR panel. Only IgG antibodies were further examined, due to the challenges of getting results from IgM measurements in CSF. In this project, one of the most important features was to determine the amount of antibodies in serum vs. CSF from the same patient, and because it was impossible to determine the IgM antibody titre in the CSF samples, the measurements of IgM antibodies in serum was omitted.

Antibodies directed against the EZMMR panel

Individual serum and CSF samples from patients diagnosed with RRMS or ON were tested against the EZMMR panel. As control samples, a cohort of healthy controls was used in the serum assays and a cohort of CSF from schizophrenia (SZ) patients was used

in the CSF assays. The CSF samples from SZ patients turned out to be an appropriate control cohort. The SZ patients were negative of OCBs, except one patient, and their IgG index is within the reference range, indicating no intrathecal synthesis of IgG. Research groups have previously demonstrated an increase of antibodies in serum directed against CMV and HSV, but not in the CSF of SZ patients.[69,70]

RRMS patients revealed significantly higher amounts of antibodies against EBNA-1 and VZV in serum, compared to the healthy controls. Measles, mumps and rubella are as mentioned viruses that children are vaccinated against in most countries.[50,71-73] The MMR vaccine consists of live attenuated viruses[50] that will activate the immune response, and for one thing cause memory B cells with affinity of the viruses. The amount of antibodies directed against measles and mumps showed significant differences between the RRMS patients and the ON patients, but no significant difference between RRMS patients and the HCs. P-values were slightly above 0,05, indicating the difference between the RRMS and HC patients almost were significant.

The difference between RRMS patients and both ON and SZ patients was evident in the measurements of antibodies within the CNS. The amount of antibodies directed against EBNA-1, VZV, measles and mumps were significantly higher in the RRMS patients compared to the SZ patients. Additionally, significant differences in the amount of VZV, measles and mumps were also shown between the RRMS and ON patients. The amount of rubella antibodies revealed no significant differences between patient groups, but by visualization of the graph, some RRMS patients have an elevated amount of rubella antibodies within the CNS as well.

The rubella antigen was suspected of not working correctly. The absorbance measurements were low compared to the four other antigens, maybe indicating a deficient adhering of the antigen to the wells. It also concerned the standard curve the patient samples were normalized to. The accuracy of the calculated units is probably acceptable, but the rubella antigen caused some doubts during the process.

The results revealed in general an elevated amount of antibodies against the EZMMR panel in RRMS patients compared to ON and control patients, especially regarding the CSF results. To quality-assure and validate the performed assays, and thereby the findings of antibodies in the patient samples, it would have been useful to include a control antigen, where no reaction and differences between patients were expected. All the included antigens in the EZMMR panel were expected to show a difference between RRMS patients and controls, according to previous studies.[20,28,56,57,74] Furthermore, inclusion of control samples with known concentrations of antibodies directed against the antigen panel could have been useful as well, for instance a known positive control and a known negative control with absorbance values that should be within a certain range. The LPC and HPC samples used in this project had no acceptance limits of absorbance, but still, they are useful to determine the inter-assay variation between the plates, which was acceptable.

Specific antibody indices (AIs)

The specific antibody index was calculated for all the RRMS and ON patients, and reveal whether the specific antibodies were synthesized within the CNS, or synthesized in the peripheral circulation and crossed the BBB. The AI values of EBNA-1 and measles indicated an intrathecal production in more than 70% of RRMS and ON patients, and the specific intrathecal antibody production of EBNA-1 was almost present in all RRMS and ON patients, supporting EBV's important role in MS. To fully validate the EZMMR panel requires a suitable control cohort, because the patient cohorts of RRMS and ON patients were both expected to have intrathecal production of antibodies. The cohort of SZ patients could have been appropriate as a control cohort, but the amount of antibodies was only measured in CSF and not in serum. Due to a deadline of this project, it was not performed. Hottenrott et al.[54] compared different subtypes of MS, with other inflammatory neurological diseases (OIND) and found a significant difference between the MS patients and the OIND patients.

The EZMMR panel compared with the MRZ panel

The frequency of a positive EZMMR reaction is determined with both requirements of two positive AIs and three or more positive AIs, and the MRZ reaction requires two or more positive AIs according to Jarius et al.[64]. Table 13 shows the comparison of positive EZMMR reactions and positive MRZ reactions in the RRMS and ON patient cohorts.

Table 13: Comparison of positive EZMMR reactions and positive MRZ reactions

Positive	EZMMR 3 pos AI	EZMMR 2 pos AI	MRZ 2 pos AI
RRMS	46,4%	85,7%	50,0%
ON	52,2%	82,6%	39,1%

If a positive EZMMR reaction requires three positive AIs, the amount of positive EZMMR and MRZ reactions are almost equal regarding RRMS patients, and the frequency of positive EZMMR reactions is slightly higher compared to positive MRZ reactions in ON patients. If the EZMMR panel only requires 2 positive AIs, the amount of positive RRMS patients is 86% and 83% ON patients, and then the EZMMR panel reveals an increased sensitivity compared to the MRZ panel. An important aspect is the specificity of the test, and due to lack of healthy control patients it could not be determined in this study. Both the sensitivity (number of true positive) and the specificity (number of true negative) are important aspects when a new test is invented, and both values must be as close to 100% as possible. Is the EZMMR panel more useful than the MRZ panel? According to the amount of positive AIs regarding EBNA-1, the EZMMR panel is more useful than the MRZ reaction. If the OCB status is included in the valuation of the EZMMR panel requiring two positive AIs, the EZMMR panel reveals pivotal results. Three of the RRMS patients were negative of OCB, but they were positive of the EZMMR reaction. It gives a sensitivity regarding RRMS patients of 100% if combining OCB and EZMMR determinations. Though, more RRMS patients with negative OCBs are necessary to determine if the EZMMR and OCB in combination have a sensitivity of 100%. Regarding the ON patients, the sensitivity is 85% with the combination of EZMMR panel and OCB presence.

A previous study[62] investigated a MRZH panel, where HSV-1 was included, but the AI index for HSV-1 were only elevated in 18% of the MS patients, compared to measles AI which they found elevated in 52% of the investigated MS patients. That substantiate the deselection of HSV-1.

The impact of different virus antibodies in MS pathophysiology

The big question is how the different viruses influence the pathophysiology of MS. The intrathecal production of antibodies requires antibody-producing B cells within the CSN, and the question of how and when they enter the CNS is still unanswered. One possible explanation could be that EBV-infected B cells are capable of entering the CNS during an acute infection with EBV, and another theory could be that the MMR vaccine with live attenuated neurotropic viruses causes an inflammation in the CNS after vaccination, and then B cells infected with EBV are invoked into the CNS as a consequence of neurological inflammation. EBV is a known impact factor of MS, and the association between infectious mononucleosis (IM), caused by EBV, and MS is reported in several studies.[56,57,74-77] Previous studies have not revealed an elevated intrathecal production of antibodies in patients suffering from other diseases with a neuroinflammatory condition, and this group of patients is often used as controls in MS studies.[54] If the MMR vaccine itself was a causative agent of why B cells entering the CNS, more people would probably reveal an intrathecal production of antibodies. The most reasonable explanation is that EBV infects B cells, and EBV is also capable of infecting brain microvascular endothelial cells (BMVECs)[15], which is a major constituent of the BBB. During an EBV infection, the BBB may be more permeable, and cells of the immune system are able to enter the CNS. In IM, EBV can infect up to 20% of an individual's B cells, and the B cells can be in different stages of differentiation (memory B cells, plasma blasts or already antibody-secreting plasma cells). The B cells that are infected with EBV can be all kinds of memory or plasma B cells, and produce different antibodies against different viruses. That depends on recently infections, vaccines, or in general which B cells that are present in the circulating system during an EBV infection. Latent EBV in the circulatory system can reactivate either spontaneously or by contact with virus particles from the outside, and it would probably also cause reactivation within the CNS, but reactivation can also influence the BBB and B cells (and other cells) from the circulating system, that can get

access to CNS. The influx of immune cells into the CNS will cause inflammation, and according to MS, maybe plaques in the white matter and relapses in the illness.

An important factor could also be the time aspect of infection with EBV, in combination with the MMR vaccine. It is known that EBV infects the majority of children in the early childhood, and children are given the MMR vaccine by the age of 15 months and again by the age of 4 years.[50] If a child that is recently infected with EBV are given the MMR vaccine, maybe the combination can influence the later risk of developing MS.

The usefulness of serum antibodies in the diagnosis of MS

Biomarkers from a blood sample are extremely relevant in the early stages of a disease, because it is easy and almost painless to take a blood sample. Are there any biomarkers that can contribute to the diagnosis of MS in a blood sample? To compare the amount of antibodies directed against the EZMMR panel between MS patients and healthy individuals, a quantitative method was invented where the average amounts of antibodies in the healthy controls were used as a cut-off value, and compared to the antibody amount in the RRMS and ON patients. The average calculated units were used, and are shown in table 14.

Table 14: The average quantity (calculated units) of serum antibodies in RRMS and ON patients, compared to the healthy controls (HC). And the amount of positive RRMS and ON patients according to a value above the average value of HCs.

Serum	EBNA-1	VZV	Measles	Mumps	Rubella
HC cut-off (units)	**31,6**	**24,0**	**37,3**	**23,3**	**42,0**
RRMS (units)	68,1	87,1	55,6	44,7	37,4
ON (units)	47,1	27,7	23,2	34,5	25,7
RRMS positive (> cut-off)	**75,0%**	**60,7%**	**60,7%**	**60,7%**	**42,9%**
ON positive (> cut-off)	**56,5%**	**52,2%**	**21,7%**	**60,9%**	**30,4%**

The applied method to compare the antibody amounts is not validated, but it can give an outline of the situation, and indicate if there is something to investigate further. Actually, the quantity of patients above the cut-off value is higher than expected. Especially regarding EBNA-1, where 75% of RRMS patients have higher amounts of antibodies

compared to the average amounts of antibodies in the HCs. In the RRMS patients, also antibodies against VZV, measles and mumps showed elevated quantities, with 60,7% of the patients above the cut-off value. In general, RRMS patients have a higher amount of antibodies in serum, and it could be interesting to investigate further. If patients with MS in general have a higher amount of EBNA-1-directed antibodies and a higher amount of antibodies directed against other neurotropic viruses in serum, a blood sample could be helpful to clinicians to confirm or deny a possible suspicion of MS in patients with newly diagnosed neurological symptoms, and maybe contribute to the diagnosis of MS. The sensitivity and specificity are important to take into consideration in this assay as well.

Conclusion

In the current study, the immune response towards EBV and other neurotropic viruses was investigated in serum and CSF samples from patients suffering from multiple sclerosis (MS) and optic neuritis (ON) compared to various control groups. An antigen panel called the EZMMR panel (EBV (EBNA-1), VZV, measles, mumps, rubella) was defined, and the results showed a significant elevated level of serum and CSF IgG antibodies directed against EBNA-1, VZV, measles and mumps in RRMS patients, but no difference regarding rubella antibodies. The specific antibody indices (AI) were calculated, to determine if the specific antibodies were intrathecal synthesized. The sensitivity of the EZMMR panel regarding RRMS patients was approximately 50% if three positive AIs were required, and approximately 85% if only two positive AIs were required. Compared with the MRZ panel where only 50% of the RRMS patients had two positive AIs, the EZMMR panel revealed improved sensitivity. Three RRMS patients with negative OCB reveal positive EZMMR, indicating a sensitivity of 100% with combination of OCB and EZMMR. More patients and further investigations are needed. Unfortunately, it was not possible to determine the specificity.

In conclusion, the results obtained in this study supports the theory of the impact of viruses according to MS etiology, and especially EBV. Numerous research groups have investigated the impact of EBV, vaccines and the MRZ reaction, but not included and compared all of the viral factors and their relations. It is still unknown if the viruses are the causative agents, and how they are related according to MS, or the increased prevalence of antibodies just is a consequence of the disease.

Future investigations and perspective

A lot of additional investigations might be relevant to the findings in this project. Further investigation of the EZMMR panel with more patients, especially MS patients with negative OCBs to determine the sensitivity of the assay. A control cohort in the calculation of AIs is important to determine the specificity of the assay.

Obviously, a bigger antigen panel could be interesting, and even more important, inclusion of control antigens with no expected reaction. An assay capable of measure IgM in CSF are very relevant, and calculation of specific IgM antibody index. Furthermore, despite the applicability of schizophrenia as CSF controls, CSF from healthy individuals would be appropriate to include in the setup. Other subtypes of MS (PPMS, SPMS and CIS), and additional patient samples from patients suffering from other neurological diseases could be useful to include, and investigate the antibody level in the different stages of MS. The list of desirable experiments in the future is long, and MS is a complex disease that occupy many researchers.

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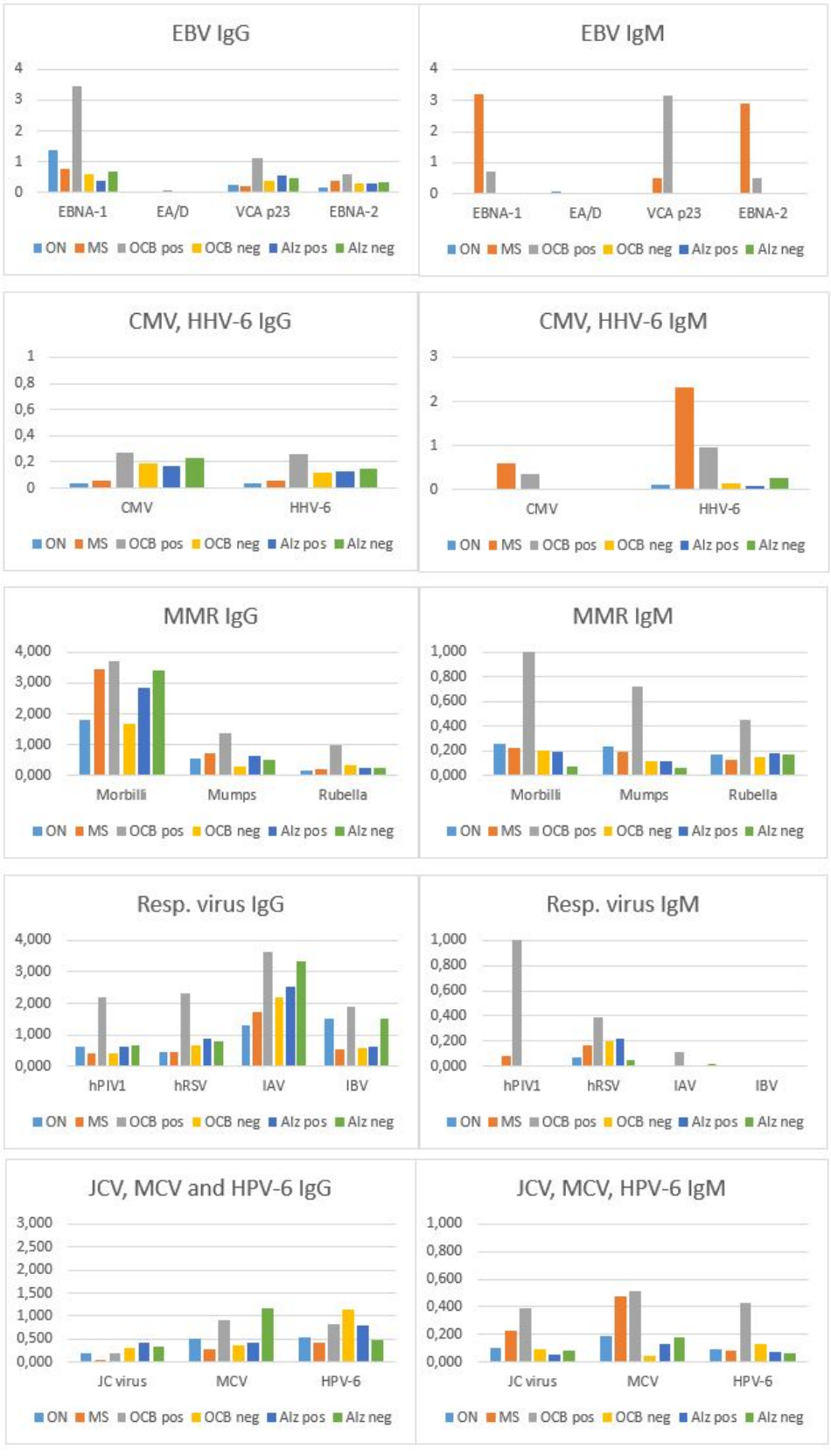
Appendix

Appendix 1: Figures from the screening phase

Serum pools: raw absorbance measurements (IgG 10 min, IgM 24 hours) 405/650 nm

CSF pools: raw absorbance measurements (IgG 30 min, IgM 24 hours) 405/650 nm



Appendix 2: Tables of calculated AI indices

Patient	EBNA-1 AI	VZV AI	Measles AI	Mumps AI	Rubella AI	QAlb	EZMMR pos	MRZ pos
MS_1	**26,15**	1,49	**2,29**	1,44	0,74	0,0047	2	1
MS_2	ND	ND	ND	ND	ND	ND	ND	ND
MS_3	0,92	1,49	**1,87**	0,64	0,00	0,0039	1	1
MS_4	**2,61**	**2,71**	**7,17**	0,11	0,00	0,0026	3	2
MS_5	0,94	**4,71**	1,26	**2,14**	1,03	0,0070	2	1
MS_6	**4,70**	**14,38**	**5,18**	**14,00**	**2,02**	0,0029	5	3
MS_7	0,82	**7,92**	**5,24**	0,36	0,00	0,0037	2	2
MS_8	1,44	1,49	**4,59**	**5,15**	**7,12**	0,0044	3	2
MS_9	**61,39**	1,10	**1,81**	**5,08**	0,86	0,0055	3	1
MS_10	**1,68**	**5,19**	**7,19**	1,19	0,48	0,0048	3	2
MS_11	**1,66**	**6,27**	**3,35**	1,14	0,69	0,0100	3	2
MS_12	**1,88**	**3,01**	**2,48**	**1,52**	0,48	0,0039	4	2
MS_13	**21,55**	**2,18**	**2,20**	**2,61**	1,22	0,0056	4	2
MS_14	**1,67**	0,55	1,28	0,68	0,56	0,0109	1	0
MS_15	**3,21**	1,40	0,91	0,00	**4,79**	0,0046	2	1
MS_16	**1,56**	**1,77**	0,74	0,57	0,60	0,0085	2	1
MS_17	**1,76**	**9,53**	**3,60**	**4,47**	1,00	0,0033	4	2
MS_18	**3,72**	**2,17**	**2,29**	1,18	**1,85**	0,0120	4	3
MS_19	**2,06**	1,22	**2,02**	0,84	1,23	0,0036	2	1
MS_20	**2,06**	**2,22**	**3,36**	0,83	0,91	0,0029	3	2
MS_21	**10,43**	0,61	1,41	1,20	0,22	0,0045	1	0
MS_22	**14,39**	**4,56**	**1,68**	0,56	0,29	0,0108	3	2
MS_23	1,08	**5,08**	**2,82**	0,77	0,54	0,0051	2	2
MS_24	0,84	1,32	1,46	0,44	0,00	0,0039	0	0
MS_25	**2,90**	1,40	0,89	0,00	**3,82**	0,0046	2	1
MS_26	**3,37**	**2,77**	**3,22**	1,29	0,72	0,0030	3	2
MS_27	**8,10**	0,65	**13,77**	0,25	0,61	0,0079	2	1
MS_28	**1,95**	0,40	0,82	1,36	**1,75**	0,0109	2	1
MS_29	1,12	0,86	**1,97**	**1,52**	0,18	0,0068	2	1
Mean	6,64	3,16	3,10	1,83	1,20		2,5	1,5
MS (28)	21 pos	15 pos	20 pos	9 pos	6 pos			

Patient	EBNA-1 AI	VZV AI	Measles AI	Mumps AI	Rubella AI	QAlb	EZMMR pos	MRZ pos
ON_1	**4,46**	0,92	0,80	1,28	0,01	0,0045	1	0
ON_2	**3,06**	**2,36**	**3,17**	**3,06**	0,00	0,0019	4	2
ON_3	**1,70**	0,54	**1,95**	0,30	0,00	0,0054	2	1
ON_4	**2,60**	1,29	**3,92**	1,40	0,83	0,0065	2	1
ON_5	**18,97**	**10,93**	**3,59**	**6,44**	1,30	0,0045	4	2
ON_6	**21,57**	**6,48**	**6,72**	1,39	0,00	0,0045	3	2
ON_7	**3,50**	1,07	**1,97**	**2,90**	**1,72**	0,0052	4	2
ON_8	**2,99**	**1,92**	**6,29**	**2,57**	**1,57**	0,0036	5	3
ON_9	**3,43**	1,15	**10,09**	**2,18**	0,00	0,0020	3	1
ON_10	**1,72**	1,49	1,31	0,80	0,50	0,0115	1	0
ON_11	ND	ND	ND	ND	ND	ND	ND	ND
ON_12	**95,87**	0,22	**4,94**	1,47	0,00	0,0042	2	1
ON_13	**2,45**	1,04	**5,83**	**2,67**	0,76	0,0100	3	1
ON_14	**5,29**	1,01	**2,59**	**3,85**	**2,49**	0,0055	4	2
ON_15	**7,28**	0,81	**4,04**	1,00	1,38	0,0027	2	1
ON_16	**35,51**	**1,79**	**1,77**	1,08	0,87	0,0073	3	2
ON_17	**11,46**	1,18	**10,92**	1,41	0,00	0,0024	2	1
ON_18	0,65	1,04	**3,80**	0,31	0,60	0,0055	1	1
ON_19	**6,13**	1,16	**1,53**	1,31	0,98	0,0065	2	1
ON_20	**4,00**	**6,20**	**2,85**	**1,69**	**1,60**	0,0051	5	3
ON_21	**2,35**	**7,79**	**76,67**	**13,35**	**2,03**	0,0047	5	3
ON_22	**8,11**	1,13	**5,27**	**1,54**	0,57	0,0059	3	1
ON_23	**20,53**	0,27	0,11	0,00	0,44	0,0039	1	0
ON_24	**14,90**	0,19	**1,56**	0,61	0,04	0,0037	2	1
Mean	12,11	2,26	7,03	2,29	0,77		2,8	1,4
ON (23)	22 pos	7 pos	20 pos	10 pos	5 pos			

Appendix 3: Methods used in the screening phase

Western blot

Western blot for observation of CMV and VZV directed antibodies

At first, a reference gel was made to control the amount of protein present in the cell lysates, in order to perform the correct dilution of cell lysates in the Western blot analyses. A 4-20% Tris-glycine gel (1,00 mm x 10 wells, Novex Life Technologies, CA, USA) was placed in a mini gel tank (Invitrogen by Thermo Fischer Scientific Inc., Mass, USA) and 1:10 dilution of running buffer (Tris glycine SDS 10x, Invitrogen by Life Technologies, Ca, USA) was poured in both chambers. CMV and VZV cell lysates were diluted 1:3 using sample buffer (0.05M Tris-HCl, 10 % glycerol, 2 % SDS, 0.1M DTT, 0.0625% Pyronin G, SSI Dianostica, Copenhagen, Denmark). Each sample was reduced 2 min at 95°C. 10 µl cell lysate were added into each well, and the gel ran for 2 h at 50 V and 250 mA, and later 30 min at 100 V and 250 mA. The gel was incubated overnight at 4°C in Coomassie Brilliant Blue (Gel code Blue Stain Pierce, PIER24592, Thermofischer Scientific, Illinois, USA), and washed with milli-Q water 5 times until all bands were coloured.

Western blot detection of CMV and VZV antibodies

After determination of the correct dilutions of the cell lysate, the Western blot for detection of CMV and VZV antibodies in patient and control samples was performed. TTN buffer (0.05M Tris, 1% Tween 20, 0.3M NaCl, pH 7.5, SSI diagnostic, Copenhagen, Denmark) was used for blocking of membrane, washing, and dilution of samples and conjugate. 10% Tris-glycine gel gel (1,00 mm x 10 wells, Novex Life Technologies, CA, USA) was placed in a mini gel tank (Invitrogen by Thermo Fischer Scientific Inc., Mass, USA) and a 1:10 dilution of running buffer (Tris glycine SDS 10x, Invitrogen by Life Technologies, Ca, USA) was poured in both chambers. Full cell lysates of CMV and VZV (CMV strain AD 169, diluted 1:20.000 and VZV-82 GA 7370-10, diluted 1:4500, grown in GMK cells, SSI Diagnostica, Copenhagen, Denmark) were diluted respectively 1:10 and 1:3 in reference to the control blot, using sample buffer (0.05M Tris-HCl, 10 % glycerol, 2 % $NaC_{12}H_{25}SO_4$, 0.1M $C_4H_{10}O_2S_2$, 0.0625% Pyronin G, SSI Dianostica, Copenhagen, Denmark). The samples were reduced by heat at 95°C in 2 minutes. 5 µl of the standard, and 120 µl of the diluted sample was added into the wells. The gel ran for 1

h at 150V and 250 mA. Afterwards the gel was blotted onto a membrane using Iblot™ (Invitrogen by Thermo Fischer Scientific Inc, Mass, USA), and afterwards the membrane was incubated overnight in TTN buffer at 4°C. The membrane was placed in a miniblotter, and the samples were added in separate wells. Serum samples were diluted 1:100 in TTN buffer, and CSF samples was diluted 1:10 in TTN buffer, and 320 µl sample were added into the respective wells and incubated 1 h at RT on a shaking table. Washes were performed for 2 x 5 min, first in wells at the mineblotter, and afterwards the membrane was washed in a Petridish. The membrane was incubated with Alkaline phosphatase (AP)-conjugated anti-human IgG, diluted 1:2000, for 1 h on a shaking table, and washed 3 x 5 min. After the wash, the membrane was incubated with the substrate BCIP/NBT (BCIP 0.5 mg/ml, NTB 0.3 mg/ml, Sigma, Saint-Louis, USA) diluted to 1 mg/ml in milliQ water, in 8 minutes, and the reaction was stopped by putting the membrane into milliQ water and dried on filterpaper.

Line blots

EBV

Line blot assay *recom*Line EBV IgG (Mikrogen Diagnostik, Neuried, Germany) were used for detection of IgG antibodies against different EBV antigens (EBNA-1, VCA (Viral capsid antigen) p18 and p23, IEA (immediate early antigen) immunodominant partial sequence of the ZEBRA protein, BZLF1 (IEA) complete ZEBRA protein, EA (early antigen) p138 and p54. The assay was carried out according to the protocol from the manufacturer, using the supplied reagents. Washing buffer (0,8 g skimmed milk powder, 16 mL wash buffer A concentrate, 144 mL milliQ water) was used for washing in all steps. Test strips were placed in 2 mL washing buffer, and 20 µl serum samples (dilution 1:100) and 50 µl CSF samples (dilution 1:40) were added into the respective wells, and incubated for 1 hour on a shaking table at RT. After 3 x 5 minutes washing, the ready to use conjugate was added, and incubated for 1 h. Washing of the blots were preformed, and “Ready to use” substrate was added and incubated 10 min for IgG and 1 h for IgM. The reaction was stopped by aspirating the liquid from the wells, and washing 3 x 1 min with milliQ water. Blots were air dried and evaluated.

To.R.C.H.

Line blot assay Anti-To.R.C.H. profile (EUROLINE, EUROIMMUN medizinische labordiagnostika AG, Lübeck, Germany) was used for detection of IgG and IgM against 5 different antigens. To.R.C.H.; Toxoplasma gondii (lysate of sonicated and gamma irradiated Toxoplasma gondii tachyzoites), Rubella virus (Inactivated cell lysates of Vero cells infected with the HPV-77 strain of Rubella virus), CMV (*E.coli* expressed CMV phosphoprotein), HSV-1 (Glycoprotein C1) and HSV-2 (Glycoprotein G2). Assays were carried out according to the protocol from the manufacturer, using the supplied reagents. Universal buffer was diluted 1:10 in milliQ water, and was used for blocking, washing and dilution of samples, controls and enzyme conjugate (for IgM; samples and positive control were diluted using IgM sample buffer, containing IgG/RF absorbent). Washing was performed 3 x 5 min, and blocking for 15 min. All incubation steps were on a shaking table at RT. Serum samples were diluted 1:51 and CSF samples 1:31, and the samples were added into respective wells. Incubated 30 min, and afterwards the blots were washed, and incubated with enzyme conjugate diluted 1:10 for 1 h. Washing of the blots was performed, and "Ready to use" substrate was added and incubated 10 min for IgG and 1 h for IgM. The reaction was stopped by aspirating the liquid from the wells, and washing 3 x 1 min with milliQ water. Blots were air dried and evaluated.