DOCTORAL THESIS

Immunodominant Antibody Epitopes Associated with the Development of Multiple Sclerosis and Type 1 Narcolepsy – A Next-Generation Phage-Display Analysis

Helle Sadam

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Helle Sadam

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Hulgiskleroosi ja tüüp-1 narkolepsiaga seotud immunodomineerivate epitoopide analüüs järgmise põlvkonna faagidisplei abil

HELLE SADAM

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List of publications

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Author's contribution to the publications

I. The author contributed to the experimental design, performed the critical experiments, analysed and interpreted the study data and wrote the manuscript.

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Sadam, H., Liivas, U., Kazantseva, A., Pruunsild, P., Kazantseva, J., Timmusk, T., Neuman, T. and Palm, K. 2016. GLI2 cell-specific activity is controlled at the level of transcription and RNA processing: Consequences to cancer metastasis. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1862 (1), 46−55. doi: 10.1016/j.bbadis.2015.10.008

Kazantseva, A., Sepp, M., Kazantseva, J., **Sadam, H**., Pruunsild, P., Timmusk, T., Neuman, T. and Palm, K. 2009. N-terminally truncated BAF57 isoforms contribute to the diversity of SWI/SNF complexes in neurons. *J Neurochem.,* 109(3):807-18. doi: 10.1111/j.1471-4159.2009.06005.x

Introduction

The immune system changes and adapts to the progressive steps of disease. Monitoring the changes in immune response over time would give feedback on the condition of the body's health. For example, detection of circulating autoantibodies holds a great promise to support diagnosis, prediction, classification and monitoring of various diseases. Here, the next generation random peptide phage display method of Mimotope Variation Analysis was used for the identification of antibody epitopes associated with diseases.

Neuroimmune diseases are a heterogeneous group of diseases including autoimmune and neuroinflammatory conditions, neuroinfections, neoplastic, neurodegenerative and neuropsychiatric disorders. Multiple sclerosis (MS), the most common neurological disease in young adults, is an immune-mediated demyelinating and neurodegenerative disease of the central nervous system with heterogeneous clinical presentations. This condition causes permanent cumulative disability, impairs quality of life, and shortens the life span. Up to 20% of MS patients experience optic neuritis (ON) as the presenting symptom, but not all ON patients will develop MS. The cause of MS is multifactorial, but often includes exposure to infectious pathogens. MS is an autoimmune disease, whilst the targeted antigens are largely unknown. Narcolepsy type 1 (NT1) as another chronic neurological disease is characterised by excessive daytime sleepiness, disturbed nocturnal sleep, and cataplexy associated with the inadequate function of the hypothalamus. The major neuropathological feature of spontaneous and Pandemrix influenza vaccine-induced NT1 (Pdmx-NT1) is the selective loss of orexin-producing neurons. Even though recent studies have identified autoantibodies against several neuronal proteins as targets, no specific antigens have been identified thus far.

In these studies, we demonstrated the antibody epitope profiles of patients with ON and the risk to progress to MS. We discovered a novel autoantibody target representing a biomarker for patients with Pdmx-NT1. The results of the studies contribute to the understanding of mechanisms underlying antibody-mediated disorders of the central nervous system.

Abbreviations

A*β*	amyloid-*β*
AChR	acetylcholine receptor
AD	Alzheimer's disease
AD2	antigen domain 2
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APC	antigen presenting cell
BBB	blood-brain barrier
BCL6	B-cell lymphoma 6 protein
BCR	B cell receptor
BCSFB	blood–cerebrospinal fluid barrier
BSCB	blood–spinal cord barrier
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
CTRL	control
DAMP	damage-associated molecular patterns
dot ELISA	enzyme-linked immunospot
DP1	D-prostanoid receptor 1
DP2	D-prostanoid receptor 2
EBNA1	Ebstein Barr virus nuclear antigen 1
EBV	Ebstein Barr virus
ELISA	enzyme-linked immunosorbent assay
gB	glycoprotein B
H1N1	influenza A virus subtype
HA	hemagglutinin
HD	Huntington's disease
HIV1	human immunodeficiency virus type 1
HHV6	human herpesvirus 6
HLA	human leukocyte antigen
HSV1	human herpesvirus 1
IEDB	The Immune Epitope Database
IFN	interferon
IFN*γ*	interferon gamma
IgG	immunoglobulin G
IL	interleukin
IL-1*β*	interleukin 1 beta
mAB	monoclonal antibody
MBP	myelin basic protein
MC	mast cell
MG	myasthenia gravis
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MRZ	measles, rubella, and varicella-zoster viruses

MS	multiple sclerosis
MS^{ON}	MS patients with ON onset
MS^{other}	MS patients with other MS onset
MV	Measles morbillivirus
MVA	Mimotope Variation Analysis
NGS	next generation sequencing
NMDA	*N*-methyl-D-aspartate
NT1	narcolepsy type 1
OCB	oligoclonal bands
ON^{MS}	ON patients later diagnosed with MS
ON^{ON}	patients with isolated optic neuritis
OX	orexin
OXR1	orexin receptor 1
OXR2	orexin receptor 2
P18 VCA	virus capsid antigen p18
PAMP	pathogen-associated molecular patterns
PD	Parkinson's disease
PD	phage display
Pdmx	Pandemrix influenza vaccine
Pdmx-NT1	Pandemrix influenza vaccine-induced narcolepsy type 1
PGD2	prostaglandin D2
PNS	peripheral nervous system
PPMS	primary progressive MS
PRR	pattern recognition receptors
PV	poliovirus
RABV	Rabies lyssavirus
REM	rapid eye movement
RRMS	relapsing–remitting MS
SFV	Semliki Forest virus
SPMS	secondary progressive MS
VZV	Varicella-zoster virus
TNF	tumour necrosis factor
WNV	West Nile virus
WB	Western blot

1. Molecular immunology of neurological disease

Our immune system is able to find everything that is foreign to our body and initiate its removal. This provides an effective shield against different infections and enables the removal of damaged or faulty cells. There are certain differences in the neuroimmune response compared to the peripheral response, e.g. the presence of physical barriers, the absence of traditional lymphatics and the lack of major histocompatibility complex (MHC) class II-expressing antigen presenting cells (APCs) (Mayne et al., 2020). The primary immune system which protects central nervous system (CNS) tissues from pathogens, is composed of CNS-resident immune cells (Beardsley & Hauser, 2014; Miller et al., 2016). However, after an injury, peripheral immune cells, including B and T lymphocytes migrate to the CNS and the following neuroinflammation is mediated by a complex crosstalk between CNS-resident and infiltrating immune cells (Mitoma & Manto, 2020). Excessive inflammatory responses can induce and accelerate pathogenesis of autoimmune demyelination, neuroinflammation, neuroinfectious, neoplastic, neurodegenerative, neuropsychiatric and traumatic conditions (Amor et al., 2014; Kempuraj et al., 2017; Stephenson et al., 2018). Depending on the immune response of affected areas, the major features include disruption of the nervous system homeostasis, neurodegeneration, loss of sensory and motor functions, impaired cognitive and behavioural functions and social skills (Mitoma & Manto, 2020). Treatment of neuroimmune diseases is most efficient when started early, but the effective biomarkers for identifying people at risk of neuroinflammation pathology, in particular the biomarkers measurable in the blood, are largely unknown (Mitoma & Manto, 2020).

1.1 Triggering pathogenic neuroimmunity

The pathogenic neuroimmune response can be elicited by a wide variety of factors including infections, neoplasms, traumas, and neurodegenerative diseases. First, immune-mediated inflammatory damage in CNS may be caused by several viral, bacterial, fungal, and parasitic infections that induce inflammation (**Table 1**) (Forrester et al., 2018; Mitoma & Manto, 2020).

Table 1. CNS Compartments Targeted by Neurotropic Pathogens *(Forrester et al., 2018; Klein & Hunter, 2017).*

	Meningitis	**Encephalitis**
Viruses	Enteroviruses West Nile virus Japanese encephalitis virus Human immunodeficiency virus* Mumps virus Measles virus Ebola virus* Cytomegalovirus*	Herpes simplex virus 1* Cytomegalovirus* Measles virus West Nile virus Japanese encephalitis virus Human immunodeficiency virus* Enteroviruses Zika virus Tick-borne encephalitis virus Varicella-zoster virus JC virus Influenza A Ebola virus* Rabies virus*
Bacteria	*S. pneumococcus* *L. monocytogenes* *H. influenza* *N. meningitidis* *T. pallidum* *M.tuberculosis**	*L. monocytogenes* Nocardia species *T. pallidum*
Fungi	*C. neoformans*	*Candida albicans* *Aspergillus niger* Zygomycetes Fusarium species *Pseudallescheria boydii* *Pigmented molds* *Histoplasma capsulatum* *Coccidioides immitis* *Penicillium marneffei*
Parasites	*Parasites Angiostrongylus* *Trichinella spiralis*	*Toxoplasma gondii** *Naegleria fowleri* *Trypanosoma cruzi* *Trypanosoma brucei* (sp) *Taenia solium*

**Can persist as latent infection within the CNS or the CNS-covering tissues.*

Infections with neurotropic pathogens typically occur in skin, mucosa, gut, or lung, via inoculation, ingestion, or inhalation with eventual dissemination to the CNS. The temporal nature of these events ensures that the initial activation of immune system occurs in the periphery and influences the generation of pathogen specific T and B cell-associated mechanisms (Klein & Hunter, 2017). On the other hand, the nervous system is also seriously affected by infections from the periphery since there are several means by which immune signals can access the CNS (Yang et al., 2020). Infectious triggers may create a pro-inflammatory state and prime the activation of both innate and adaptive immune response and may contribute to the development of neuroimmune diseases

(Forrester et al., 2018). For example, human herpes simplex virus 1 (HSV1) can migrate into the CNS and cause herpes simplex encephalitis (HSE), a devastating disease of the CNS (Menendez & Carr, 2017). Additionally, increasing evidence supports the hypothesis that infectious agents may provide an initial trigger to generate CNS-specific autoimmune response by molecular mimicry, epitope spreading and bystander activation (Mentis et al., 2017). Antibodies mostly generated towards pathogens in the periphery mediate neuroimmunity by intruding into CNS and recognising neural proteins (cell surface, cytoplasmic or nuclear antigens) (Mentis et al., 2017; Platt et al., 2017) (more about this in chapters 1.5 and 1.6). These autoantibodies may induce neurological disease via various pathogenic mechanisms including disruption of protein-protein interactions, receptor internalization, antibody-dependent cell toxicity, complement-mediated cell lysis, increased phagocytosis and opsonisation by microglia and mononuclear phagocytes (Lehmann-Horn et al., 2017).

Second, tumours may trigger immune activation in the brain. The immune response directed against onconeural antigens that closely resemble neural antigens contributes to tissue damage and leads to the development of neuroimmune conditions (Melzer et al., 2013). In case of *N*-methyl-D-aspartate receptor (NMDAR) encephalitis (NMDARE) in patients with ovarian teratomas, the tumours expressing NMDAR trigger peripheral immune response against the named onconeural antigen (Graus & Dalmau, 2008). Subsequent blood brain barrier (BBB) permeability (more about this in the chapter 1.2) would then allow anti-NMDAR antibodies to target glutamatergic synapses also containing this receptor (Dalmau et al., 2011). However, not all NMDARE patients have a cancer-related onset – the tumours rate is approximately 50% for NMDARE patients (Dalmau et al., 2011).

Third, head traumas (Kipnis, 2016) and neurodegenerative diseases (Scheiblich et al., 2020) are associated with activation of innate immunity through damage-associated molecular patterns (DAMPs). DAMPs include damaged, misfolded and aggregated proteins as amyloid-β (Aβ) and tau in Alzheimer's disease (AD), α-synuclein in Parkinson's disease (PD), and huntingtin in Huntington's disease (HD) (Scheiblich et al., 2020). This implies that the impaired clearance of deteriorated proteins from the brain could be a shared phenomenon in traumatic brain injury and neurodegeneration by inducing inflammation. For example, a rare variant in gene triggering receptor expressed on myeloid cells 2 (*TREM2*) and dysfunction in TREM2 were recently reported to increase the risk of AD and other neurodegenerative diseases (Bertram, 2015). TREM2 is a cellular receptor that recognises DAMPs and is a key factor participating in the clearance of dying cells, myelin debris and aggregated proteins (Bertram, 2015).

1.2 CNS barriers

The differential susceptibility of anatomical regions to neuroinflammatory events is associated with different barriers (Wilhelm et al., 2016). Namely, the CNS is separated from periphery by the blood-brain barrier (BBB) or blood-spinal cord barrier (BSCB), the blood-cerebrospinal fluid barrier (BCSFB) at the choroid plexus, and the arachnoid barrier (pial-CNS barrier), which is a part of the BCSFB separating the CSF-filled subarachnoid space from brain parenchyma. Within the CNS, the BBB and BCSFB restrict the migration and diffusion of cells (e.g. peripheral myeloid cells), pathogens, and macromolecules (e.g. large molecular weight serum proteins) into the brain parenchyma (Wilhelm et al., 2016). Differences in the structure of the barriers, as well as differences in the cranial and spinal meninges, in white and grey matter, and other regional

differences may explain site-specific disease-sensitivity patterns. CNS barriers effectively prevent the entry of most bacterial, fungal, and viral pathogens. However, neurotropic viruses, fungi, and certain parasites (**Table 1**) are able to readily access the CNS parenchyma (Mitoma & Manto, 2020). Pathogens can enter the CNS via multiple routes: i) viruses may directly infect the cells comprising the barriers, followed by release into the parenchymal space (for example, neurotropic RNA viruses, including poliovirus (PV), measles virus (Measles morbillivirus; MV) and some flaviviruses) (Koyuncu et al., 2013); ii) alternatively, viruses may diffuse across permeable regions of the BBB. For example, peripheral West Nile virus (WNV) acts through the engagement of Toll-like receptor 3 (TLR3) to induce the synthesis of cytokines, including tumour necrosis factor (TNF) by circulating antigen presenting cells. TNF reduces BBB integrity by loosening tight junctions, allowing for WNV migration through the less-restrictive BBB (Wang et al., 2004). Notably, not all cytokines induce the permeability of barriers. For example, interferons beta (IFNβ) and lambda (IFNλ), which are also produced in infected hosts, help to keep the barrier intact (Daniels et al., 2014). Thus, the relative types and ratios of cytokines that are synthesized in response to various infections will affect barrier integrity differently; iii) In the 'Trojan horse' mode viruses can traffic across the BBB or BCSFB into the brain parenchyma by infecting lymphocytes or monocytes (including macrophages)(McGavern & Kang, 2011). A classic example of this mode of invasion is provided by the human immunodeficiency virus type 1 (HIV1): $CD16^+$ monocytes, permissive for HIV1, traffic across the BBB and release virions that can then infect CNS microglia (McGavern & Kang, 2011); iv) transneuronal migration of viral particles, which involves the transport of viral genomes and associated proteins via microtubules and molecular motors in peripheral nervous system (PNS) neurons (Kramer & Enquist, 2013; Ugolini, 2011). For example, rabies virus (Rabies lyssavirus; RABV) from the muscle can move across the neuromuscular junction (Ugolini, 2011) and HSV, after infecting epithelial cells in the oral mucosa, spreads from there to sensory and autonomic ganglia, establishing lifelong latency (Kramer & Enquist, 2013).

Antibodies and immune cells reach the brain parenchyma *via* four distinct routes: i) systemic cytokine-associated breakdown of tight junctions within the BCSFB (for example, TNFα from the systemic inflammation in periphery); ii) olfactory ensheathing glia facilitate transport of IgGs or immune cells along sensory axons exiting the olfactory mucosa; iii) inflammatory cytokine-associated damages of tight junctions between endothelial cells; iv) Fc (Ig fragment crystallisable region) receptor directionality reversing enables shuttling IgGs from vessels into brain parenchyma (Platt et al., 2017). The BCSFB is less tightly regulated than BBB and can become more permeable to immune cells or antibodies during disease. For example, in mouse models for systemic lupus erythematosus (SLE), the cell adhesion molecules vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) show elevated expression in the choroid epithelium and this promotes entry of large amounts of cellular infiltrates (T and B cells) into the CSF to initiate damaging immune attacks (Zameer & Hoffman, 2003). Dysfunction of CNS barriers is well known to occur in neuroinflammatory disorders, including multiple sclerosis (MS), PD, AD, stroke, epilepsy and traumatic brain injury (Abbott et al., 2010), and is associated with activated endothelial cells that display an altered phenotype and a decrease in tight junction proteins. These changes that are also observed during ageing (Gorlé et al., 2016) explain the increased susceptibility to neuroinflammation and neurodegenerative disorders of the elderly.

The BBB can also be a target of pathogenic immune response like in neuromyelitis optica (NMO), where autoantibodies to aquaporin 4 (AQP4) damage astrocytes that otherwise protect the BBB integrity (Stephenson et al., 2018).

1.3 Innate immunity in the CNS

The biggest challenge in neuroimmunity is the fact that the host cannot tolerate the loss of neurons. HSV1 infection of epithelial cells results in massive cell death (Paludan et al., 2011). However, the lost cells are readily replaced, as observed in the healing that follows a cold sore. If lysis of irreplaceable neurons would occur in the same manner, neural circuits could become compromised, and, depending on the magnitude of damage, and cause permanent impairment. Thus, the activated immune response in the CNS must be very strictly controlled and efforts must be made to save neurons even if they carry an infection. This may also be the reason why protein aggregates are not correctly cleared in the CNS and neurons are more sensitive to pathologies associated with protein aggregates (Stephenson et al., 2018).

Innate immunity is the first line of defence in infection, but also plays a key role in tissue repair, clearance of apoptotic cells and cellular debris, as well as in response to tumours (Stephenson et al., 2018). Regional or cellular heterogeneity in innate immune response of neurons and astrocytes might underlie observed differences in pathogen spread, persistence, and immunopathology in different regions of the CNS. For example, cortical neurons have low levels of factors (including RIG-like intracellular pattern recognition receptors (RIG-I, MDA5), and IFN-induced proteins (ISG54 and ISG56)) that activate antiviral defence programs and therefore are more permissive to WNV entry than these in the hindbrain (Daffis et al., 2007). Thus, differences in the baseline expression of genes involved in responsiveness to cytokines (type I IFNs) and in susceptibility to viral infections might also help to explain the tropism of viruses for different cell types and regions.

While the key innate immune cells in the CNS are microglia and astrocytes, other cells such as macrophages, natural killer cells and mast cells (MC) as well as oligodendrocytes and neurons all contribute to innate immune responses in the CNS (Stephenson et al., 2018). Microglia are responsible for maintaining brain homeostasis by supporting neurons with growth factors and neurotrophic factors (Boza-Serrano, 2018). In addition, microglia cells scan their microenvironment to recognise and defend the brain against external challenges (Colonna & Butovsky, 2017). To perform this function, microglia are equipped with specialised pattern recognition receptors (PRRs) that sense a variety of microbial molecules called pathogen-associated molecular patterns (PAMPs) and host-derived DAMPs. Thus, microglia quickly adapts to immunological challenges by inducing a finetuned inflammatory response (Scheiblich et al., 2020). Innate immune receptors such as TREM2 are key to aiding clearance of dying cells, myelin debris and aggregated proteins (Bertram, 2015). Microglia secrete pro-inflammatory as well as anti-inflammatory factors, which can either be beneficial or detrimental in neuroinflammatory diseases (Wes et al., 2016). For example, microglia depletion in a mouse model of AD reduced neuronal loss without affecting AD pathology (Spangenberg et al., 2016). Microglia appears to have different transcriptomic profiles dependent on the region of the brain, age, (Grabert et al., 2016) and also neuropathological state (Wes et al., 2016).

All the other neural cell types, including neurons, astrocytes and oligodendrocytes, are capable of initiating and responding to inflammatory responses during infection (Nair & Diamond, 2015). All these cells express TLRs, Nod-like receptors (NLRs), RIG-like

receptors (RLRs), mitochondrial anti-viral signalling (MAVS) proteins, AIM2-like receptors (ALRs), C-type lectin receptors, and cytokine receptors (Kigerl et al., 2014). Activation of many of these innate immune pathways converges on expression of type I IFNs (Faul et al., 2010) and mononuclear cell chemoattractants, and there are many studies that illustrate the relevance to CNS infection (Miller et al., 2016).

The complement system is an ancient part of the immune system, which protects from microbes, removes debris, and promotes cell survival, and is often considered a link between the innate and adaptive immune response. There are high constitutive levels of complement proteins present in the CNS, which contribute to normal neuronal function and microglial activity (Veerhuis et al., 2011). For example, microglia are activated after WNV infection, leading to upregulation of complement receptor 3 (CR3) and expression of complement component C1qa (Schartz & Tenner, 2020; Vasek et al., 2016). Activation of the complement cascade is also a main response to antigen-bound autoantibodies (IgG1 and IgG3 class immunoglobulins) (Tüzün & Christadoss, 2013). For example, the extracellular domain of the α1 subunit of the skeletal muscle acetylcholine receptor (AChR) is the target of autoimmune response in patients with myasthenia gravis (MG) (Kordas et al., 2014). When bound to antigens, anti-AChR antibodies can activate the complement cascade via their Fc domains through interactions with complement proteins C1 and C1q (Tüzün & Christadoss, 2013). This complement cascade culminates in the formation of the membrane attack complex which insertion into the muscle end plates disrupts the phospholipid bilayer, resulting in loss of the junction folds and AChRs from the surface (Tüzün & Christadoss, 2013). Anti-complement therapy has shown effectiveness for the treatment of MG in a clinical trial (Gilhus et al., 2019).

After the initial response of innate immunity, innate sensors direct the local recruitment of myeloid cells (commonly neutrophils, T cells, B cells, monocytes, and dendritic cells) for the pathogenicity control (Klein & Hunter, 2017; Platt et al., 2017).

1.4 Adaptive immunity in the CNS

The adaptive immune system is an important component of the host defence against pathogens through recognition of non-self antigens. This defensive mechanism is mediated by B and T lymphocytes, which display a diverse range of specific antigen receptors in conveying the humoral and cellular immunity (Mayne et al., 2020). Traditionally the CNS was considered an immune-privileged site, whereas more recent studies have indicated the role of the adaptive immune system in the CNS for immune-surveillance and defence against neurotropic viruses (Ellwardt et al., 2016). Studies have also highlighted the role of adaptive immunity in maintaining CNS homeostasis and integrity by promoting neurogenesis and improving cognitive functions (Radjavi et al., 2014; Ziv et al., 2006). In healthy individuals, these immune system-CNS interactions are highly regulated to maintain the beneficial relationships. However, during pathology (infections, neurodegeneration, etc) when BBB is disrupted, increased infiltration of the peripheral immune cells into the CNS further potentiates neuroinflammation and facilitates tissue regeneration (Mayne et al., 2020). The role of adaptive immunity in neuroimmunology is supported by changes in T and B cell subsets and levels of (auto)antibodies in the blood, CSF and brain tissue during different neuroinflammation-associated diseases (Stephenson et al., 2018). The initial priming of T and B cell response that contributes to pathogen control occurs in the periphery. As a consequence of pathogen replication in the brain, pathogen-specific T and B cells and/or

antibodies traffic to the CNS through BBB and BCSF, and are able to gain parenchymal access (Platt et al., 2017).

T cells can penetrate into the damaged CNS with altered activation of glial cells and degeneration of neurons (Mundt et al., 2019). The activation of T cells, however, depends on the APCs, whereas the CSF lacks professional APCs. In this case, APCs are recruited to the CSF like in neurodegenerative diseases, they start to present peptide antigens through MHC class II molecules to further activate infiltrating T cells while their surface T cell receptors (TCR) bind these presented antigen peptides. The TCR-MHCI/II interactions induce the production of $CD4^+$ T cells and $CD8^+$ T cells (Chen et al., 2018). Undifferentiated $CD4^+$ T cells activated by APCs differentiate into different functional cell phenotypes under different mediators. $CD4^+$ T cells tend to become T-helper 1 (Th1) and Th17 cells with inflammatory phenotype, closely related to neuroinflammation and neuronal damage (Peck & Mellins, 2010). Furthermore, $CD4^+$ T cells could differentiate into the functional T-helper 2 (Th2) and regulatory T (Treg) cells. These phenotypes play a fundamental role in inhibitory effects on the inflammatory functions of T cells and the reduction of neuroinflammation (Peck & Mellins, 2010). Recent studies found that Th1 and Th17 cells secreted pro-inflammatory factors, such as TNFα and INFγ, to induce microglia activation and the subsequent release of other pro-inflammatory factors (Prajeeth et al., 2017), whereas Th2 cells and Tregs produced interleukins 4 (IL-4) and 10 (IL-10) to promote the shift of microglia from pro- inflammatory to anti-inflammatory phenotypes (González & Pacheco, 2014).

Most neurons do not express MHC class I antigens under non-inflammatory conditions (Joly et al., 1991; Neumann et al., 1995). Thus, the T cell effector functions, including cytokine production, may not be triggered by the infected neuron directly but rather by adjacent MHC class I-expressing cells (for example activated microglia) that display antigenic peptides via cross-presentation (Calzascia et al., 2003). Resident CNS cells may not only be invisible to immune cells as a result of reduced expression of MHC recognition molecules but also by expressing immunomodulatory molecules, such as programmed cell death ligand 1 (Jeon et al., 2013).

Lately, the dogma about immune privilege of CNS provided by different barriers has been challenged by the identification of lymphatic vessels in the dura mater (Aspelund et al., 2015). The latter are crucial to clear waste products like Aβ peptides and tissue debris that accumulate during AD (Iliff et al., 2012). Moreover, these vessels also stimulate the adaptive immunity in the CNS by linking it with the deep cervical lymph nodes, the site of T cell activation and proliferation (Aspelund et al., 2015; Louveau et al., 2015). These studies indicated to the mechanism of how antigens and professional APCs could exit the CNS to alert immature T cells in the lymph nodes.

Aberrant activation of the cellular immune response pathways is described for all neurodegenerative diseases (Mayne et al., 2020). For example, although adaptive immunity is not the central pathogenic mechanism of amyotrophic lateral sclerosis (ALS), T lymphocytes displaying pro-inflammatory features contribute to ALS progression and severity as infiltrated T lymphocytes were found in the post-mortem spinal cord of ALS patients (Chen et al., 2014). Compared to the CNS, peripheral blood of ALS patients showed significantly lower proportions of $CD4^+$ T cells with unchanged or elevated $CD8^+$ T cell proportions (Chen et al., 2014; Jin et al., 2020). Despite this decreased CD4:CD8 T cell ratio, only a higher $CD4^+$ T cell percentage correlates with disease severity and progression in ALS patients (Chen et al., 2014; Shi et al., 2007). This correlation was associated with a shift towards a Th1/Th17 cell-mediated pro-inflammatory immune

response (Jin et al., 2020; Saresella et al., 2014). Even though the percentages of anti-inflammatory Th2 cells and Tregs were decreased (Jin et al., 2020; Saresella et al., 2014), higher percentages of IL-13, a Th2-related cytokine, producing $CD4^+$ T cells, were detected in ALS patients, correlating with the disease severity (Shi et al., 2007) (more about cellular immunity in neuroimmuno diseases in chapters 1.5 and 1.6).

B cells involved in the adaptive immunity are considered to be an important component in the development of neuroinflammation (Prüss, 2021). Besides, B cells perform various functions, including the antigen presentation to T cells, production of pro-inflammatory factors and secretion of anti-inflammatory cytokines (Wortel & Heidt, 2017). Specifically, auto-reactive B cells have a key role in neurological autoimmune disorders and in neuroinflammation that occurs by generation of autoantibodies directed against components of CNS (Prüss, 2021). For example, the molecular mimicry between microbial antigens and neuronal antigens represents a significant trigger for neuroinflammation in genetically susceptible individuals (see chapters 1.1, 1.5, 1.6). B cells release cytokines like IL-6 and TNFα to promote inflammatory responses and granulocyte-macrophage colony-stimulating factor (GM-CSF) to promote the differentiation of pro-inflammatory factors (Vazquez et al., 2015). On the other hand, B cells (named regulatory B cells) that secrete TGFβ1, IL-10 and IL-35 exert anti-inflammatory effects and thus control the progression of neuroinflammation (Staun-Ram & Miller, 2017).

Numerous human CNS infections (**Table 1**), including those caused by MV, PV, VZV, HSV and flaviviruses, are characterised by the presence of intrathecal antibodies in the CSF (Phares et al., 2013). Humoral response against virus antigens seems to be associated with protective rather than pathogenic functions, as observed for Japanese Encephalitis virus (JEV) and some neurotropic retroviruses (Phares et al., 2013). Antibodies may be particularly beneficial for those infections that result in extracellular infectious virus production. However, as already described infectious agents may provide an initial trigger to generate autoimmune response targeting neural protein antigens and further escalate the immunopathology in the CNS. The CNS-infiltrated B cells form cellular structures along with Th and T follicular helper (Tfh) cells known as leptomeningeal ectopic lymphoid follicle-like structures (Serafini et al., 2004). These structures are common in MS, experimental autoimmune encephalomyelitis (EAE), optic neuritis (ON) and neuromyelitis optica spectrum disorder (Haugen et al., 2014). These local lymphoid structures support intrathecal antibody production (Haugen et al., 2014; Serafini et al., 2004). Future research will have to show how B cell follicle-like structures relate to these recently discovered meningeal lymphatic vessels in CNS.

B cell population has been shown to decline with age, contributing substantially to immunosenescence, a process of the gradual deterioration of the immune system brought on by natural age advancement (Cancro et al., 2009). B cell immunosenescence induces lower antibody specificity (Tabibian-Keissar et al., 2016). Thus, the senescence of B cells affects the pathological process of neurodegenerative diseases (Cancro et al., 2009). For example, a decrease in the peripheral B cell subsets was detected in AD patients (Jiang et al., 2017). Furthermore, differences in the levels of anti-Aβ antibodies in serum or CSF between AD patients and healthy controls were found although these findings have been very inconsistent (Söllvander et al., 2015). These findings might be related to the observed increased binding of anti-Aβ antibodies to Aβ in AD patients (Maftei et al., 2013; Söllvander et al., 2015) which supports a potential therapeutic

benefit of anti-Aβ antibody therapy. Indeed, intracerebral administration of anti-Aβ antibodies in AD-prone animals led to clearance of Aβ plaques, which was at least in part due to effects on microglia (Wilcock et al., 2003).

1.5 Multiple sclerosis

Multiple sclerosis (MS) is one of the most frequent neurological autoimmune disorders in young adults accompanied by a serious physical nontraumatic disability affecting more than 2 million individuals worldwide. The clinical course of MS differs, but approximately 90% of patients present relapsing–remitting MS (RRMS), in which relapses – discrete self-limited episodes of neurological dysfunction – are followed by periods of remission (Filippi et al., 2018). Approximately 50% of patients with RRMS eventually develop progressive disability independent of relapses, which are commonly referred to as secondary progressive MS (SPMS) (Filippi et al., 2018). The remaining 10% of patients present with an insidious course, in which neurological disability worsens from onset without discrete relapses, referred to as primary progressive MS (PPMS). To what extent disease progression to PPMS and SPMS involves shared or distinct pathophysiological mechanisms is currently unclear (Filippi et al., 2018; Tremlett et al., 2008).

A characteristic hallmark of MS is the presence of a well-defined inflammatory demyelinating lesion and neurodegeneration within the CNS (Filippi et al., 2018). In 85% of patients, MS starts when the adaptive immune system attacks the CNS leading to the infiltration of perivascular and parenchymal inflammatory cells (T and B lymphocytes, natural killer cells, and dendritic cells) into the brain and spinal cord through leaky BBB (Frischer et al., 2009). Both genetic and environmental risk factors related to immunity increase the risk for development of MS. Multipoint linkage screens of families with MS show significant linkage with the MHC region, highlighting the importance of antigen presentation and the adaptive immune system in MS genetic risk (Genet, 2005). This is further supported by genome-wide association studies with the discovery of MS risk alleles within the MHC region relating to T cell differentiation (The International Multiple Sclerosis Genetics Consortium & The Wellcome Trust Case Control Consortium 2, 2011). Associations with human leukocyte antigen (HLA) HLA-DRB1*15:01 (HLA class II) and HLA-A*02:01 (class I) along with more than 200 genetic variants related to the risk of MS have been reported (International Multiple Sclerosis Genetics Consortium (IMSGC), 2013). Female gender, Scandinavian/Celtic descent, low childhood vitamin D status, adolescent obesity, smoking, and infection background – all include major risk factors of MS (Lassmann, 2019). The onset of MS has also been associated with several other neurological diseases (Filippi et al., 2018). For example, as the optic nerve is one of the major targets in MS, about 20% of MS patients present with optic nerve inflammation as one of the first symptoms, whereas during the course of the disease ON may occur in 50% of patients. In a follow-up of a large cohort of ON patients the risk of developing MS after ON was 30% at 5 years, close to 40% at 10 years, and 50% after 15 years (Sergott, 2009).

Because of its strong association with the MHC class II alleles, MS was traditionally recognised as a predominantly T cell-mediated autoimmune disease. This association was further supported by the findings of: i) autoreactive T cells showing inter- and intra-molecular epitope spreading against myelin proteins; ii) elevated levels of IFNγ, IL17 and other cytokines/chemokines in the CSF at relapse: iii) increased number of Th1 and Th17 cells at relapse; iv) different immune-treatments of MS affect the number and phenotype of T cells (Sabatino et al., 2019). An infiltration of peripheral T lymphocytes,

with both $CD4^+$ T cells and $CD8^+$ T cells was found at the edge of demyelinating lesions and at lesser extent at chronic inactive lesions (Traugott et al., 1983). The T cell-mediated pathogenic mechanism has been thought to involve a Th1 response, due to the expression of IFN*γ* within lesions and the association of macrophages as the effector cells of Th1 immunity and demyelination (Traugott & Lebon, 1988). Active demyelinating lesions showed high heterogeneity, affirming the complexity of the pathogenesis of MS (Lucchinetti et al., 2000).

1.5.1 The role of B cells and antibodies in MS

Although B cells are rare in the CNS parenchyma, they exist in the perivascular areas and leptomeninges (Machado-Santos et al., 2018) and are now claimed as major contributors to MS pathogenesis (Sabatino et al., 2019). Various findings in patients with MS suggest the involvement of B cells in the pathogenesis, including: i) the presence of oligoclonal bands (OCBs) and clonal expansion of B cells in the CSF; ii) antigen-dependent affinity maturation of antibodies; iii) Ig and complement deposition in lesions; iv) B cell follicle-like structures; and v) B cell-supportive environment (Sabatino et al., 2019).

The first indication that B cells might contribute to MS was discovered decades ago with the identification of unique IgG fractions in the CSF (Yahr et al., 1954) that represented antibodies produced by clonal B cell populations, which are referred to as OCBs. Intrathecal B cells were later confirmed as the source of the antibodies that give rise to OCBs (Obermeier et al., 2011). OCBs are found in ~90% of patients with MS (Dobson et al., 2013) and are a diagnostic hallmark of the disease. B cell numbers which are indicative of acute inflammation and BBB breakdown are generally increased in the CSF of patients with MS, in particular in those with contrast-enhanced lesions in MRI (Eggers et al., 2017). The levels of antigen-experienced class-switched memory B cells and antibody-producing plasma cells are high in the CSF of patients with MS (relative to their levels in the blood). B cells can be detected in CNS lesions in early to late stages of MS, but are most abundant in active lesions of patients with RRMS compared with levels in inactive lesions and in patients with progressive MS (that is, SPMS or PPMS) (Machado-Santos et al., 2018). It has been found that leptomeningeal ectopic lymphoid follicle-like structures of MS patients contain $CD20^+$ B cells, $CD138^+$ plasma cells and follicular dendritic cells (Howell et al., 2011; Magliozzi et al., 2006; Serafini et al., 2004). The levels of $CD138^+$ plasma cells are increased (particularly in the meninges) in patients with progressive MS (Machado-Santos et al., 2018). These findings highlight a possible pathophysiological link between meningeal B cell inflammation and cortical pathology in all phenotypes of MS (Schirmer et al., 2019). Several lines of evidence suggest that the CNS of patients with inflammatory diseases like MS provides a unique environment with increased levels of cell survival factors as B cell activating factors (BAFF), chemokine (C-X-C motif) ligands 12 (CXCL12) and 13 (CXCL13) that supports long-term B cell lineages (Meinl et al., 2006, 2008).

The importance of B cells in MS relates to the fact that anti-CD20 monoclonal antibodies (mAbs), such as rituximab, ocrelizumab, and ofatumumab, that deplete B cells are highly effective for MS treatment (Hauser et al., 2008). Peripheral, CSF and CNS-specific B cells in patients with MS express increased levels of T cell co-stimulatory proteins and MHC class II molecules (Fraussen et al., 2016). Activated B cells can present myelin and other CNS antigens to inflammatory autoreactive T cells in MS. On the other hand, Tfh cells promote autoimmunity by supporting B cell proliferation and differentiation, isotype switching and antibody production in geminal centres (Fan et al.,

2015). Thus, bidirectional B cell – T cell communication is crucial in both for tissue homeostasis and disease progression.

The multi-directional exchange of antibody forming cells across the BBB and BCSF in individuals with MS and antigen-driven clonal expansion has previously been suggested from sequencing data of B cell receptor (BCR) (von Büdingen et al., 2012) and IgG heavy chain variable region studies (Bankoti et al., 2014; Colombo et al., 2000). Importantly, clonal B cells persist in the CSF of patients with MS and do not seem to be affected by immunomodulatory therapies (Stern et al., 2014). The immune-repertoire sequencing of the BCRs from different B cell populations has provided further insights into the clonal characteristics of B cells in the blood, CSF and CNS of patients with MS. Indeed, clonally expanded B cells in the CSF of patients with MS show somatic hypermutation indicative of localized antigen-driven affinity maturation (Bankoti et al., 2014; Beltrán et al., 2014; Colombo et al., 2000).

Regardless of tremendous efforts, the target antigen(s) of B cells in MS have yet to be identified. Owning to the demyelinating nature of the disease, myelin is considered a putative autoantigen in MS. Despite extensive investigation, however, studies have yielded dubious results of whether patients with MS have increased levels of antibodies against myelin antigens and/or other specific CNS antigens (**Table 2**). Additionally, CSF antibody reactivity against antigens of measles, rubella and varicella-zoster viruses (MRZ) has long been noted (Jarius et al., 2017). The results of at least one study have suggested that OCB antibodies are not specific for CNS proteins but instead target ubiquitous self-antigens (Brändle et al., 2016). Collectively, these investigations suggest that the adaptive immune response in patients with MS is not directed towards a single antigen or even a single CNS cell type. Rather, the humoral B cell response in patients with MS seems to be directed at a heterogeneous array of self-antigens and non-self antigens, which can differ greatly from one individual to another. The diversity of antigenic targets might be due to a combination of epitope spreading and secondary immune reactions to CNS cellular debris rather than a primary pathogenic response (Winger & Zamvil, 2016).

Table 2. Most studied autoantibodies in MS *(from Immune Epitopes Database (www.iedb.org; accessed 09.2021)).*

Antigen	Name	Uniprot ID
Myelin basic protein	MBP	P02686
Myelin proteolipid protein	PLP	P60201
Myelin-oligodendrocyte glycoprotein	MOGP	Q5SSB8
2',3'-cyclic-nucleotide 3'-phosphodiesterase	CN37	P09543
Oligodendrocyte-myelin glycoprotein	OMGP	P23515
Aquaporin-4	AQP4	P55087
Actin, cytoplasmic 1	ACTB	P60709
Myelin-associated oligodendrocyte basic protein	MOBP	Q13875
Keratin, type I cytoskeletal 18	KRT18	P05783
Alpha-enolase	ENO1	P06733
Myelin-associated glycoprotein	MAG	P20916
Transaldolase	TALDO1	P37837
Claudin-11	OSP	O75508
Neurofilament light polypeptide	NEFL	P07196
Heat shock protein HSP 90-beta	HS90B	P08238
Heat shock 70 kDa protein 1-like	HSPA1L	P34931
Alpha-crystallin B chain	CRYAB	P02511
Transketolase	TKT	P29401
Contactin-2	CNTN2	Q02246
Inward-rectifier potassium channels KIR4.1	KCJ10	P78508
Neurofascin	NFASC	O94856
Anoctamin-2	ANO2	Q9NQ90
Mitogen-activated protein kinase kinase kinase 7	MAP3K7	O43318
Tubulin beta-1 chain	TBB1	Q9H4B7
Tubulin alpha-1A chain	TBA1A	Q71U36

1.5.2 MS and infections

Different infections are considered as triggers and relate to the progression of MS. The “hygiene hypothesis” argues that improved hygiene in childhood leads to the development of autoimmune disease, also atopic/allergic inflammation conditions (Yazdanbakhsh, 2002). For example, the rate of *Helicobacter pylori* (*H. pylori*) infections is significantly lower in patients with MS than in healthy individuals (Fabis Pedrini et al., 2015). *H. pylori* infection occurs in infancy, when the mucosal barrier of the stomach is immature, and persist for life (Graham, 1991). The protective effects of *H. pylori* infection could be explained according to the hygiene hypothesis; repeated childhood infection induces maturation of the immune system, whereas improved sanitation and the resulting scarcity of childhood infections hamper its development (Yazdanbakhsh, 2002). This subsequently leads to an inability of the immune system to suppress autoimmune flares and inflammation in adulthood (Yazdanbakhsh, 2002).

Another example for the “hygiene hypothesis” is the delay in herpes virus 4 (Epstein Barr virus (EBV)) infection in childhood (Mitoma & Manto, 2020). EBV has the strongest epidemiological and serological connection to MS (Hassani et al., 2018) and EBV infection is more prevalent in MS patients than in healthy controls in countries with high sanitary conditions (Bjornevik et al., 2022; Levin et al., 2010), where it is suggested that a more hygienic environment during childhood predispose individuals to later EBV infections

(Lucas et al., 2011). This delay in EBV infection increases the risk of MS, because mononucleosis, a common manifestation of EBV infection is associated with occurrence of MS (Ascherio & Munger, 2007). The risk of MS is extremely low in EBV-seronegative individuals (Ascherio & Munger, 2015). EBV DNA has been detected in the post-mortem brain tissue of MS patients and viral reactivation has been localized to acute lesions and ectopic B cell follicles in the meninges (Serafini et al., 2007).

EBV infection with periodic reactivations is mainly found in memory B cells. It remains mostly latent during the rest of an infected individual's lifetime, expressing a single gene (nuclear antigen 1 (EBNA1)) within memory B cells which allows its episome to be copied when the cells divide (Thorley-Lawson, 2015). Even though the EBV load in the CNS of MS patients is currently unclear, in the scenario when EBV infected memory B cells in the CNS reactivate, expand and differentiate into plasma cells, viral synthesis would occur, eliciting a strong immune response (Hislop & Taylor, 2015). While some studies suggest that EBV aids in breaking immune tolerance to CNS myelin antigens through molecular mimicry (Lanz et al., 2022; Morandi et al., 2017), others advocate on the ability of the virus to promote immortalization of antibody secreting B cells (Serafini et al., 2007). Previous EBNA1 immunoblot analyses have shown the increased aberrant EBNA1 IgG reactivity in MS serum and CSF suggesting a role for latent EBV antigens in MS. Links between disease's course and different phases of EBV infection were also shown in a study revealing that the latent virus was more prevalent in MS brains, while lytic virus was associated with chronic MS lesions (Moreno et al., 2018). In this regard, it is conceivable that efficacy of anti-CD20 therapies might depend not only on suppression of B cell functions, but also on the control of EBV infected cell populations (Hart et al., 2013). It has been suggested that when EBV-infected B cells from the periphery migrate into the CNS, they play a crucial role in propagating CNS-compartmentalized neuroinflammation (Bar-Or et al., 2020). Given that the general opinion for the development of MS pathology is thought to involve interactions between T and B cells, whether EBV-infected B cells can also activate T cells in the periphery is an interesting question (Bar-Or et al., 2020).

An adequate immune response to another herpes virus – herpes virus 5 (cytomegalovirus (CMV)) reflects the body's immune fitness whereas aberrant immune response to it may cause subsequent chronic re-activation of the virus (Huygens et al., 2014). The virus usually targets the myeloid lineage cells (e.g., macrophages, dendritic cells) followed by subsequent replication in the salivary gland (Berry et al., 2020). Additionally, the virus may traverse the brain directly or indirectly (Vanheusden et al., 2017). CMV can potentially alter the brain microenvironment by influencing the CNS resident cells (Horakova et al., 2013). An association between CMV infection and MS risk has been made in the past but with inconsistent results (Langer-Gould et al., 2017; Vanheusden et al., 2017). CMV DNA and CMV-specific IgG antibody titres in sera/plasma were found to be higher in MS patients compared to healthy (Sanadgol et al., 2011). Anti-CMV seropositivity was associated with relapses, time to relapses and enhanced brain atrophy (Weinstock-Guttman et al., 2013; Zivadinov et al., 2014). Overall, CMV as a grey matter neurotropic agent might cause MS-associated neurodevelopmental disorders, like intellectual disability, hearing loss, etc (Tsutsui et al., 2005). Most studies have found that CMV seropositivity is negatively associated with MS (Bjornevik et al., 2022; Maple et al., 2020; Sundqvist et al., 2014) with reports suggesting that CMV infection modulates the immune response to alter MS pathophysiology. B cells from CMV(–) MS patients induced an enhanced pro-inflammatory profile compared to CMV(+)

MS cases, suggesting that persistent CMV infection might reduce the inflammatory responses of B cells in MS (Zabalza et al., 2020) – a finding similar to what has been previously described for chronic CMV infection carriers (Das et al., 2012). Another hypothesis of how CMV may associate with milder MS symptoms could be that in patients who are CMV/EBV double seropositive, there is a balance in the immune response between these two viruses (Maple et al., 2020). However, in CMV seronegative patients, EBV could drive the immune system towards a more aggressive MS disease phenotype (Maple et al., 2020).

MS association with infectious agents is also proved with the findings on the immune reactivity of OCBs and intrathecal antibodies with bacterial (e.g., *Chlamydia pneumoniae* (Derfuss, 2001)), human herpesvirus 6 (Derfuss et al., 2005) and other viral (measles, rubella, and varicella-zoster) (Felgenhauer et al., 1985) antigens.

1.6 Type 1 Narcolepsy

Classic or typical narcolepsy, now termed narcolepsy type 1 (NT1), a chronic neurological disease is characterised by an irresistible or imperative excessive daytime sleepiness and brief episodes of loss of muscle tone or control triggered by emotions and accompanied by a preserved state of consciousness (variously termed ataxia, catalepsy and cataplexy) (Bassetti et al., 2019). The prevalence of NT1 in Europe is about ~200–500 cases per million individuals (Bassetti et al., 2019). NT1 usually starts in adolescence, and a small second peak of onset occurs at around age 35 years (Bassetti et al., 2019). Narcolepsy can have an acute course, in which symptoms develop within a few days or weeks after a triggering event, such as vaccination, stress or head trauma, a chronic course, in which the onset of symptoms is difficult to determine, or a progressive course, in which the onset of different symptoms is separated by years or even decades (Pizza et al., 2014b). These differing presentations probably reflect different pathophysiological mechanisms.

The major neuropathological features of NT1 are loss of ~90% (ranging between 75 to 95%) orexin (OX) (gene name hypocretin (HCRT))-producing neurons in the lateral hypothalamus and increased gliosis in the posterior hypothalamic nuclei (Bassetti et al., 2019). The demonstration of low levels (less than 200pg/mL) of orexin A in the CSF (and shortly thereafter of the selective loss of orexin-producing neurons in the lateral hypothalamus) in patients with NT1 highlighted these cells as the targets of such a pathological process (Nishino et al., 2000). Orexin A and orexin B are small neuropeptides that activate target neurons through orexin receptors type 1 and type 2, respectively (Sakurai, 1998). The orexin peptides are produced only by a cluster of neurons in the lateral hypothalamus but are released by projections of these neurons to the other parts of CNS, from the cortex to the spinal cord. Loss of this crucial system disrupts the functioning of multiple frontal, limbic, diencephalic and brainstem networks and results in the symptoms of narcolepsy (Burgess & Scammell, 2012). Today, NT1 is considered to arise from multiple hits: the co-occurrence of genetic predisposition, environmental factors and triggering events eventually lead to the selective, immune-mediated destruction, dysfunction or silencing of orexin-producing neurons (Bassetti et al., 2019). More than 90% of individuals with NT1 bear the HLA class II allele *DQB1*06:02* (Nishino et al., 2000). The *DQB1*06:02* allele increases the risk of developing NT1 and disease rarely develops in people lacking this allele (Tafti et al., 2014). However, HLA-DQB1*06:02 is also expressed in 5–38% of the general population, and only 1 in 1,000 carriers of this allele will develop narcolepsy (Tafti et al., 2014). Other studies have identified associations between NT1 and polymorphisms in other immune-related genes

(Partinen et al., 2014). As appeared, there is a plethora of data that genetic or experimental alterations of the OX system are associated with NT1, however, OXs are not restricted to the CNS and together with their receptors OX1R and OX2R are also widely expressed in peripheral tissues (Voisin et al., 2003).

Season of birth was associated with the risk of narcolepsy in some but not all studies, which suggested that exposure to viruses, bacteria or toxins early in life might alter the development of the immune system and thereby predispose individuals to NT1 (Picchioni et al., 2004). Narcolepsy has occasionally been reported to occur after traumatic brain injuries (Silber, M. H, 2005).

The loss of OX signalling seems sufficient to explain the major features of NT1 (Crocker et al., 2005) but some symptoms such as fragmented night-time sleep, rapid eye movement (REM) sleep behaviour disorder and resolution of hypersomnia over time cannot be easily explained by the current models. The loss of OX neurons might trigger compensatory responses that are helpful in some ways but harmful in others. For example, two groups have shown substantially increased numbers of histaminergic neurons in the tuberomammillary nucleus of people with NT1 (John et al., 2013; Valko et al., 2013). Histamine is a key wake-promoting transmitter and, in contrast to the other monoamine-producing neurons, histamine neurons remain active during cataplexy (John et al., 2004). Thus, increased histamine signalling might help to counter a tendency towards hypersomnia and help to maintain consciousness during cataplexy by preventing full transitions into REM sleep (Mahoney et al., 2019). Mast cells (MCs) can release histamine and other factors that affect sleep and the immune response in the brain. Accumulating evidence shows that MCs play a role in the regulation of sleep and behaviour (Chikahisa et al., 2013). MCs are most abundant in young individuals under the age of 19, after which their counts decline with age (Turygin et al., 2005). Most significantly, the maturation of MCs is influenced by prostaglandin D2 (PGD2) and the receptor D-prostanoid receptor 1 (DP1) (Taketomi et al., 2013). PGD2 is the most abundantly expressed prostaglandin in the brain, synthesized in the leptomeninges, choroid plexus and oligodendrocytes in the brain and secreted to the CSF (Urade & Hayaishi, 2011). This prostaglandin has two types of receptors: D-prostanoid receptor 1 (DP1) and DP2 (CRTH2). Prostaglandins play a key role in the inflammatory response and their synthesis is significantly increased after tissue injury and cell stress (Ricciotti & FitzGerald, 2011). Studies on AD showing that the neuroinflammation accelerates the progression of the disease revealed that PGD2 is involved in this acceleration (Mohri et al., 2007). It was shown that the level of the mRNA of the DP1 was increased in AD brains compared with non-AD brains. *In situ* hybridization studies revealed that DP1 mRNA was specifically localized in microglia and reactive astrocytes within senile plaques of AD brains (Mohri et al., 2007). Besides its role in inflammation, this prostaglandin can act to inhibit inflammation, as virus-induced inflammasome activation is suppressed by PGD2/DP1 signalling (Vijay et al., 2017). Specifically, PGD2/DP1 signalling is necessary for optimal microglia/macrophage activation and IFN expression after infection with neurotropic coronaviruses (Vijay et al., 2017). In addition to histamine, DP1 signalling may also influence the levels of adenosine that is known to regulate sleep (Urade & Hayaishi, 2011). The precise role of MCs and PGD2/DP1 signalling in NT1 warrants further studies.

1.6.1 Immunological aspects in NT1

Although, the pathophysiological events leading to the destruction of hypothalamic OX-producing cells in NT1 are presently unclear, the environmental and genetic factors discussed in the preceding chapter provide circumstantial evidence that an aberrant immune process contributes to narcolepsy. Moreover, inflammatory findings in the CSF of NT1 patients can occasionally be observed. Accordingly, it has been considered that in particular cell-mediated autoimmunity, but also autoantibodies against neuronal structures, or cytotoxicity caused by cytokines and inflammatory cells in the CNS could lead to the destruction of OX-producing neurons (Sarkanen et al., 2018b).

However, even after the description of OX-producing neurons as the putative autoimmune target, attempts to demonstrate narcolepsy-associated autoimmune responses have largely been unsuccessful (Kornum et al., 2017). Most recently Latorre *et al*. showed an increased $CD4^+$ T cell response to OX in NT1 patients as compared with very limited response in controls (Latorre et al., 2018). As resident cells of CNS express only MHC class I and not class II molecules under normal physiological conditions, cytotoxic $CD8^+$ T cells are the most likely effector cells in the autoimmune destruction of OX-producing neurons (Degn & Kornum, 2015). A role of these cells is also supported by the association studies linking several HLA class I alleles with narcolepsy (Tafti et al., 2016), and also by the finding that a higher frequency of autoreactive $CD8^+$ T cells was detected in the blood of NT1 patients compared with healthy controls (Pedersen et al., 2019). Despite these findings, it is currently not clear if T cells are primarily involved in the pathogenic process or if they are generated as a consequence of the destruction of the OX-producing neurons (Liblau, 2018).

Since $CD4^+$ T cells-mediated response is usually associated with B cell involvement, investigators have long searched for autoantibodies directed against OX-producing neurons. Currently, a limited set of potential autoantigens has been reported (**Table 3**). Evidence that the humoral response attacking the CNS is derived from different peripheral tissue antigens is provided by findings that antibodies from sera samples of NT1 patients can bind to brain and muscle structures (Ahmed et al., 2014; Smith et al., 2004). In relation to peripheral antigens, B-cell lymphoma 6 protein (BCL6; anti-BCL6 (Zandian et al., 2017)), another NT1 antigen, is a master regulator of mature B cells required for germinal centre reactions (Pei et al., 2017). Neurexin 1 (NRXN1α; anti-NRXN1 (Zandian et al., 2017)) has been isolated from brain and heart tissues suggesting a role in heart development (Nagase, 1998). Tribbles homolog 2 (TRIB2; anti-TRIB2 (Cvetkovic-Lopes et al., 2010)) is present in many cell populations both in and outside the nervous system, including the immune cells (Eder et al., 2008). Gangliosides (including GM3; anti-GM3 (Saariaho et al., 2015)) are abundant in the brain, but also in extraneural tissues with relatively high concentrations in bone marrow, erythrocytes, intestine, liver, spleen, testis, kidney, and in embryonic stem cells (Kolter, 2012). However, it remains elusive what pathogenic role these autoantibodies may exert within the periphery. Furthermore, possible autoantibodies against different neuronal structures that have a low specificity for narcolepsy were also found in other sleep disorders and also in healthy people.

Table 3. List of autoantigens associated with NT1 *(from Immune Epitopes Database (www.iedb.org; (accessed 09.2021)).*

Antigen	Name	Uniprot ID
Orexin	OX	O43612
Transcription factor RFX4	RFX4	Q33E94
Tribbles homolog 2	TRIB2	Q92519
Neurexin 1	NRXN1	Q9ULB1
Orexin receptor type 2	OX2R	O43614
Orexin receptor type 1	OX1R	O43613
Endonuclease 8	NEI	P20382
Melanocyte-stimulating hormone	aMSH	P01189
B-cell lymphoma 6 protein	BCL6	P41182
Spectrin alpha chain, non-erythrocytic 1	SPTN1	Q13813
Serine/threonine-protein kinase Nek9	NEK9	Q8TD19
Alpha-enolase	ENOA	P06733
Dysferlin	DYSF	O75923
Intermediate filament family orphan 2	IFFO2	Q5TF58
Coiled-coil domain-containing protein 6	CCDC6	Q16204
Glutamate receptor ionotropic, NMDA 2B	NMDE2	Q13224
Orexigenic neuropeptide QRFP	OX26	P83859
Piezo-type mechanosensitive ion channel component 1	PIEZ1	Q92508
Proenkephalin-B	PDYN	P01213
Neurofilament medium polypeptide	NFM	P07197
Sodium Voltage-Gated Channel Alpha Subunit 5	SCN5A	Q14524
SRY-Box Transcription Factor 11	SOX11	P35716
Reticulon 4	RTN4	Q9NQC3
Contactin 2	CNTN2	Q02246
Retinoid X receptor gamma	RXRG	P48443
ABL Proto-Oncogene 2, Non-Receptor Tyrosine Kinase	ABL2	P42684

1.6.2 H1N1 infection- and Pandemrix vaccine-induced NT1 (Pdmx-NT1)

As already described in the previous chapters, infections can induce autoimmunity through a wide variety of mechanisms, with a growing body of evidence suggesting that pathogens can trigger narcolepsy (Mentis et al., 2017). NT1 onset has been suggested to be triggered by upper airway infections during winter (Han et al., 2011). Streptococcal throat infection has been associated with a 5.4-fold increased risk of narcolepsy (Koepsell et al., 2010) and anti-streptococcal antibodies have been detected in 65% of patients with recent narcolepsy onset compared with age-matched controls (Aran et al., 2009). The most informative connection between narcolepsy and an infectious agent occurred in China, where the incidence of NT1 increased 3-fold following the 2009 H1N1 winter influenza pandemic, and then decreased to the normal rate after the pandemic had subsided (Han et al., 2011). Also, a link between NT1 and H1N1 infection has been seen in immunocompromised mice. Specifically, in recombinant activating gene 1-deficient mice, which lacked T and B cells, infection with the H1N1 influenza A virus spread to CNS and targeted OX-producing neurons in the hypothalamus, which led to a NT1-like syndrome in these animals (Tesoriero et al., 2016).

The most direct evidence that NT1 can be caused by an autoimmune process stemmed from findings where clinicians noticed a surge of individuals developing NT1

after vaccination with AS03-adjuvanted Pandemrix® (Pdmx, GlaxoSmithKline), a brand of influenza vaccine that was mainly used in northern Europe (Partinen et al., 2012). In contrast, no elevations in the rate of narcolepsy were reported in the US where only non-adjuvanted vaccines were used (Duffy et al., 2014), or elsewhere in Europe, where the closely related MF59 adjuvant was used in the H1N1 vaccine Focetria® (Novartis Vaccines & Diagnostics, 2007) (Ahmed et al., 2014). Pdmx inoculation in Nordic countries was associated with an 8–12-fold increase in new cases of NT1 in children and adolescents and a 3–5-fold increase in adults (Partinen et al., 2012; Vaarala et al., 2014). Epidemiologic and clinical studies conducted in different countries including Finland, Sweden, Ireland, England, Norway, and France have later confirmed the association of NT1 in children and adolescents with the AS03-adjuvanted Pdmx (Partinen et al., 2012; Sarkanen et al., 2018a). Importantly, all these affected individuals carried *DQB1*06:02* allele and developed NT1 a few weeks to months after vaccination. Why NT1 was triggered by Pdmx but not by other influenza vaccines remains unclear, but altered viral nuclear proteins in Pdmx may have contributed (Vaarala et al., 2014). Although the results suggested that the adjuvant AS03 could be problematic, no elevation in narcolepsy rates were observed in Canada where AS03 was also used as a component of the H1N1 vaccine Arepanrix® (GlaxoSmithKline, 2009) (Montplaisir et al., 2014). However, although Pdmx and Arepanrix were produced by the same manufacturer and administered with AS03, different protocols for antigen isolation were used (Montplaisir et al., 2014). This has led to the hypothesis that different composition of the vaccines might have contributed to the increased incidence in narcolepsy in the affected populations (Ahmed & Steinman, 2017). Some researchers now hypothesize that NT1 arises from a process of molecular mimicry (Ahmed et al., 2015).

There are numerous findings proving a link between Pdmx vaccine and NT1: i) strong and consistent epidemiological connection as increased incidence of NT1 was shown separately by different study groups and authorities in all the countries where the Pdmx was used at large scale (Partinen et al., 2012; Sarkanen et al., 2018a, 2018b); ii) immunological connection with high immune response against H1N1 viral nucleoprotein (NP) in narcoleptic patients (Vaarala et al., 2014); cross-reactivity between NP and OX2 (Ahmed et al., 2015); iii) link between NT1 and natural H1N1 infection (Han et al., 2011). Search for potential cross-reactive epitopes between Pdmx viral antigens and host components led to the identification of a potentially cross-reactive epitope between influenza NP and OX2 (Ahmed et al., 2015). Antibodies against the NP epitope, which cross-reacted with a similar epitope from OX2, were found in sera collected from children and adolescents who developed NT1 after the vaccination with Pdmx (Ahmed et al., 2015). Peptide absorption studies confirmed the specificity of autoantibodies against the shared epitope of NP and OX2 (Ahmed et al., 2015). Most recently, it was shown that paediatric Pdmx-NT1 patients had enhanced T cell immunity against the epitope of the H1N1 neuraminidase protein antigen that mimicked the epitope of protein-O-mannosyltransferase 1 (POMT1). Moreover, high levels of anti-POMT1 serum autoantibodies were found in Pdmx-vaccinated children or adolescents, thus providing evidence of POMT1 as a potential autoantigen recognised by T and B cells in NT1 (Vuorela et al., 2021). Additional information on the presence of autoantibodies against neuronal structures in Pdmx-NT1 was obtained from the immunohistochemical study of rat brain sections, revealing acid-isoleucine/α–melanocyte-stimulating hormone (NEI/αMSH) as targeted antigens (Bergman et al., 2014).

Some interesting differences have been reported in the clinical features of Pdmx-related NT1 and non-vaccine-associated narcolepsy (Pizza et al., 2014a; Sarkanen et al., 2016). A few studies have reported a higher mean age at the time of diagnosis or onset of symptoms in Pdmx-NT1 compared with spontaneous narcolepsy (Pizza et al., 2014a). However, the clinical picture of Pdmx-associated NT1 seems to be very similar to spontaneous narcolepsy (sNT1) (Sarkanen et al., 2018b).

2. Technical advances towards analysis of antigen repertoire

The immune system changes and adapts during the normal ageing and during the progression of different diseases. Previous chapters describe its association and change during neuroimmune diseases including MS and NT1. Screening the changes of the immune system activity gives important information on the condition of the human body. Thus, detection of circulating antibodies against self and non-self proteins holds a great promise to support diagnosis, prediction, classification and monitoring of various diseases. In addition, antibodies are thought to be the major mechanism through which most vaccines work (Piot et al., 2019) and epitope profiling would help to identify neutralising antibodies that provide immunity by preventing the initiation of the viral infection in the host (Abbott & Crotty, 2020). Furthermore, monoclonal antibodies have proven to be remarkably versatile therapeutics for treatment of cancer, but also other inflammatory disorders (Yasunaga, 2020). A good example of the situation is reflected in the fact that tens of antibody therapeutics were in development for COVID-19 during the last two years (Kaplon & Reichert, 2021).

The antibody-antigen interactions can be studied from different sides: studying the immune response input value by describing the repertoires of naïve B cells and plasmablasts or studying the output values of immune response by describing the repertoires of the antibody paratopes and antigen epitopes. Each direction has its advantages according to the research question. Different analytical tools are in use to study antibody-antigen interactions: immunofluorescence assays, radioimmunoassays (RIAs), enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot assay (ELISpot), luciferase immunoprecipitation systems (LIPS), western blot (WB), antibody-antigen co-crystallization, nuclear magnetic resonance (NMR) and computational docking, as well as site-directed mutagenesis of relevant target proteins together with high-throughput epitope mapping analysis (see chapter 2.1) (Burbelo et al., 2019). For example, antibodies against myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) and myelin associated glycoprotein (MAG) were the first to be discovered in MS patients by using solid-phase radioimmunoassays and ELISA (Fraussen et al., 2014) methods. ELISA analysis was also first used to show that sera from narcolepsy patients with cataplexy had higher anti-Trib2-specific antibody titres compared with either normal controls or patients with idiopathic hypersomnia (Cvetkovic-Lopes et al., 2010) whereas an immunohistochemical study in rat brain sections revealed NEI/αMSH as targeted antigens in NT1 (Bergman et al., 2014).

2.1 High-throughput epitope mapping analysis

Recent advances in immunomics, e.g. studies of the immune system regulation and response to pathogens and self using genome-wide approaches, have made it possible to study the humoral immune response in great detail and at a high resolution (Ayoglu et al., 2016b). New assays provide a high-throughput and multiplex analysis of several hundreds of body fluid samples against several thousands of antigens. High-throughput paratope/epitope mapping analysis include BCR sequencing, peptide/protein arrays and different display technologies (Valldorf et al., 2021) (**Table 4**).

Table 4. High-throughput epitope mapping in analysis and examples of their implementation.

Techno-logy	Capture	Assay	Analytes	Clinical model	References	Company
BCR	Naive B cells	Sequencing	Millions	Influenza vaccines	(Briney et al., 2019; Soto et al., 2019)	ImmunoSEQ
	Plasma-blasts	Sequencing	Millions	H1N1/2009 influenza infection	(Wrammert et al., 2011)	
Protein arrays	Proteins	Bead-analysis	Up to 500	Psychotic disorder	(Jernbom Falk et al., 2021)	Luminex Corporation
	Proteins	Solid phase	Hundreds of thousands	MS/NT1	(Zandian et al., 2017)	PEPperPRINT/ PEPperCHIP
Protein displays*	Proteins	Bacterial display	Hundreds of thousands	Chagas disease	(Kamath et al., 2020)	Serimmune
	Proteins	Phage display	Millions	MS/NT1	(Sadam et al., 2018; Xu et al., 2015)	PhIP-seq/ Protobios

** This category also includes yeast surface, mammalian cell display and cell-free in vitro ribosome display which are not specifically addressed here.*

The humoral immune response begins with BCR of naïve B cells (i.e., not antigen-experienced) recognising the antigen, thus the repertoire of B cells responding to antigen is the starting material from which antibodies eventually arise (Abbott & Crotty, 2020). BCR rearrangement during B cell development can yield an extraordinary level of diversity (predicted to be about 10^{12}-10^{15}) (Briney et al., 2019). While an impressive diversity of BCR sequences exists *in vivo,* all epitope specificities are not equally abundant in the repertoire (Havenar-Daughton et al., 2018). Profiling the repertoire of BCR will give important knowledge as to how many naïve B cells exist with an epitope specificity against targets of interest. This knowledge is very important, for example, in the design of new and effective vaccines (Lee et al., 2019). Customarily, a combination of fluorescently labelled antigen probes and flow cytometry has been used to identify HIV antigen-specific B cells (Havenar-Daughton et al., 2018). Next generation BCR repertoire sequencing was first described by Glanville *et al*., 2009 (Glanville et al., 2009). As it is the third complementarity determining region that mostly determines antibody binding properties with the antigen, many studies have focused only on sequencing this BCR region and have revealed largely unique repertoires for each individual studied and also a subpopulation of universally shared antibody clonotypes (Briney et al., 2019). Although today's methods allow very fast and very precise sequencing of millions of BCRs, some complexity remains in the description of potential binders (epitopes/antigens) directly from BCR repertoire data (Marks & Deane, 2020). One possible approach is to compare repertoires in naïve and *in vivo* antigen-treated samples. This approach can be effectively used to monitor the immune response during the development of influenza vaccines (Lee et al., 2019).

In one outcome, upon exposure to the antigen, B cells differentiate into a short-lived antibody producing plasma cells (Cyster & Allen, 2019). Plasmablasts thus represent an accessible and measurable subset of B cells only detectable during acute immune response (Carter et al., 2017). Measurements of plasmablasts by ELISpot technology

have been used extensively to describe the humoral immune response following vaccination (Saletti et al., 2013) and profiling plasmablast BCRs could be useful for vaccine development or to develop therapeutic monoclonal antibodies (Mitchell et al., 2014).

In addition to the studies of BCRs, the antibody repertoire can also be described by the antigens/epitopes to which they bind – so called undirect antibody profiling or epitope profiling. There are two high-throughput analyses for describing the repertoire of protein epitopes: peptide/protein arrays and different display technologies. Peptide/protein arrays may be planar or bead-based and typically comprise hundreds to thousands of distinct protein/peptide sequences used for epitope and antigen mapping. A large number of different solid support options are available, offering different surface chemistry choices for planar arrays. For example, nitrocellulose-coated surfaces allow for a random orientation of the proteins, affinity tag coated surfaces allow for a uniform orientation of the proteins (Ayoglu et al., 2016b). To date the most common approach to designing peptide microarrays has been to tile sequences from a known protein or proteome of interest and find sequences that bind the target (Forsström et al., 2014). There are different strategies to the synthesis of protein arrays: recombinant protein or protein extracts immobilized on planar or bead-based surfaces, *in situ* expression on spot (or on bead) using cell-free expression systems (Ayoglu et al., 2016b). Although protein arrays have the great advantage of mimicking discontinuous epitopes, peptide arrays are even more widely used because the synthesis of short peptides is significantly less complex and cheaper (Ayoglu et al., 2016b). A good example for protein array is the in-house generated antigen arrays, where a total of 11,520 human protein fragments representing 7644 protein-encoding genes were utilized for autoantibody profiling in a MS-related serum sample collection (Ayoglu et al., 2013). The more targeted follow-up work of this study recently led to the identification of the ion channel protein anoctamin 2 (ANO2) as an autoimmune target in MS, which might contribute to the characterisation of a patient subgroup (Ayoglu et al., 2016a). Furthermore, high-density peptide array representing the whole proteome, hosting 2.2 million 12-mer peptides with a six amino acid lateral shift was used to study autoantibody epitope profiles in MS and NT1, revealing two potentially new autoantigens: MAP3K7 in MS and NRXN1 in narcolepsy (Zandian et al., 2017). Another example is for pathogen-associated antigens where the tiling of overlapping peptides from sets of *a priori* known antigens from eight tick-borne pathogens enabled construction of planar peptide arrays with 170,000 features enabling detection of infections (Mucci et al., 2017). Among known protein arrays random-sequence peptide arrays are also used to increase the detection of previously unknown antigens. Random peptide arrays of up to 330,000 members have proven effective to detect antibodies towards a range of organisms (i.e., viruses, bacteria, fungi) (Richer et al., 2015).

The principle of bead-based assays relies on immobilization of capture reagents on distinguishable microsphere sets as solid supports and detection of the captured targets on each microsphere set by means of a flow cytometric read-out system (Ayoglu et al., 2016b). There are complementary advantages to each array, both planar and bead-based, and so they are often used together in research (Jernbom Falk et al., 2021). Planar array platform theoretically offers the possibility to spot tens of thousands of antigens with one slide whereas the number of studied samples is limited. Therefore, it is highly suitable for initial determination of the antigens and described antigens can be verified further on technological platforms offering much higher sample throughput

capacity, such as the bead-based arrays (Ayoglu et al., 2016b). For example, precise autoantibody profile was associated with specific psychopathology symptoms using a planar protein array containing 42,100 human antigens representing 18,914 proteins and a 380-plex bead-based array for autoantibody profiling of individuals with established psychotic disorder diagnosis (Jernbom Falk et al., 2021).

Beside the technically challenging BCR repertoire sequencing and considerably more expensive protein/peptide arrays, protein display methods are widely used to describe antibody repertoire (Anand et al., 2021; Valldorf et al., 2021; Weiss-Ottolenghi & Gershoni, 2014). Different protein display technologies are commercially available: phage, bacterial, yeast surface, mammalian display and cell-free *in vitro* ribosome display, with the phage display (PD) being the most implemented one (Valldorf et al., 2021). For example, a random peptide library consisting of 10^{10} random 12-mers displayed on the outer surface of *E. coli* bacteria was used for serum epitope repertoire analysis (SERA) to discover shared but highly specific immunogenic epitope motifs associated with Chagas disease caused by the protozoan parasite *Trypanosoma cruzi* (Kamath et al., 2020; Reifert et al., 2021). Protein PD technology is the most frequently employed because of the high diversity of molecules that can be displayed and ease of handling compared to bacterial, yeast and eukaryotic displays. PD has its origins in 1985, when George P. Smith was able to display and affinity purify short peptides (recombinantly) fused to the pIII minor coat protein of M13 filamentous phage (Smith, 1985). Recognised with the Nobel Prize for Chemistry in 2018, the method allows the isolation of high-affinity ligands for diverse substrates, ranging from recombinant proteins to cells, organs, and even whole organisms, non-organic substances, like metals, alloys, semiconductors, toxins and plastic surfaces (Valldorf et al., 2021). Similar to the great technological advances in BCR profiling, the introduction of next generation sequencing (NGS) platforms significantly changed the possibilities of PD, and instead of single high affinity epitopes, it is now possible to describe repertoires containing millions of analytes (Pupina et al., 2022; Sadam et al., 2018, 2021). In the current COVID-19 pandemic, the description of target epitopes of neutralising antibodies and the search for neutralising antibodies by PD is highly relevant for developing new vaccines and therapeutic candidates against novel SARS-CoV-2 virus (Anand et al., 2021).

There are different PD systems available but the F pilus-specific phage, encompassing the strains f1, M13 or fd, and T7 lytic bacteriophages have been most extensively used (Anand et al., 2021). PD (but also other displays) technology allows the construction of libraries in which various peptides and proteins are presented: phage antibody libraries (the engineered nucleotide fragments of immunoglobulins encoding the antigen-binding region are used for library construction), phage proteome libraries (randomly generated fragments of targeted pathogen DNA is used for library construction) and random peptide libraries (randomly generated oligonucleotide inserts are used for library construction) (Weiss-Ottolenghi & Gershoni, 2014). Using phage antibody libraries has highly contributed to antibody-based drug discovery from various perspectives, such as the development of human monoclonal antibodies, affinity enhancement of monoclonal antibodies, and the identification of therapeutic targets for antibody drugs (Nagano & Tsutsumi, 2021). Several antibody formats, such as single-chain variable fragment (scFv), fragment antigen-binding (Fab), and variable fragment (VHH) derived from heavy chain antibodies of *Camelidae* have been reported to be displayed on the phages (Nagano & Tsutsumi, 2021). A broad-based description of viral protein epitopes was made using a phage display library of 93,904, 56-mer peptides spanning 208 human virus proteomes,

and after following immunoprecipitation and DNA sequencing, seropositivity towards each virus was inferred (Xu et al., 2015). Similar approach has been used to detect autoantigens, in which overlapping peptides of human proteome were used for construction of T7 phage library (Larman et al., 2011). Furthermore, recent scientific research profiled the serum antibody response using peptide array method of 997 healthy individuals against 244,000 rationally selected peptide antigens derived from gut microbiota and pathogenic and probiotic bacteria, revealing a wide breadth of individual-specific as well as shared antibody responses against microbiota that associate with age and gender (Vogl et al., 2021). Depending on the method used for precipitation of antibody-phage complex, different Ig molecules can be analysed with the IgG and IgA isotypes being the most studied ones (Shrock et al., 2020).

Targeted proteome libraries are very useful because the immune profile obtained is easily annotated and straightforward. However, because these methods are biased towards specific organisms, they probably do not enable comprehensive immune evaluation. One strategy to overcome the limitations is to use random peptide libraries. The use of random peptide phage libraries became particularly informative as NGS became widely available (Ryvkin et al., 2012). The output of such an analysis is a vast collection of thousands to millions of peptide sequences that have been affinity purified by the antibodies in a given serum sample (more about this in chapter 2.2).

2.2 Mimotope Variation Analysis

Mimotope Variation Analysis (MVA) is a high-throughput next generation epitope profiling technology that describes immune response towards antigens from different organisms (autoantigens, microbiome-associated antigens, pathogen-associated antigens) (Pupina et al., 2022; Sadam et al., 2018, 2021). It applies parallel advancements in random peptide phage library technology, NGS, and computational discovery algorithms. The MVA workflow includes the following steps of i) immunocapture of IgG-phage complexes from a sample using peptide phage library, ii) subsequent high-throughput Illumina short DNA sequencing of amplicon libraries; iii) bioinformatical data processing resulting in epitope/mimotope identification; iv) validation of selected candidate epitopes; v) generation of discriminative immunoprofiles (**Fig. 1**). More specifically, to effectively mimic the diverse epitopes from many different organisms, a modified version of the random peptide library consisting of 10^9 random 12-mers displayed on the outer surface of bacteriophage M13 is used (PhD12, NEB, UK). In order to significantly increase the specificity of the results obtained, additional measures have been introduced to ensure that any nonspecific binding was avoided using preblocking by pIII mutant M13 phage lysate with enriched irrelevant protein load. The resulting protocol is patented (Patent ID US9672324B1). The IgG fraction of antibodies is purified from sera or other fluids by immobilizing onto a solid support of protein G-coated magnetic beads exhibiting high affinity for IgG molecules. The purified antibody fraction is then used in affinity selection of peptide displaying phages and phage particles binding to IgG can be removed easily by separating the beads from the solution by using a magnet. Next, the DNA of bound phages is extracted and the variable fragment encoding the displayed peptides is amplified and barcoded for sample multiplexing by polymerase chain reaction. The experimental part of the MVA technology is completed by obtaining the DNA sequence data. Data from the DNA sequencing is subsequently analysed using proprietary bioinformatics tools resulting in the epitope profile of the studied sample. The peptide profiles allow us to identify the individuality and heterogeneity in the

antibody immune response to antigens. The selected candidate epitopes are then annotated against human proteome and metagenomes and different independent analytic methods are used for further antigen validation (ELISA, dot ELISA, IHC, ect.). MVA approach has been successful in mapping epitopes of polyclonal antibodies and mAbs, IgG factions from different biological solutions (blood sera and plasma fractions, CSF and urine), as well as various biomaterials, including metals and polymers (unpublished data, Jaago et al., 2019, 2020; Palm et al., 2020; Pupina et al., 2022; Sadam et al., 2018, 2021; Vrana et al., 2020).

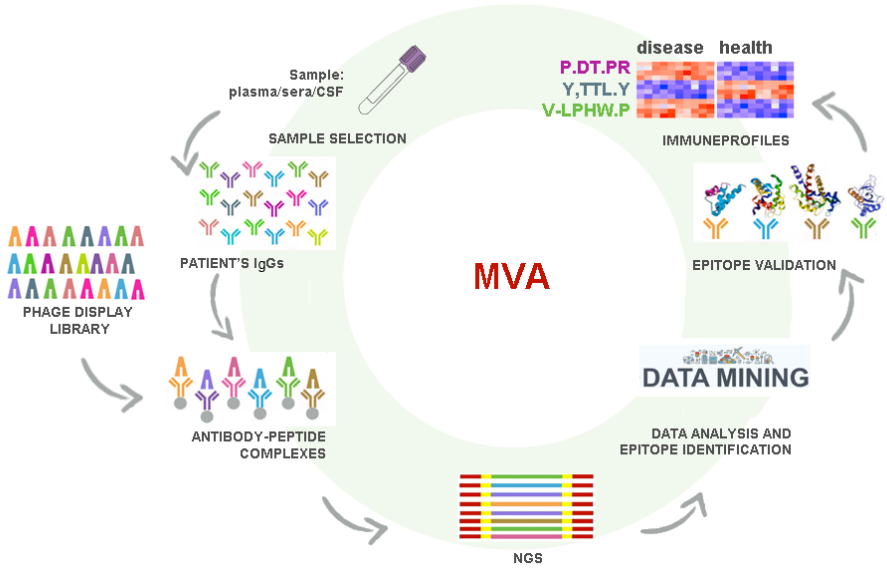

Fig 1. Scheme of the humoral immune response studies using the Mimotope Variation Analysis (MVA) method. *For IgG mimotope mapping, modified random 12-mer peptide M13 phage library is used with complexity of ~5 × 10^9 different peptides. MVA workflow comprises of different sequential steps: i) immunocapture of IgG-phage complexes from a sample using peptide phage library, ii) subsequent high-throughput Illumina short DNA sequencing of phages; iii) bioinformatical data processing resulting in epitope/mimotope identification; iv) validation of selected candidate epitopes; v) discriminative immunoprofiles. Grey lines indicate the order of the steps.*

As previously described, there are a wide variety of approaches (direct, indirect) and methods (BCR sequencing, protein/peptide arrays, display methods) for studying the antibody repertoire, each of which has its own advantages and shortcomings. Significant differences concern the following characteristics of high-throughput technologies for antibody mapping: i) nature and number of analytes (probes); ii) number, volume, and treatment of samples; iii) interpretation of results; iv) overall complexity and cost of the technology. Current estimates of the adult human antibody repertoire size, although controversial, have been suggested to be ~10^{13} members for the naïve repertoire (Rees, 2020). In terms of the number of possible analytes, MVA certainly stands out from other technologies because it employs a random peptide phage library with a significantly higher number of peptides displayed compared to either the peptide/protein arrays (Ayoglu et al., 2016b) or the peptide libraries used in other display technologies (Weiss-Ottolenghi & Gershoni, 2014). Technologies also vary in terms of what they

present to antibodies (nature of analytes). The advantage of MVA compared to many other methods is that the peptides displayed are 12-mer. Previous studies of antibody binding epitopes have reported that 95% of linear epitopes span fewer than 12 amino acids (Forsström et al., 2014) and longer peptide sequences (>15 amino acids) can contain a larger number of distinct epitopes, thereby increasing opportunities for peptide cross-reactivity with antibodies with divergent specificity. The potential false positive phenomena described should be considered when analysing the results obtained by using longer (>15 amino acid long peptides) analytes including protein arrays (Zandian et al., 2017) and longer sequencies (56-mer peptides) using display methods (Xu et al., 2015)). Phage peptide libraries, like synthetic peptide arrays, have some drawbacks compared with protein arrays: the lack of the post translational modifications and the conformational structures (Anand et al., 2021). Still, studies of epitope-paratope interactions in crystal structures have shown that 85% of all epitopes contain at least one continuous stretch of five amino acids (Kringelum et al., 2013) which makes the study of linear epitopes also important for the detection of immune response against structural antigens. Furthermore, linear peptides may mimic structural epitopes (mimotopes) and these epitopes can be annotated against databases containing information on protein conformational structures (Weiss-Ottolenghi & Gershoni, 2014). The same mimicking ability of phage displayed peptides is also described for post-translational modifications of proteins. For example, phage display peptide analyses of sugar specific antibodies have proven feasible as often the affinity purified peptide is a mimetic of a non-amino acid structure (Barenholz et al., 2007).

Given the size of the antibody repertoire, BCR high-throughput sequencing is also a very suitable method for whole repertoire analysis. Still, there are some significant deficiencies in the BCR profiling method. First, while only one drop of serum sample (2 mL) is required for MVA, significantly more patient blood (>10 mL) is required to collect enough circulating B cells (Lee et al., 2016). Second, while MVA provides direct information about epitopes recognised by antibodies, it is easy to identify the organism that triggered the immune response. At the same time, BCR provides information about paratopes, and antigen prediction certainly requires prior knowledge of the expected response (for example vaccination analysis) (Lee et al., 2016).

The complexity of implementing different technologies is also important in conducting research because it determines the time and cost of the analysis. The complexity of the technology is determined, for example, by the need to pre-process samples before analysing, such as the sorting of B cells before BCR sequencing (Lee et al., 2016). The direct use of serum or plasma in methods such as protein/peptide arrays and peptide displays, including MVA is certainly less complex (Zandian et al., 2017). It is important to consider how easy or difficult it is to synthesise analytes/probes for a technology. For example, any potential antigen must be synthesised and attached to a carrier prior to making the planar or beads-based protein array (Ayoglu et al., 2016b) which is more labour intensive than cloning and using display libraries (Valldorf et al., 2021). Finally, the result-reading tools have to be considered, where the devices for microarray scanning are needed, as well as the NGS services, where the analysis of millions of sequences is needed.

In conclusion, MVA is a suitable method for examining broad immune system responses mediated by antibodies. It provides a comprehensive profile of an individual's immunoglobulin G response and therefore enables to get a detailed overview of a person's current health conditions, including chronic (pathogen) disease burden.

3. Aims of the study

Although a large number of scientific studies have been published in recent years on MS and NT1 pathologies, there are still a number of aspects that remain to be addressed. It is clear that B cells and the antibodies they produce play an important role in the development of these neuroinflammation-associated diseases. Effective biomarkers are lacking for the assessment of neuroinflammation pathologies, in particular those measurable in blood. In the current studies, two immune-associated brain diseases were analysed – ON developing to MS and Pdmx-NT1. The aims of the current thesis were to employ a hypothesis-free approach of Mimotope Variation Analysis to

i) determine specific antibody epitope profiles of subjects with ON who progressed and who did not progress to MS;

ii) delineate antibody epitope profile characteristics of sNT1 and Pdmx-NT1.

4. Materials and methods

I used the following experimental methods during the studies that are described in the indicated publications:

- Mimotope Variation Analysis (publications I, II)
- Bioinformatic analysis for epitope mapping (publications I, II)
- ELISA, dot ELISA (publications I, II)
- Semiquantitative RT-PCR analysis (publication II)
- Construction of expression vectors (publications I and II)
- RNA extraction (publication II)
- Cell differentiation (publication II)
- Preparation of nuclear extracts and western blotting (publications I, II)
- Immunocytochemistry (publication II)

5. Results and discussion

5.1 Antibody epitope profiling of the serum and CSF of patients with neuroimmune pathologies by MVA (Publications I and II)

To identify antibody epitopes that associate with prognosis of ON or NT1, we characterised the antibody epitope repertoire in six independent clinical cohorts: patients with optic neuritis (n=24; serum and CSF samples; ON), patients with multiple sclerosis (n=20; serum and CSF samples; MS), patients with Narcolepsy type 1 (n=16; serum samples, NT1) and corresponding controls (n=500; serum and CSF samples; CTRL or HC) (**Table 5**) using MVA, a next generation phage display technology (**Publications I and II**). Fifteen of the ON patients were subsequently diagnosed with relapsing MS by McDonald 2005 and 2017 criteria (denoted as ON^{MS}) during the median follow-up time of 52 months (ranging from 38 to 69 months), whereas nine patients did not develop MS (denoted as ON^{ON}). Another study cohort for MS studies contained samples from the diagnostic phase of treatment-naïve patients with relapsing MS (by McDonald 2005 and 2017 criteria) either with ON onset (n=10; denoted MS^{ON}) or other MS onset (n=10; denoted MS^{other})**.** Intrathecal oligoclonal bands were present in all CSF samples of patients with ON and MS. For NT1 studies, we used a cohort of serum samples that were collected from patients with H1N1-induced NT1 (n=10; denoted Pdmx-NT1) or spontaneous NT1 (n=6; denoted sNT1). Four out of 6 sNT1 patients were vaccinated with Pdmx after they had been diagnosed with NT1. All narcolepsy patients had NT1 as defined by the American Academy of Sleep Medicine international criteria of sleep disorders version 3. Control cohort contained samples of healthy individuals, and samples of subjects with different ICD-10 diagnosis codes, but without any notification of demyelinating disease or any type of narcolepsy disease. In addition, samples of H1N1-infected (denoted as H1N1-HC) and Pandemrix-vaccinated healthy controls (denoted as Pdmx-HC) were recruited in the NT1 study (**Table 5**).

Table 5. Description of samples analysed (Publications I and II).

Study cohort	Size	Type	Diagnose	Ethnicity	Average age at sampling (NA)	Female/ Male (NA)
ON	24	serum/CSF	ON	Fin*	33	16/9
MS	20	serum/CSF	MS	Fin	32	16/4
ON_CTRL_1	38	serum/CSF	No ON/MS	Fin/Est	47	15/23
ON_CTRL_2	414	serum	No ON/MS	Fin/Est	41	239/172 (10)
NT1	16	serum	NT1	Fin	18	10/6
Pdmx-HC	16	serum	No NT1	Fin	NA	12/2 (2)
H1N1-HC	16	serum	No NT1	Fin	21	0/16
NT1-HC	16	serum	No NT1	Est	34	10/6

*ON – optic neuritis (including patients with stable ON (ON^{ON}) and patients with subsequently diagnosed with MS (MS^{MS})); MS – multiple sclerosis (including patients with ON onset (MS^{ON}) or patients with other MS onset (MS^{other})); CTRL/HC – controls; NT1 – narcolepsy type 1(including Pdmx-induced NT1 samples (Pdmx-NT1) and spontaneous NT1 (sNT1) samples); Pdmx-HC – Pandemrix-vaccinated healthy controls; H1N1-HC – H1N1-infected healthy controls; NA – not available; Serum – serum or plasma samples; CSF – cerebrospinal fluid; * also contains 3 samples with non-native Finnish background*

Using MVA we generated a database containing millions of peptides for the ON and NT1 study groups (**Table 5**) that allowed a fine molecular description of the peptide antigenic repertoires of ON and NT1 patients (**Publications I and II**). The data analysis steps included: i) data pre-processing and classification; ii) group-based data filtration and statistical analysis; iii) data annotation and validation (**Fig. 2a**). Data pre-processing included sample demultiplexing by 4-bp tags of NGS sequence reads (50 bp), quality control (discarding the reads with mismatches in the flanking 4 bases of the constant region and sequences from wild type phages M13), *in silico* translation into 12-mer peptides (all untranslated sequences were discarded), data trimming to average reads and finally linking the data with the clinical classificators. On average, MVA generated individual immunoprofiles containing 3 million peptide sequences per sample, out of which 350,000 peptides, on average, were unique. The reproducibility of data by Pearson correlation coefficient was >0.9 by comparing two independent replicas of the same or different sequencing outputs (**Fig. 2b**). The data structure analysis of three different data fractions (the most frequent 5,000 (Top5000), the most frequent 20,000 (Top20000), and the entire peptide dataset) revealed that although most of these peptides were largely individual-specific (**Fig. 2c, top**), the study cohorts shared a substantial fraction of common characteristics across all datasets (**Fig. 2c, bottom**).

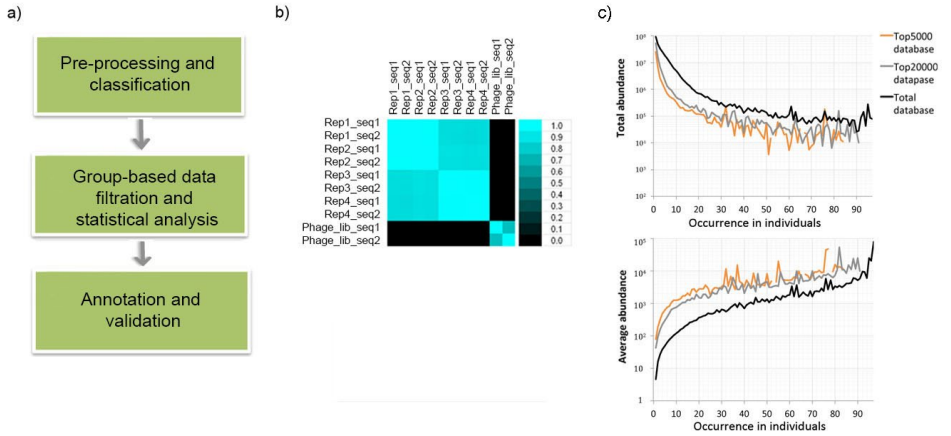

Fig. 2. Overview of MVA data processing. a) *MVA data processing consists of the following steps: data pre-processing and classification (NGS sequence quality control and translation), group-based data filtration and statistical analysis (peptide filtration, peptide clustering with SPEXS (https://github.com/egonelbre/spexs2), correlation analysis, group comparisons, logistic modelling), and finally data annotation and validation (aligning on antigens (IEDB, UNIPROT), ELISA, dot ELISA, WB, ICC, etc.).* ***b)*** *Pearson correlation coefficient of independent experimental replicas. One control sample (HC_1) was replicated in four independent MVA experiments (Rep1-4). All samples were sequenced twice by NGS (seq1-2). Lib – sequencing of modified 12-mer peptide phage library (Ph.D.-12, New England Biolabs).* ***c)*** *Data structure analysis plots of unique peptides across all samples defined by MVA shown as total (top) and average abundance (bottom) distribution values (range – number of peptides described in 1 to 97 samples including serum and CSF samples of ON, MS and ON_CTRL_1 (**Table 5**)). Three different data fractions were analysed: the most frequent 5,000 (Top5000), the most frequent 20,000 (Top20000) and the entire peptide dataset. y-axes – total or average abundance values of peptides; x-axes – 97 individual samples; abundance – frequency of peptides (Sadam et al., 2021).*

Next, group-specific peptide selection (**Fig. 2a**) with the highest sensitivity and/or the best discrimination in abundance resulted in sets of peptides that were enriched in different study groups. For example, 1300 unique peptides highly enriched in NT1 cohort ($p<0.001$, ANOVA) were described when the dataset of this disease group was compared with that of controls (**Publication II**). Since antibodies require only a few amino acids to bind to the antigen, constituting a minimal epitope (Sykes et al., 2013), several 12-mer peptides containing the same epitope consensus were described. To characterise these consensuses further we clustered ON-enriched peptides into a reduced set of epitopes using an exhaustive pattern search algorithm SPEXS2 and delineated 1669 group-discriminative epitopes ($p<0.005$ (hypergeometric test)) (**Publication I**). For epitope discovery, when using unique peptides or counts of peptides containing the epitope as input values, different statistical tests were applied, including ANOVA, t-test, correlation analyses, Receiver Operating Characteristic (ROC), Chi-square test, distribution profiles (**Fig. 2a**). Finally, reference sequence databases (IEDB, NCBI, Uniprot) were used for peptide/epitope alignments and antigen annotation (**Fig. 2a; Publications I and II**). Various independent molecular methods including ELISA, dot ELISA, WB, ICC and others were used to validate the results obtained by MVA (**Fig. 2a; Publications I and II**). We confirmed that different MVA-defined viral epitopes were associated with seropositivity of infections ($p<0.001$ ANOVA; EBV, CMV and influenza) (**Publications I and II**) and validated MVA leads by using dot ELISA at accuracy of 95% or above (ROC analysis; **Publication I**).

The MVA analysis of the individual samples from different study groups (ON, MS, NT1 and CTRLs) described unique immunoprofiles containing both individual and common subsets of peptides (**Publications I and II**). Furthermore, analysis of the most abundant fractions resulted in defined sets of peptides that were common to samples of serum/plasma and CSF from the same individual ($r>0.7$, Pearson correlation coefficient; $p<0.001$, ANOVA) (**Publication I**). By demonstrating close similarity of the immunodominant antibody epitope profiles of blood and CSF, our analysis also argued for the free exchange of antibodies across the BBB in individuals with ON and MS. Using dot ELISA method, we measured the total amounts of IgGs and proteins in CSF samples of the ON patients (ON^{ON} and ON^{MS}), MS patients (MS^{ON} and MS^{other}) and controls (CTRL) (**Publication I**). As expected, we described increased (ANOVA $p<0.01$) IgG fraction in ON (average 0.14 ug/ul) and MS (average 0.18 ug/ul) patient compared with the control sample cohort (average 0.05 ug/ul) (**Publication I**). Although the levels of total protein and IgGs in the ON and MS samples were similar, the OCB analysis described a clear group-differentiating result (**Publication I**). Using standard isoelectric focusing and agarose gel electrophoresis, OCBs were detected in all CSF samples of the discovery and validation cohorts. In the ON group the average number of CSF-specific OCBs was 14, whereas MS group average number of CSF-specific OCBs was significantly higher, 20 ($p<0.005$, ANOVA) (**Publication I**). Furthermore, normalizing the dot ELISA findings from the CSFs of MS patients to total IgG content yielded a highly positive correlation in the immunoreactivity characteristic of the CSF and peripheral blood to epitopes of gB of CMV and VCA of EBV (more about the epitopes of VCA and gB in the following chapter) ($r=0.95$, and $r=0.71$, Pearson correlation coefficients, respectively) (**Publication I**). According to our knowledge, this is the first broad comparison of the IgG repertoires of the CSF and peripheral blood. The identification of circulating biomarkers with immunological significance presents an attractive solution for early diagnosis and prognosis of

neuroimmunology diseases. Questions that still remain include the origin and also of role of these antigenic epitopes in the normal physiology and pathology of the brain.

In conclusion, our results of ON and NT1 studies showed that MVA, a hypothesis-free high-throughput NGS-based epitope mapping from body fluids is a suitable method for examining the breadth of immune response mediated by antibodies. It provides a comprehensive profile of an individual's IgG response and therefore enables to get a detailed overview of the person's current health conditions, including the status of the disease. Numerous scientific publications on neuroinflammation, in which B cells play a very important role, have shown that antibodies are associated with a wide range of neurological diseases, including MS and NT1 (Ahmed et al., 2015; Fraussen et al., 2014; Sabatino et al., 2019). The free flow of antibody secreting B cells across the BBB in individuals with ON and MS has previously been suggested from the sequencing data of B cell receptors (von Büdingen et al., 2012) and IgG heavy chain variable regions (Bankoti et al., 2014). The levels of antigen-experienced, class-switched memory B cells and antibody-producing plasma cells are particularly elevated in the CSFs (relative to their levels in blood) of patients with MS (Obermeier et al., 2008). Therefore, it is crucial to study the disease-specific antibody repertoires in the intrathecal compartments of the CNS. In our ON and NT1 studies, we performed MVA immunoprofiling of CSF and serum samples demonstrating the power of the MVA method for high-throughput profiling of antibody response at the epitope precision for the early phase of MS and with ON onset.

5.2 Antiviral antibody epitopes associated with MS and NT1 (Publications I and II)

Data from group comparative clustering analysis of ON-specific peptide profiles resulted in case-discriminating antigenic repertoire. Herein, we identified a specific epitope mimicking the EBV VCA p18 (the viral capsid antigen p18; $_{161}$GGQ**P**HD**T**A**PR**GARKK$_{175}$: **Fig. 3**) that was highly recognised in both CSF and serum samples of ON diseased (ANOVA $p<0.05$) (**Publication I**). Immunodominant region in the p18 VCA of EBV encompassing amino acids 153 to 176 was initially described by Grunsven and colleagues, as a useful biomarker for EBV IgG and IgM diagnostics (Fachiroh et al., 2006; van Grunsven et al., 1994). However, the delineated epitope has not, to our knowledge, been previously mentioned in the neurological disease literature. Even though the EBV load in the CNS of MS patients is currently unclear, in the scenario when EBV-infected memory B cells reactivate, expand and differentiate in the CNS, this would lead to viral synthesis and elicitation of the strong anti-viral immune response (Hislop & Taylor, 2015). This could cause a permanent loss of immune tolerance to CNS-specific antigens. In this regard, the finding of the increased anti-capsid protein (anti-VCA) immune response and not against EBNA1 (anti-EBNA1), a protein expressed during EBV latency, in ON samples was particularly interesting because it suggested the reactivation of EBV infection in memory B cells (**Publication I**). Based on our results, we hypothesised that the viral reactivation might still be the primary causative agent of the disease because the response was detected at the prediagnostic phase of MS (at the first clinical episode of ON). Our data are in good support with previous findings (De Paschale, 2012) and would argue for acute, late primary infection or reactivation of EBV as a trigger in ON to MS transition in immunocompromised individuals. Moreover, a very recent study about samples of pre- and post-onset of disease collected from 995 MS patients also showed a high anti-VCA response in the diagnostic phase of MS (Bjornevik et al., 2022). Future studies are needed to determine the dynamics of viral re-activation/infection and the related development of MS.

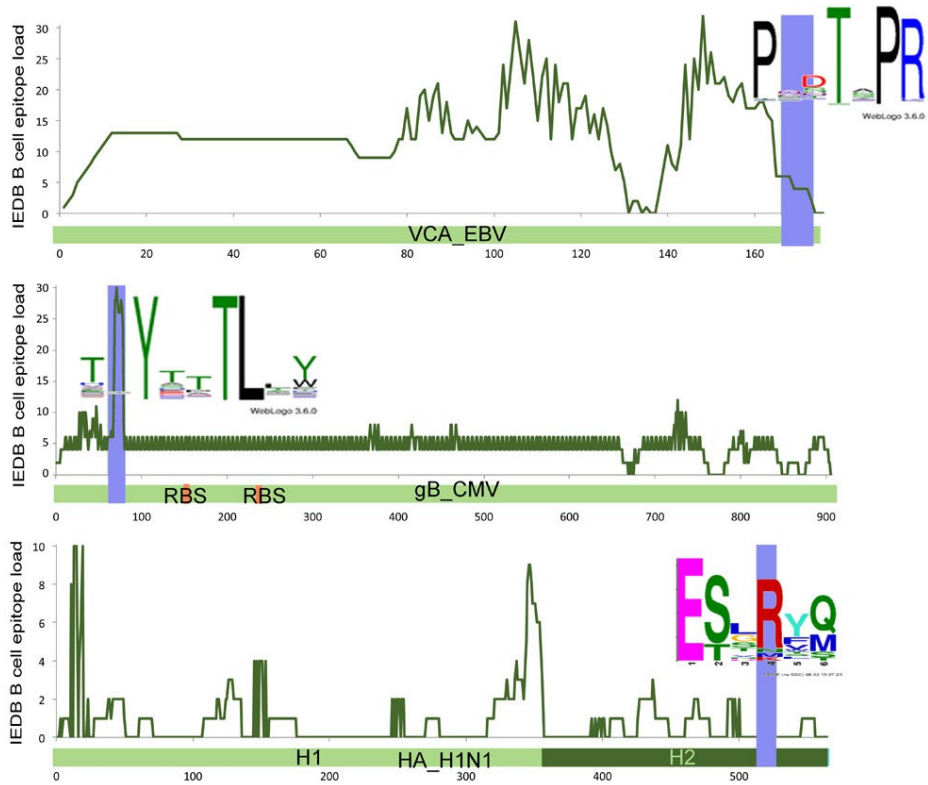

Fig 3. MVA-defined epitopes mimic highly antigenic epitopes of EBV (VCA, P14348), CMV (gB, F5HB53), and H1N1 (HA, P03452). *Referred B cell epitopes of EBV VCA (altogether 27 characteristics), CMV gB (119 characteristics), and H1N1 HA (394 characteristics) antigens retrieved from Immune epitope database (http://www.iedb.org/) were aligned on the respective protein sequences. Purple highlights indicate epitopes studied in current research (epitopes CMV gB ($_{70}$ETI**Y**NT**TLKY**$_{80}$), EBV VCA p18 ($_{161}$GGQ**P**HDT**APR**GARKK$_{175}$) and H1N1 HA ($_{120}$L**ES**MGI**YQ**IL$_{131}$)). To define characteristic sequence logos for each epitope, position weight matrices were generated and visualised as sequence logos using in-house "MotifTree" algorithm. y- axes – IEDB B cell epitope mapped per amino acid; x -axes – protein sequences of EBV VCA (P14348), CMV gB (F5HB53), and H1N1 HA (P03452).*

Further, we described changes in the antibody response to CMV antigens in ON patients who later developed MS (ON^{MS}) (**Publication I**). In more detail, from group-discriminating analysis we described an immunodominant epitope within the domain 2 (AD2) of the neutralising epitope of CMV gB ($_{70}$ETI**Y**NT**TLKY** $_{80}$; **Fig. 3**) (**Publication I**). A high titre antibody immune response was observed in patients with stable ON and in controls ($p<0.05$, ANOVA), but not in ON^{MS} patients. More interestingly, although the overall seropositivity was also slightly lower in the ON^{MS} compared to the ON^{ON}, (**Publication I**). Interestingly, the binding site of antibodies to the AD2 are predominantly encoded by germline V-genes *IGHV3-30*18*, and *IGKV3-11*01*, in a process that has been proposed to favour reproductive success by limiting CMV infections and protecting the fetus (Ohizumi et al., 1992; Thomson et al., 2008). Thus, our data would predict that individuals with these alleles have a lower risk of developing MS. Although the association between CMV infection and development of MS has not precisely described, a recent large-scale

study provides convincing evidence that MS risk is negatively correlated with CMV-seropositivity (Bjornevik et al., 2022) and moreover, that the immune response to CMV may attenuate the adverse effect of EBV (Grut et al., 2021). Current study is the first study describing associations between the highly antigenic epitopes of gB CMV and VCA p18 EBV with MS, also suggesting direct involvement of these two herpesviruses in the pathophysiology of MS.

From epidemiological studies, there is substantial evidence that upper airway infections, most likely influenza A and *β*-hemolytic streptococcal infections (Aran et al., 2009), can precipitate the onset of NT1 (Kornum et al., 2017). In **Publication II** we showed that the antibody titres to seasonal flu (A/H1N1 and A/H3N2) were lower in NT1-diseased as compared to Pdmx-HC individuals as determined by using commercial ELISA tests ($p < 0.001$) (**Publication II**). This finding was in slight contrast to earlier studies reporting that Pdmx-NT1 patients had higher median levels of anti-H1N1 antibodies than controls (Lind et al., 2014). Our data showed that the most commonly shared epitopes targeted by the anti-Pdmx/anti-H1N1 immune response were located in the conserved C-terminal region (H2, stem domain) of H1N1 hemagglutinin (HA; C4RUW8; $_{120}$L**ES**MGI**YQ**IL$_{131}$; **Fig. 3**) between amino acids 521 to 531. This encompassed a proven T cell antigenic region of HA of A/California/04/2009 mapped to amino acids 527–541 (Schanen et al., 2011) (**Publication II**). We defined a new epitope with E[ST].R.[QM] consensus that was highly recognised in H1N1-HC, and relatively infrequent in Pdmx-HC and NT1 samples as compared with HCs ($p<0.05$, ANOVA; **Publication II**). The most common human neutralising antibody response to influenza HA is directed to the N-terminal head domain (H1), especially the receptor binding site (RBS) (Murin et al., 2019). The challenge for vaccine development associated with the head domain is that protein sequence surrounding the RBS is highly variable among different influenza strains (Crowe, 2019). Although antibodies against the stem region exhibit lower potency in virus neutralisation than antibodies directed against the head domain, anti-stem antibodies can achieve broader cross-reactivity of influenza A viruses (Corti et al., 2011). In addition, we described immunodominant epitopes also for the nucleoprotein (H1N1/NP, B4URE0), another surface glycoprotein, and to polymerase acidic protein (H1N1/PA, I6THC5), some of which described the known immunogenic epitopes from IEDB (http://www.iedb.org/) (**Publication II**). These results indicate that MVA can provide detailed data at epitope level associated with vaccinations against viral antigens.

As described in the following chapter, we discovered prostaglandin D2 receptor DP1 as a novel molecular target of autoimmune response in NT1 (**Publication II**). In relation to this, we looked for the mimicry of the newly defined epitope of DP1 (sequence of $_{94}$RVLAPALD$_{101}$) in the NCBI protein database (accessed 05.2021). Although we did not detect high homology (100%) matches with the epitope RVLAPALD, there was a reasonable similarity with multiple microorganismal proteins (>75% similarity, **Table 6**). Interesting hypotheses were raised based on the BLAST analysis data as several of these organisms carrying antigens with similarity to the DP1 epitope have previously been described in relation to neuroinflammation (Forrester et al., 2018; Klein & Hunter, 2017a). For example, we described mimicry of the epitope with three different HSV-1 proteins (**Table 6**). HSV-1 is a neurotropic human pathogen (**Table 1**) that infects and establishes latency in the peripheral sensory neurons of the host. From latency, HSV-1 can re-activate and cause peripheral pathologies as a result of anterograde trafficking from the sensory neurons. Under rare circumstances, HSV-1 can migrate into the CNS and cause herpes simplex encephalitis, a devastating disease of the CNS (Menendez &

Carr, 2017). Furthermore, although rhinoviruses are not neurotropic, it has been shown that respiratory viral infections contribute to asthma and autoimmune diseases by inducing cross-reactive T and B cell responses (Pusch et al., 2018). Coordinately, NT1 onset has been suggested to be triggered by seasonal upper airway infections at wintertime (Han et al., 2011).

Table 6. Potential mimicry between epitope RVLAPALD and antigens of neurotropic pathogens.

Organism	Antigen	Sequence NCBI ID	Epitope
Rhinovirus A	polyprotein	ALT21484.1	$_{87}$LAPALD$_{92}$
HSV1	Capsid triplex subunit 1 (UL38)	YP_009137113	$_{336}$RGLEPALD$_{343}$
HSV1	Tegument serine/theonine protein kinase (UL13)	YP_009137087.1	$_{433}$RVLHPAL$_{439}$
HSV1	Transcriptional regulator ICP4 (RS1)	YP_009137135.1	$_{1156}$VL-PALD$_{1161}$
Borreliella burgdorferi	excinuclease ABC subunit UvrA (UvrA)	WP_002657284.1	$_{665}$VLYPALD$_{671}$

In conclusion, we reported on the prognostic value of viral antibody epitopes as novel biomarkers for the risk of MS after the first episode of ON and NT1. Primary infections (also vaccinations) and reactivation of latent infections of neurotropic pathogens (i.e. EBV, CMV and influenza) are the known main factors eliciting pathogenic neuroimmune response in CNS. Different infections and vaccinations have been associated as initial triggers and have been related to the progression of MS and NT1 (Han et al., 2011; Langer-Gould et al., 2017; Partinen et al., 2012). We described the association between the highly antigenic epitopes of gB CMV and VCA p18 EBV with MS, suggesting direct involvement of these two herpesviruses in the pathophysiology of MS. Why and how the humoral response against these two viral epitopes affects the course of the disease remains an open question. Nonetheless, linking different infections to the development of neuroimmune diseases provides important information on the disease course and provides opportunities to develop epitope-specific blood-based diagnostics. Furthermore, we showed that the most shared epitopes raised by the anti-Pdmx/anti-H1N1 immune response were located in the conserved C-terminal stem domain of H1N1 HA. The major challenges in vaccine development are to achieve protective antibody response and to induce highly cross-reactive response against different strains of viruses (Crowe, 2019). Thus, knowledge of the epitopes shared between different strains of influenza viruses may be important for the development of new vaccines. However, the neutralising properties of the delineated novel epitopes need to be confirmed in the future.

5.3 Novel autoantibody targets in MS and in NT1 (Publications I and II)

By today, many potential autoantibodies have been associated with MS (**Tabel 2**). Moreover, a recent comprehensive study showed that the development of autoantibodies in MS may be linked to the mimicry of the anti-pathogen response (Lanz et al., 2022). Specifically, high-affinity molecular mimicry between the EBV transcription factor EBNA1 and the CNS protein GlialCAM was shown and cross-reactive CSF-derived anti- EBNA1/GlialCAM antibodies were more prevalent on patient with MS (Lanz et al., 2022). Our annotation analysis against epitopes in the IEDB database revealed that the

epitope mapped on VCA p18 also mimicked the epitope on human septin 9 ($_{37}$ET**P**NS**T**P**PR**$_{45}$, IEDB ID 704012; **Publication I**). Most interestingly, a recent study revealed that antibodies to the small capsid protein of EBV cross-react with human septin-9 (Lindsey, 2017). Septin-9 is a cytoplasmic filament-forming protein and may function in cytokinesis, exocytosis, neurite growth and cell cycle control and deviations in Septin-9 function may contribute to the pathological processes of MS (Lindsey, 2017). For CMV it has previously been shown that the CMV-UL86 antigenic region located between amino acids 981–1003 shares a cross-reactive T cell epitope with the encephalitogenic MOG peptide encompassing amino acids from 34 to 56. Additional alignment analysis using NCBI protein database (accessed 05.2021) performed with epitopes mapped to CMV gB and EBV VCA p18 (**Publication I**) to detect the mimicry between viral epitopes and autoantigens revealed potential associations with human polypeptide N-acetylgalactosaminyltransferase 9 isoform A (NP_001116108.1; $_{458}$V**YN**N**TL**T**Y**G$_{466}$) and apolipoprotein L6 (NP_085144; $_{171}$I**YN**LRN**TLKY**$_{180}$) for epitope of gB of CMV, and human amiloride-sensitive sodium channel subunit beta (NP_000327; $_{591}$Q**P**-**DTAPR**$_{597}$ and HHIP-like protein 1 isoform a precursor (NP_001120730; $_{558}$**G**E**P**SA**TAPRG**$_{567}$) for epitope of VCA p18 of EBV. These mimics between viral- and auto-antigens are highly interesting leads, although further validations are needed.

Antibody epitope profile differences between NT1 and healthy controls revealed that patients with NT1 exhibited a specific immune response to epitopes located in the second extracellular loop region of the human prostaglandin D2 (PGD2) receptor protein (DP1; $_{94}$**RVLAPALD**$_{101}$; $p<0.0001$, ANOVA; **Publication II**). This region in DP1 was predicted to function in recognition of the ligand prostaglandin D2 (Avlani et al., 2007; Nagata et al., 2017). Interestingly, four MHC-I binding epitope regions encompassing amino acids 132-140 (ID: 716767), 145-156 (ID: 637966), 195-203 (ID: 727099) and 303-311 (ID: 697995) have previously been defined according to the immune epitope database (www.iedb.org/). The latter (303-311) encoded another extracellular domain of DP1 that our study defined as another potential target of B cell response. In line with previous reports (Urade & Hayaishi, 2011), we observed a broad expression of *DP1* mRNA across different regions of human brain, in human glioma (hGC), and normal mesenchymal stem cells (hMSCs). Treatments of the mesenchymal stem cells with the ligand prostaglandin D2 increased *DP1* expression, whilst the pro-inflammatory cytokines *IL-1β* and *IFNγ* either slightly increased or decreased its expression, respectively. In contrast, PGD2 strongly reduced *IL-1β* and *IFNγ* expression, suggesting that an intact PGD2-DP1 signalling induced anti-inflammatory effects in the studied cells (**Publication II**). Data combined from the western blot and immunocytochemistry analysis suggested that the peptide **RVLAPALD**SWGT could embed a part of structural as well as a linear epitope given that upon competition studies the peptide interfered with DP1-specific serorecognition of globular as well as denatured antigens (**Publication II**). This finding highlights the potential importance of the PGD2-DP1 pathway in the functioning of sleep-wake homeostasis as suggested by the role of DP1 in slow-wave sleep (Terao et al., 1998). Besides its role in inflammation, PGD2 can also act to inhibit inflammation, as virus-induced inflammasome activation is suppressed by PGD2/DP1 signalling (Vijay et al., 2017). Among cells expressing DP1, MCs can release histamine and other factors that affect sleep and the immune response in the brain. Accumulating evidence show that MCs play a role in the regulation of sleep and behaviour (Chikahisa et al., 2013). However, the precise mechanisms by which PGD2-DP1 signalling may influence orexinergic neurons and immune regulation in NT1 require further studies.

We also examined the immune response to the previously found autoantigens in the NT1 samples, for which we used MVA-based immunoprofiling combined with the interrogation of the presence of known autoantigens as previously identified in Pdmx-NT1 disease (**Table 3**). Although we identified epitopes resembling those in OX and OX1R/2R NTSC1A, MAP3K7 and BCL6 proteins, immune response to none of the previously identified antigens was prevalent across the NT1 cohort and was also observed in HC if less stringent statistical power criteria were used (**Publication II**). These data allowed to conclude that, apart from the BCL6 related peptide subset, peptides corresponding to the previously identified autoantigens had relatively little discriminative power, suggesting also that these antigens were either rare or recognised promiscuously in these patient groups with heterogeneous clinical and ethnical backgrounds (Sarkanen et al., 2018a). This underscores the complexity of NT1 disease with different autoimmune targets and involved pathways (Sarkanen et al., 2018b). Because DP1 is important in the escalation and also in reduction of neuroinflammation (Mohri et al., 2007; Vijay et al., 2017), our hypothesis was that the increased response against DP1 was associated with a specific NT1 phase. Of note, majority of the DP1 and BCL6-positive NT1 samples had undetectable OX findings from the related CSFs (with average values of 6.1 pg/ml), whereas those 4 that were negative by our dot ELISA measurements had OX levels in respective CSF samples low but in the detectable range (with average values of 77 pg/ml) (**Publication II**). Thus, we suggested that the development of the autoantibodies against DP1 and BCL6 is associated with the further progression of NT1 pathologies. In summary, our data of immunoprofiling supports the findings of immune defects in NT1 in pathways of i) DP1 and PDG2/histamine signalling; ii) BCL6-related functions; iii) orexin/OX1/OX2 signalling; iv) stress and inflammation-associated mitogen-activated pathways (including MAP3K7, also known as transforming growth factor (TGF)-*β*-activated kinase 1 (TAK1)); v) adenosine signalling (involving NT5C1A) (**Publication II**). Together these results provide a comprehensive map of potential targets that may contribute to NT1 and as such can be of help in designing future strategies for the treatment of the disease.

In conclusion, we found a strong autoimmune-like response to prostaglandin D2 receptor DP1, suggesting a firm link between DP1/PDG2 and histamine pathways and the immunological basis of NT1. In the brain, PGD2 regulates sleep, body temperature, and nociception, and its levels exhibit marked changes in different neuropathologies (Urade & Hayaishi, 2011). Together our results justify further investigation of the autoimmune response associated with the PDG2 pathways as antibodies to it might modify the function of pharmaceutical compounds aimed at treating NT1. Furthermore, our data highlighted the epitopes of common viral antigens (VCA p18 of EBV and gB of CMV) as conveyers of the pathological processes due to the mimicry with human proteins (Lindsey, 2017). Given the significant clinical severity and heterogeneity of the neuroimmune diseases (Mitoma & Manto, 2020), it is crucial to describe the disease-associated autoantigens that may reflect the pathogenic mechanisms or could present valuable markers for predicting the disease progression and facilitate individualised treatments. The present studies shed light onto those aspects to aid better diagnosis and disease management.

5.4 Concluding remarks (Publications I and II)

Understanding the antibody-mediated immune response – how its homeostasis is maintained, how B cell memory is affected by immunisation, how it encounters with pathogens, physiology, and ageing – is essential for the development of novel effective vaccines, diagnostics, and therapies. To meet these challenges, one must profile the antibody repertoire in a manner that is high-throughput and still cost effective and expedient. Only in this way would we be able to run antibody screens routinely during personalised medical diagnosing and treatment. New assays provide a high-throughput and multiplex analysis of several hundreds of body fluid samples against several thousands of antigens (**Tabel 4**). Peptide-expressing phage display libraries are widely used for the antibody response profiling, where affinity selected peptides are analysed to discover epitope mimics (Valldorf et al., 2021) (**Publications I and II**). We have used the next generation peptide phage display MVA technology for characterisation of the antibody epitope repertoire from body fluids (**Publications I and II**). MVA i) employs a random 12-mer peptide phage library with a significantly high number of peptides displayed (10^9); ii) needs only a drop (2 ml) of serum or plasma per analysis without any major pretreatments; iii) is hypothesis free and useful for antigen identification, also at organism level; iv) is based on easy handling of phage library and widely available NGS services. Our current studies demonstrated the power of the MVA method for high-throughput profiling of antibody response at the epitope precision of the clinical samples from the early phase of MS with ON onset (**Publication I**) and the clinical samples from vaccination-associated NT1 (**Publication II**). Despite the highly individual top immune response profiles, we observed that paired serum and CSF samples share highly similar antigenic features, arguing for the common robust antibody response both in the peripheral blood and in the CNS potentially associated with MS (**Publication I**).

Because of the significant clinical heterogeneity of MS, biomarkers that reflect pathogenic mechanisms may be valuable for predicting disease progression and facilitate individualised treatments. The identification of circulating immunological biomarkers presents an attractive solution for early diagnosis and prognosis of MS. Despite the abundance of potential biomarker candidates, thus far there is no definitive biofluid test available for accurately diagnosing MS or monitoring its progression (Filippi et al., 2018). Since EBV and CMV infections are so common, the overall seropositivity to named viruses would not constitute a good biomarker for MS risk assessment. In our study, we found that different demyelinating inflammatory conditions (ON^{ON}, ON^{MS}, MS^{ON} and MS^{other}) were characterised by the immune response to specific antibody epitopes (**Publication I**). Based on these promising data, one would argue that epitopes of gB of CMV and VCA of EBV detect MS with different onsets. Biomarker risk score model with 74.61% accuracy established epitopes of A and B as blood-based biomarkers of MS with ON onset. For stratifying MS from other non-myelinating disease, the clinical accuracy of these two biomarkers reached as high value as 76.62% (**Fig. 4a**; **Publication I**). In the future, it would be needed to investigate the additional diagnostic value that epitopes A and B would carry for stratifying patients with first MS symptoms and with high initial neurofilament light chain levels as serologic biomarkers for the disease course prognosis.

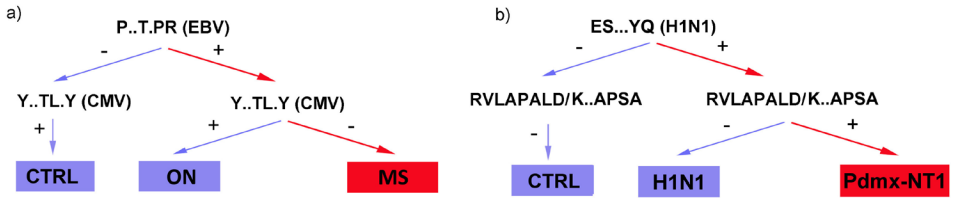

Fig 4. Proposed models of epitope-based diagnostics for risk assessment of (a) MS and (b) NT1. ***a)*** *Herein we showed that antibodies against a predominant C-terminal epitope of VCA p18 EBV (P14348; epitope* $_{161}$*GGQ**P**HD**T**A**PR**GARKK*$_{175}$*) distinguished ON patients from healthy individuals (CTRL). Concurrent seropositive immunoreactivity to AD-2 of gB CMV (F5HB53; epitope* $_{70}$*ETI**Y**NT**TL**K**Y***$_{80}$*) was a characteristic feature of control (CRTL) and ON*^{ON} *groups, but not of the ON*^{MS} *group.* ***b)*** *Herein we showed that antibodies against a H1N1 HA (P03452; epitope* $_{120}$***LES**MGI**YQ**I*$_{131}$*) distinguished H1N1 and Pdmx-H1N1 individuals from healthy individuals (CTRL). Seropositive immunoreactivity to human DP1 (Q13258; epitope* $_{94}$***RVLAPALD***$_{101}$*) and BCL6 (P41182; epitope* $_{280}$***LK**PA**APSA**R*$_{290}$*) distinguished NT1 patients from samples of natural H1N1 infection.*

Despite extensive research using biomarkers and neurophysiological approaches, known heterogenic background of NT1 disease is not always coinciding with serologic marker-based subtype classification (Kornum et al., 2017). We used the MVA method to immunoprofile autoantibody repertoires in patients afflicted by NT1 and in controls. Prominent immune response was observed to a peptide epitope derived from DP1, as well as to peptides with high homology to BCL-6 protein. Further validation confirmed that these can act as true antigenic targets in discriminating NT1 diseased along with a novel epitope of HA of H1N1 to delineate exposure to H1N1. We used peptides containing H1N1/HA-specific linear consensus epitopes that discriminated H1N1 infected from healthy individuals, consensus of the epitope on DP1 antigen and the epitope on BCL6 antigen, and distinguished correctly the NT1 group from the HC (ANOVA $p<0.001$) and 11 (7 Pdmx-NT1, 3 NT1) out of 16 individual NT1 samples while combining data with dot ELISA analysis ($p<0.001$ Chi-squared test, **Fig. 4b**; **Publication II**).

In conclusion, large-scale antibody epitope profiling revealed the broad scope and high heterogeneity of disease-associated antibody response (**Publications I and II**). Describing novel antibody epitopes associated with neurological diseases is significant for a number of reasons: i) mapping new virus infections associated with neurological diseases have therapeutic implication, especially in case of herpesviruses against which multiple antiviral agents exist; ii) epitope-based biomarkers that are detectable in the blood can be used as biomarkers for the prognosis and diagnosis of disease; iii) detection of disease-specific antigens can reveal novel drug targets; iv) the immunogenicity of antigens determined at epitope level will contribute significantly to the development of novel vaccines. Future research will reveal the whole power of MVA to describe antibody immune response at epitope resolution in different clinical samples.

6. Conclusion

Here, we present a methodology for antibody epitope repertoire analysis to rapidly discover the immunodominant epitopes and to develop high performance epitope-specific diagnostic tests.

Using hypothesis-free MVA immunoprofiling approach we described antibody epitope repertoires in patients diagnosed with ON, MS and NT1. The principal findings of this study are summarised below:

- The results show that the MVA method is well-suited for the high-throughput hypothesis-free studies of neuroimmune diseases, establishing antigenic epitope profiles from peripheral blood and CSF samples.

- Distinct epitope profiles of ON, MS and the relevant controls were described, and the described epitope repertoires of serum and CSF were found to be highly similar.

- MS case-discriminating epitopes included determinants of gB of CMV and VCA p18 of EBV antigens.

- In validation analyses, these epitopes were confirmed as potential serologic biomarkers of MS.

- Antibody epitope profile differences between NT1 and healthy controls were established.

- Prostaglandin D2 receptor DP1 was defined as a novel autoantigenic target of NT1.

- Use of a 3-biomarker ELISA assay to assess exposure to H1N1 influenza virus and susceptibility to NT1 was demonstrated.

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Abstract

Immunodominant antibody epitopes associated with the development of multiple sclerosis and type 1 narcolepsy – a next-generation phage-display analysis

The immune system changes and adapts during the normal ageing and in response to different diseases. Recent advances in immunomics have made it possible to study the humoral immune response in great detail and at a high resolution. Mimotope Variation Analysis (MVA) is a high-throughput next generation epitope profiling technology that describes the immune response towards different antigens. It applies parallel advancements in random peptide phage library technology, next generation sequencing, and computational discovery algorithms. Herein, we used hypothesis-free MVA immunoprofiling approach and described antibody-targeted epitope repertoires of patients diagnosed with optic neuritis (ON), multiple sclerosis (MS) and narcolepsy type 1 (NT1). MS with ON onset is the most common neurological disease in young adults, which causes demyelination, axonal loss and progressive disability. NT1, including influenza vaccine Pandemrix-induced NT1, is a chronic neurological disease characterised by irresistible daytime sleepiness, disturbed nocturnal sleep, and cataplexy associated with the inadequate function of the hypothalamus. In our research we described the prognostic value of viral antibody epitopes as novel biomarkers for the risk of MS after the first episode of ON. These epitopes with cross-reactivity to antigens of common viral pathogens (Epstein-Barr virus, Cytomegalovirus) potentially reflect pathogenic mechanisms in the development of MS. Furthermore, we found a strong autoimmune-like response to prostaglandin D2 receptor DP1, suggesting a firm link between DP1/PDG2 and histamine pathways and the molecular basis of NT1. In conclusion, our results showed that the MVA method is suitable for the studies of different neuroimmune diseases, establishing antigenic epitope profiles of peripheral blood and cerebrospinal fluid.

Lühikokkuvõte

Hulgiskleroosi ja tüüp-1 narkolepsiaga seotud immunodomineerivate epitoopide analüüs järgmise põlvkonna faagidisplei abil

Immuunsüsteem muutub ja kohaneb organismi normaalse vananemise ajal ja vastusena erinevatele haigustele. Hiljutised molekulaartehnoloogilised arengud võimaldavad antikehalist immuunvastust uurida väga paljude tunnuste ja proovide osas üheaegselt. *Mimotope Variation Analysis* (MVA) on uudne suureskaalaline epitoopide profileerimise tehnoloogia, mille abil on võimalik kirjeldada väga erinevate antigeenide vastast immuunvastust. Nimetatud meetod seob tehnoloogilisi edusamme faagi pinnal juhuslike eksogeensete peptiidide kuvamise meetodis (ik. *random peptide phage display library technology*), uudses mass-sekveneerimises (ik. *next generation sequencing* (NGS)) ja kaasaegsetes andmeanalüüsi meetodites.

Käesolevas doktoritöös rakendati hüpoteesivaba MVA immunoprofileerimise meetodit ja kirjeldati antikehade epitoopide repertuaare patsientides, kellel oli diagnoositud nägemisnärvi põletik (optiline neuriit), hulgiskleroos (lk. *sclerosis multiplex*) või narkolepsia tüüp 1. Optilise neuriidiga algav hulgiskleroos on kõige levinum neuroloogiline haigus noortel täiskasvanutel, mis põhjustab aksoneid ümbritseva müeliinkihi ja aksonite hävimist ning selle tulemusena patsientide progressiivset invaliidsust. Narkolepsia tüüp 1, sh. Pandemrix gripivaktsiini-indutseeritud narkolepsia tüüp 1, on krooniline neuroloogiline haigus, mida iseloomustavad une-ärkveloleku häired ja katapleksia, mis on seotud hüpotaalamuse alatalitusega. B-lümfotsüütide vahendatud immuunvastus omab olulist rolli nii hulgiskleroosi kui ka narkolepsia tüüp 1 patoloogiate tekkimisel, seega on see heaks sihtmärgiks varajaste diagnostiliste markerite tuvastamisel.

Teadustöös uuriti üle 600 erineva kliinilise proovi (sh. nii vere- kui ka liikvoriproove). Tuvastatud epitoopmarkerid, mis on seotud optilise neuriidi patsientidega, kirjeldavad immuunvastust, mis on arvatavasti tekkinud viiruspatogeenide (herpesviirused Epstein-Barri viirus (EBV) ja tsütomegaloviirus (CMV)) antigeenide EBV VCA p18 (ik. *capsid antigen p18 protein*) ja CMV gB (ik. *glycoprotein B*)) vastu, peegeldades seega patogeenseid mehhanisme hulgiskleroosi arengus. Lisaks leiti narkolepsia tüüp 1 patsientides tugev autoimmuunne reaktsioon prostaglandiini D2 retseptori DP1 vastu, mis viitab seosele DP1/PDG2 ja histamiini signaaliradade ning narkolepsia tüüp 1 patoloogia vahel. Kokkuvõtvalt võib öelda, et MVA meetod sobib erinevate neuroimmuunhaiguste uurimiseks. Täpsed biomarkerid, mis peegeldavad haiguste patogeenseid mehhanisme, on väga väärtuslikud ennustamaks haiguse kulgu ja hõlbustamaks erinevate haiguse ravi.

Appendix 1

PUBLICATION I

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Research paper

Identification of two highly antigenic epitope markers predicting multiple sclerosis in optic neuritis patients

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ABSTRACT

Background: Optic neuritis (ON) can occur as an isolated episode or will develop to multiple sclerosis (MS) a chronic autoimmune disease. What predicts ON progression to MS remains poorly understood.
Methods: We characterised the antibody epitope repertoire in three independent clinical cohorts (discovery (n = 62), validation (n = 20) and external cohort (n = 421)) using mimotope variation analysis (MVA), a next generation phage display technology to identify epitopes that associate with prognosis of ON.
Findings: We observed distinct epitope profiles for ON, MS and the controls, whereas epitope repertoires of sera and CSF were highly similar. Two unique and highly immunogenic epitopes A and B were detected in subjects with ON progressing to MS. These epitopes A and B were strongly associated with herpesviral antigens (VCA p18 of Epstein-Barr virus (EBV); gB of Cytomegalovirus (CMV)). ROC addressed 75% of MS subjects with ON onset correctly (at 75% sensitivity and 74.22% specificity) based on the two-epitope biomarker analysis.
Interpretation: This is the first report on epitope diagnostics for MS employing the unbiased strategy of MVA for identification of novel immunological features of disease.
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1. Introduction

Multiple sclerosis (MS) is the most prevalent chronic inflammatory disease of the central nervous system (CNS). Most patients present with relapsing MS, with episodes of relapse and remission phases, whereas ~10% have a progressive course from the onset.

The causes of MS are not known. Associations with HLA-DRB1×15:01 (HLA class II) and HLA-A*02:01 (class I) along with more than 200 genetic variants have been reported to influence the risk of MS [1]. Female gender, Scandinavian/Celtic descent, low childhood vitamin D status, adolescent obesity, smoking, and infectious background – all include major risk factors of MS (reviewed in [2]). MS is considered an autoimmune disease, but the antigens of CNS targeted by T and B cells are currently largely unknown (reviewed in [3]). About 90% of patients with MS have IgG oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) [4]. OCBs are also common in other types of CNS inflammation [4,5]. Reactivity of OCBs and intrathecal antibodies against bacterial (e.g., *Chlamydia pneumoniae* [6-8]), human herpesvirus (Epstein-Barr virus (EBV), Human Herpesvirus 6 (HHV-6)) and other viral (measles, rubella, and zoster ("MRZ") [9-14]) antigens have been reported in patients with MS, although underlying mechanisms remain unclear.

The optic nerve is one of the major targets in MS. About 20% of MS patients present with optic neuritis (ON) as one of the first symptoms, whereas during the course of the disease ON may occup in 50% of patients. In a follow-up of a large cohort of ON patients the risk of developing MS after ON was 30% at 5 years, close to 40% at 10 years, and 50% after 15 years [15].

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Research in Context

Evidence before the study

Multiple sclerosis (MS) is the most common neurological disease in young adults, affecting more than 2 million individuals worldwide. About 20% of MS patients experience optic neuritis (ON) as the presenting symptom, but not all ON patients develop MS. Thus, it is important to identify prognostic biomarkers of the development of MS after ON. B cells are now recognised to play a central role in MS. The MS community has traditionally focused on well-defined candidate autoantigens, whereas novel techniques can expand the number of target antigens in an unbiased manner.

Added value of this study

Here, we present a general methodology for antibody epitope repertoire analysis to rapidly discover the immunodominant epitopes, and to develop high performance epitope-specific diagnostic tests. Using next generation phage display technology (MVA) we provide a broad, high-resolution view on humoral immunity associated with MS and report on the prognostic value of viral antibody epitopes as novel biomarkers for the risk of MS after the first episode of ON. These epitopes with cross-reactivity to antigens of common viral pathogens (EBV, CMV) potentially reflect pathogenic mechanisms in the development of MS. In addition, we show a high clinical potential of these epitopes as blood biomarkers in addressing MS correctly for 76.6% of cases.

Implication of all the available evidence

Because of the significant clinical heterogeneity of MS, biomarkers that reflect pathogenic mechanisms may thus be valuable for predicting disease progression and facilitating individualised treatments. The identification of circulating biomarkers' immunological significance presents an attractive solution for early diagnosis and prognosis of MS.

Although some immunological factors have previously been associated with MS, virtually nothing is known whether different sites of onset have different predictive markers for MS. Here we employed a hypothesis-free approach of mimotope variation analysis (MVA), a next generation phage display method to analyse antibody epitope profiles of subjects with ON who did or did not progress to MS. Furthermore, we identified novel epitope biomarkers for assessing ON progression to MS from blood.

2. Methods

2.1. Study population

The discovery cohort included CSF and sera/plasma samples of 24 treatment-naïve Finnish patients who initially received the diagnosis of ON and 38 controls (**Table 1**). Fifteen of the ON patients were subsequently diagnosed with relapsing MS (ON^{MS}) during the median follow-up time of 52 months (range 38–69 months), whereas 9 patients did not develop MS (denoted ON^{ON}). Using standard isoelectric focusing and agarose gel electrophoresis OCBs were found in all CSF samples of the discovery cohort. In the ON^{ON} group the average number of CSF-specific OCBs was 12; in the ON^{MS} group the average number of CSF-specific OCBs was 15 (the numbers of CSF-specific OCBs observed are in **Figure S1a**).

The validation cohort included, sera/plasma and CSF samples collected at diagnostic phase (treatment-naïve) of 20 Finnish patients with relapsing MS, out of which 10 presented with ON (MS^{ON}) and 10 with other symptoms (MS^{other}) (**Table 1**)**.** All patients with MS in the discovery and validation cohorts fulfilled McDonald 2005 and 2017 criteria, their clinical features on baseline EDSS, visual functional score and brain MRI findings are summarised in **Table S1** and OCBs in the CSF on **Figure S1a**.

The diagnostic model using ROC analysis was tested in an independent external cohort of Estonians including healthy individuals (n = 229, **Table 2**) and in subjects with different ICD-10 diagnosis codes, but without any notification of demyelinating disease (n = 192, **Table 2**).

2.2. Ethics statement

Patients for this study were recruited at the Department of Neurology and Department of Ophthalmology of Helsinki University Hospital, Finland. Written informed consent was obtained from all study participants. This study was pre-approved by the regional ethics committee at the Helsinki University Hospital (Dno 83/13/03/01/2013), Ethics Review Committee on Human Research of the University of Tartu, Estonia: 212T-24 (issued 13.03.12), 177/T-2 (issued 15.12.08), 211/M-22 (issued 23.01.12) and 281/T-5 (issued 16.04.18) and from Tallinn Medical Research Ethics Committee 1161 (issued 13.09.2007). The healthy control (HC) samples were from donors of the North Estonia Medical Blood Centre (Tallinn, Estonia).

2.3. Mimotope variation analysis (MVA)

For qualitative and quantitative characterisation of antibody epitopes from blood sera/plasma and CSF samples, we used the MVA method as described previously [16]. Analysis included discovery (sera/plasma and CSF samples; n = 62), validation (sera/plasma and CSF; n = 20) and independent external (sera/plasma; n = 421) cohorts. On average, MVA generated 3 million peptide sequences per sample,

Table 1
Description of clinical samples studied by MVA.

Characteristics	Discovery cohort ON^{ON}	ON^{MS}	Controls CTRL	CTRL	Validation cohort MS^{ON}	MS^{other}
Group size (n)	9*	15**	27	11	10	10
Diagnose on sample collection	ON	ON	-	-	MS	MS
ON development into MS	No	Yes	-	-	Yes	NA
Sample type	CSF(9), serum(5)	CSF(15),serum(10)	serum	CSF	CSF (10)/serum (10)	CSF (10)/serum (10)
Gender (female/male)	3/6	13/3	7/20	8/3	7/3	9/1
Age (average)	35	32	64	31	32	32

Serum=serum or plasma.
* 1 sample with non-native Finnish background.
** 2 samples with non-native Finnish background.

Table 2
External cohorts used for ROC analysis.

Characteristics	Samples from subjects with no demyelinating disease	Samples from healthy donors
Group size (n)	192	229
Sample type	sera/plasma	sera/plasma
Gender (female/male (NA))	141/41(10)	98/131
Age (average) (NA)	43 (10)	39

out of which 350,000 peptides, on average, were with unique amino acid sequence (data structure shown on **Figure S1b**). Altogether, the size of the described antigenic repertoire of the discovery cohort encompassed about 20 million unique peptide sequences (data not shown). Although the majority of these peptides were largely individual-specific as observed by the data structure analysis of the most frequent 5000 and 20,000 peptides from each sample (top 5000 and top20000 peptides, respectively), the studied samples shared a substantial fraction of common peptide antigen characteristics across all datasets (**Figure S1c** shows the heatmap image of MVA profiles from the CSF samples of patients in the discovery cohort).

2.4. Data processing and statistical analysis

Data processing was performed as described previously [16]. Statistical analyses (ANOVA, *t*-test, correlation analyses, Receiver Operating Characteristic (ROC), distribution profiles) and corresponding visualisations were done using MedCalc Statistical Software (version 17.0.4, MedCalc Software Bvba, Belgium). For clustering and hypergeometric test SPEXS2 Software was used. In-house data analysis scripts were used to perform peptide divergence, peptide abundance, and coefficient of variation computations, as well as motif alignments and sequence annotations against user-defined reference sequence database (IEDB.org (01.08.2019)). Excel VBA (Visual Basic for Applications) scripts were used for these data visualisations.

2.5. Measurements of total IgG and protein

Total amount of IgGs in sera/plasma and CSF were measured using ELISPOT method. In brief, diluted sera/plasma samples and CSF samples were printed onto nitrocellulose film slides (Amersham Bioscience) by SpotBot® 4 Personal Microarrayer (Arrayit). Purified human IgG (Sigma, i4506) was used for standard curve preparation with rabbit anti-human IgG (HRP) (Abcam, ab6759; dilution 1:1000) as a secondary antibody. Results were scanned by using Ettan™ DigeImager (GE Healthcare Life Sciences) and quantified by ImageQuant TL (GE Healthcare Life Sciences). Total protein concentration in CSF was measured using Pierce BCA Protein Assay kit (Thermo Scientific) according to the kit protocol. Bovine serum albumin (Naxo) was used for standard curve preparation. absorbence was measured at 450 nm with SpectraMax Paradigm (Molecular Devices).

2.6. ELISPOT of peptide displaying phages

In vitro mutagenesis method was used to generate recombinant phages displaying peptides of interest (TLPMDTSPRAHW (vector for cluster B), TLPMDASPRAHW (control vector of cluster B) and DYKDDDDK (FLAG tag)) in the N-terminus of the pIII of M13. In addition, peptides of cluster A - NETIYNTTLKYGGGGDYKDDD(LYS(BIOTIN)); control peptides of cluster A - NETIANAAAKAGGGGDYKDDD (LYS(BIOTIN)) were synthesised by Genescript (US). For ELISPOT, peptides or peptide-displaying phages printed onto nitrocellulose filter pads (Amersham Bioscience) by SpotBot® 4 (Arrayit) were exposed to human precleared sera/plasma (dilution 1:100) or CSF (dilution 1:2) for 1 h at room temperature (RT) and then incubated with rabbit anti-human IgG (HRP) (Abcam, ab6759; dilution 1:1000) as a secondary antibody. Images were scanned using Ettan™ DIGEImager (GE Healthcare Life Sciences).

2.7. Western blot analysis

For western blot (WB) analysis, recombinant phages displaying sequences of interest (ELEKAYKTTLSY (vector of cluster A), TLPMDTSPRAHW (vector of cluster B) and DYKDDDDK (FLAG tag)) at the N-terminus of the pIII of the M13 were generated with in vitro mutagenesis. For WB, 30 μg of protein lysate of 1×10^{13} phages with the following primary antibodies: anti-Flag (Sigma-Aldrich F3165; 1:3600), precleared human sera (dilution 1:750) and precleared human CSF (dilution 1:7,5) samples were incubated with secondary rabbit anti-mouse (Abcam, ab6728; dilution 1:10 000) or rabbit anti-human IgG antibodies (Abcam, ab6759; dilution 1:10 000). The ECL Femto kit (Amersham) was used for detection of target proteins.

2.8. CMV and EBV seropositivity

Human Cytomegalovirus- (CMV) and EBV-specific IgGs were determined by the ISO 17,025 accredited enzyme-linked immunosorbent assay (ELISA). For CMV, for analysis of sera/plasma anti-CMV IgG ELISA kit (EUROIMMUN EI 2570 9601 G) was used. Anti-EBV IgG ELISA kit (EUROIMMUN EI 2731 9601) was used for measuring EBV seropositivity in sera/plasma samples. Analyses were carried out in accordance with the manufacturer's specifications. absorbence was measured at 450 nm with SpectraMax Paradigm (Molecular Devices).

2.9. Role of the funding source

We confirm that all funders played no role in study design, data collection, data analysis, interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

3. Results

3.1. Discovery study of antibody epitope profiles differentiating ON from MS

Effective biomarkers for assessment of ON prognosis, in particular those measurable in blood, are largely lacking. MS is driven by systemic immune activation of autoimmunity against CNS, thereof CSF has been the primary object of biomarker exploration. Regarding this, we performed MVA immunoprofiling of CSF and sera samples on the cohort of 24 subjects i) with isolated optic neuritis ON^{ON} and ii) with initial diagnosis of ON, who were later diagnosed with MS ((ON^{MS}), **Fig. 1a**).

A total of 20 million unique peptides from the discovery cohort were included in downstream analyses. This allowed a fine molecular description of the antigenic repertoires of ON patients (data structure analysis shown in **Figure S1b** and heatmap image of MVA profiles in the CSF samples of the patients shown in **Figure S1c**). Analysis of the most abundant fractions resulted in defined sets of peptides that were common to samples of CSF and sera/plasma from the same individual ($r>0.7$ (Pearson); $p<0.001$ (ANOVA); **Fig. 1b-c**). Subsets of

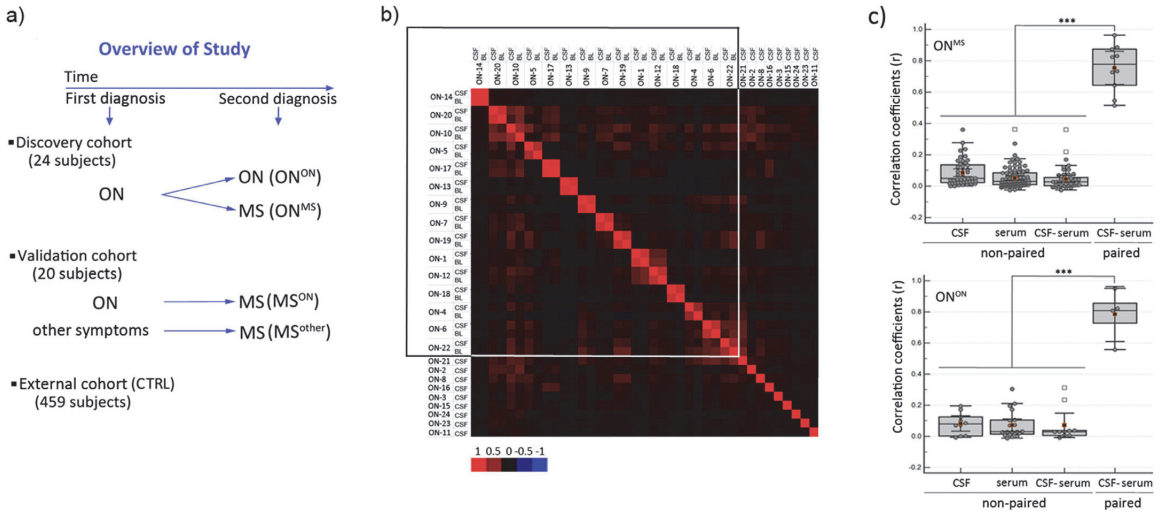


Fig. 1. Highly individual but similar immunoreactive epitope profiles of matched sera and CSF samples as observed by MVA. a) Clinical study design: for discovery cohort, CSF and sera/plasma samples were collected from 24 ON patients, of which 15 were later diagnosed with MS (ON^{MS}), whereas the remaining nine patients were not (ON^{ON}). 27 sera samples and 11 CSF (CTRL) samples from individuals without ON or MS diagnosis were used as controls (see Table 1-2). For validation cohort, independent samples from 10 MS patients with ON onset (MS^{ON}) and 10 with MS but with other symptoms onset (MS^{other}) were collected at diagnostic phase. For ROC analysis of biomarker performance, another independent external cohort including healthy individuals ($n = 229$) and subjects without any known diagnosis of the demyelinating disease ($n = 192$) was added to CTRLs. b) Pearson correlation matrix analysis of Top5000 peptides from sera/plasma and CSF samples of 24 individuals diagnosed with ON revealed high similarity of epitope repertories in paired CSF and sera/plasma samples. The range of correlation coefficient values is shown below the figure by the colour-coded panel; *boxed samples* - paired CSF and sera/plasma samples. c) Box plots of ANOVA analysis of Pearson correlation values (r) in ON^{MS} ($n = 15$) (*top*) and ON^{ON} ($n = 9$) (*bottom*) peptide datasets are shown. Epitope profiles were dissimilar in non-paired CSF and sera/plasma samples as compared to paired samples. *y-axes* – correlation coefficients (r); *x-axes* – non-paired samples of CSF (1) and sera (2), non-paired CSF and sera/plasma (3) and paired CSF and sera/plasma samples (4)); ***- ANOVA $p<0.001$.

peptides were also shared between individuals ($r<0.3$ (Pearson); $p<0.001$ (t-Test); **Fig. 1b-c).** The exchange of antibody forming cells across the blood brain barrier (BBB) in individuals with ON and MS has previously been suggested from sequencing data of B cell receptors [17] and IgG heavy chain variable regions [18]. Our analysis, by demonstrating close similarity of the immunodominant antibody epitope profiles of blood and CSF, also argues for the free exchange of antibodies across the BBB in individuals with ON and MS.

3.2. Two immunodominant epitopes discriminate between ON^{ON} and ON^{MS}

To examine the group-discriminating features, using unsupervised clustering of the most abundant (immunodominant) peptides from each study cohort (altogether 1.4 million peptides) we delineated 1669 group-specific epitopes ($p<0.005$ (hypergeometric test)). The overall data analysis scheme is shown in **Figure S2a. Figure S2b** shows the distribution of these 1669 epitopes across study cohorts. Correlation analysis that followed revealed two major consensus motifs, clusters A and B (coefficient of variation >1.2, **Fig. 2a** and top sequence motifs are listed in **Table S2**). Cluster A contained peptides with Y..TL.Y amino acid patterns, whereas cluster B was formed by P..T.PR pattern-containing peptides. The heatmap analysis of median peptide abundance as shown in **Fig. 2b** further confirmed the differential distribution of peptides forming clusters A and B in different clinical cohorts.

Next, we evaluated the association of clusters A and B with clinical diagnosis using ANOVA. In blood samples, as shown in **Figs. 2c**, cluster A epitopes were most abundant in ON^{ON} and controls ($p<0.05$ (ANOVA) as compared to ON^{MS}), whereas a trend of cluster B epitopes being more abundant in ON^{MS} and ON^{ON} as compared with controls was observed ($p = 0.077$ (ANOVA)). Furthermore, the group-discriminating features of clusters A and B were similarly detected in the CSF of ON^{MS} and ON^{ON} as compared with controls (cluster A CTRL vs. ON^{ON} $p<0.005$ (ANOVA); cluster B CTRL vs. ON^{MS} $p<0.05$ (ANOVA); **Fig. 2c**).

3.3. Immunodominant epitopes A and B mimic highly antigenic epitopes of CMV and EBV

Epitopes of clusters A and B harvested the most abundant antibody immune response, suggesting their possible association with common human pathogens. Reviewed epitopes of human pathogens from IEDB database were aligned to consensus sequences of clusters A and B. Peptides of cluster A unequivocally defined the antigen domain 2 (AD2) of the neutralising epitope of CMV glycoprotein B (gB), whereas peptides of cluster B were close mimics of the C-terminal epitope of capsid antigen (VCA) p18 protein of EBV (**Fig. 3**). As shown in **Fig. 3a** and **3b** and further corroborated by western blot analysis by using recombinant phages displaying prototype peptides, epitopes derived of AD2 ($_{70}$ETI**Y**NT**TL**K**Y**$_{80}$) of CMV gB and of EBV VCA p18 ($_{161}$GGQ**P**HD**T**A**PR**GARKK$_{175}$) could act as true antigens mimicked by clusters, i.e., epitopes of A and B, respectively. Further validation using ELISPOT analyses confirmed the MVA detected seropositivity of epitopes A and B at accuracy of 95%. Comparative analysis of seropositivity by MVA and ELISPOT are shown in **Figure S3a**. The findings of the herpesviral antigenic background of these two epitopes were further strengthened by clinical ELISA testing. The highest anti-CMV seroresponse was observed in the cohorts of ON^{ON}, MS^{other} and CTRL, whereas all study subjects in the discovery cohort were seropositive for anti-VCA EBV (percentages of CMV and EBV seropositive and -negative subjects in study groups are shown in **Figure S3b**). Moreover, all study subjects, who showed antibodies against clusters A or B epitopes in MVA, were seropositive for CMV and EBV, accordingly (**Figure S3c and S3d**).

Interestingly, the alignment analysis of other MVA-defined consensus motifs (**Fig. 2a**) resulted in delineating epitopes associated with Epstein–Barr nuclear antigen 1 (EBNA1) and also with other

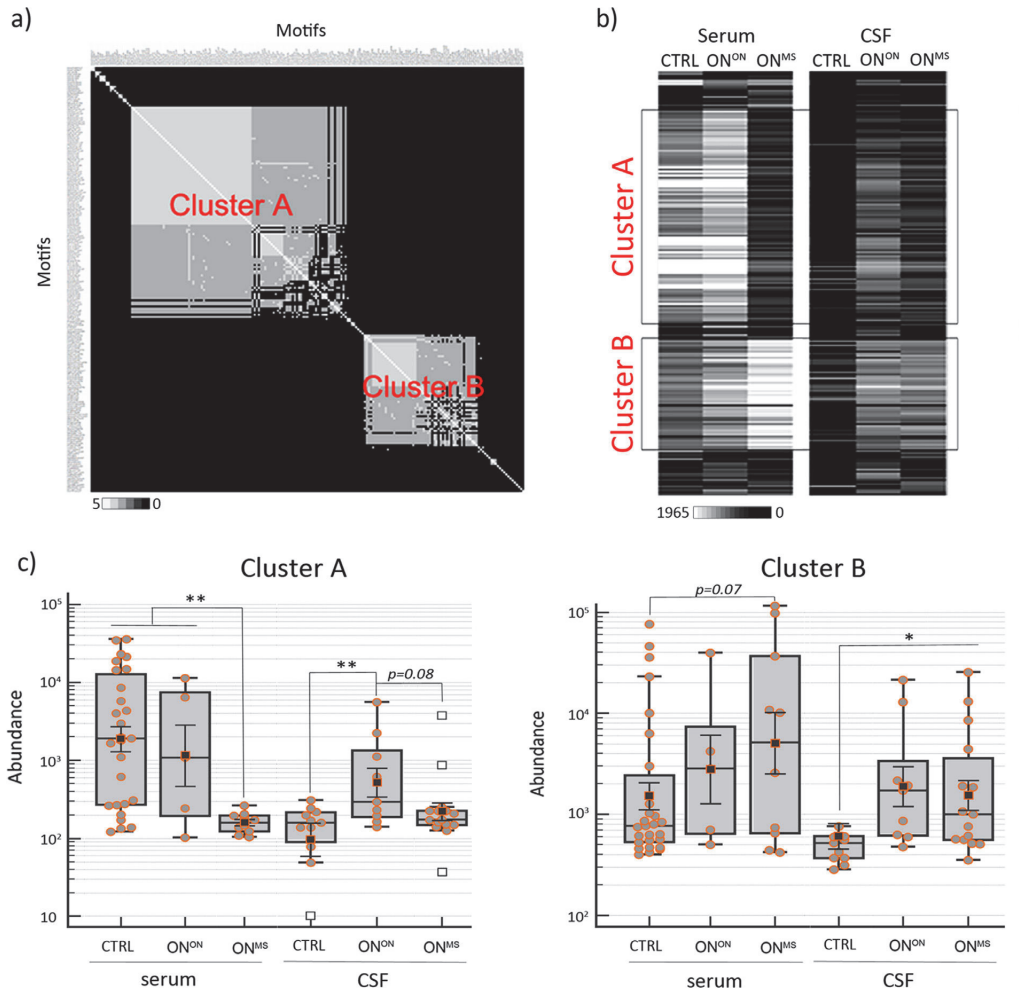


Fig. 2. Epitope clusters A and B with group-specific features discriminate between ON^{MS}, ON^{ON} and CTRL. a) Strong sequence associations in peptides forming epitope clusters A and B became evident by the amino acid positional identity count matrix analysis of 205 motifs underlying these clusters (Figure S2). *White* – high correlation; *black* – low correlation. b) The heatmap depicts distribution of the same 205 motifs as in B according to the median values of motif abundance across different study groups (n = 64). *White* – maximum median value (1965); *black* – minimum median value (0). Study cohort acronyms are provided on the top of the heatmap. *CSF, BL* - samples of discovery cohort; *framed boxes* - median values of clusters A and B motifs across different study cohorts. c) Box plot analysis of peptides containing clusters A and B epitopes as detected by MVA in the CSF and sera samples across different study groups (n = 64). *y- axes* - peptide abundance (in $\log_{10}$); *x-axes* – study groups; *p*- ANOVA on log-transformed data p value, *- $p<0.05$; **- $p<0.01$.

epitopes of gB of CMV (**Table S3**). However, these were detected by MVA at considerably (50 to 135 times) lower abundances as compared to epitopes of A and B. Nonetheless, the epitopes aligning to EBNA1 were clearly more frequent in ON^{MS} as compared to controls (**Table S3**).

3.4. Value of epitopes A and B as clinical biomarkers

We next tested epitopes of clusters A and B in the independent validation cohort of 20 subjects with relapsing MS and in 448 controls (**Fig. 4** and **Tables 1** and **2**). MVA analysis of the blood samples of the validation cohort showed that MS patients either with ON onset (MS^{ON}) or other MS onset (MS^{other}) had roughly similar patterns of response to peptide epitopes of A and B, although these of MS^{ON} differed slightly more from the controls (**Figure S4a**). **Fig. 4a** shows that by combining sera findings from ON^{MS} and MS^{ON} groups, clusters A and B epitopes specifically discriminated between these diagnostic groups and controls ($p<0.05$ (ANOVA), **Fig. 4a**). Furthermore, normalising the ELISPOT findings from CSF of MS patients to total IgG content yielded a high positive correlation between CSF and peripheral blood in the immunoreactivity to epitopes of A and B (r = 0.95 (Pearson), $p<0.0001$ (t-Test); r = 0.71 (Pearson), $p<0.0001$ (t-Test); **Fig. 4b).** The measurements of total amounts of proteins and IgGs in the CSF samples of study cohorts are in **Figure S4b**.

We used ROC analysis to assess the predictive value of epitopes of clusters A and B as blood-based diagnostic biomarkers for MS after ON (**Fig. 4c** and **S4c**). The prediction value of these two epitope biomarkers was high in stratifying subjects with ON^{MS} and MS^{ON} (n = 20) from healthy controls (n = 256) (**Table 1** and **2**) with balanced accuracy of 74.61 at 75% sensitivity and 74.22% specificity (AUC 0.796,

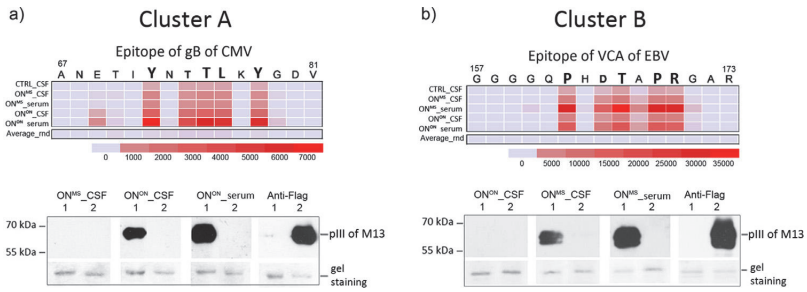
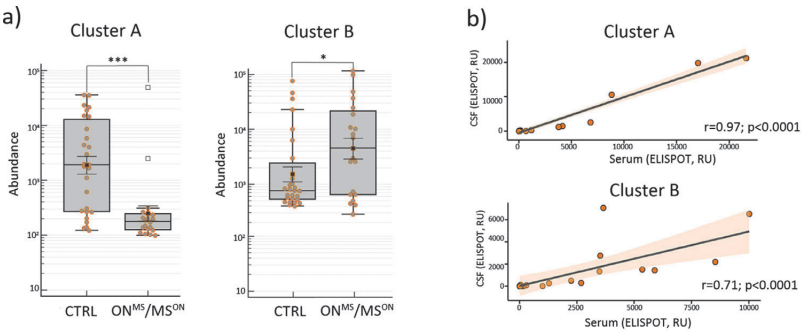


Fig. 3. Epitope clusters A and B mimic highly antigenic epitopes of CMV and EBV. a-b) Heatmap images of epitopes of clusters A and B alignments to IEDB epitopes (01.08.2019). The criteria for homology searches were set to 4 amino acid similarity matches and group median values for each epitope motif were used in alignment calculations. On top of the heatmap panels primary sequences of gB CMV (Uniprot code Q2FAM8) and VCA p18 EBV (Uniprot code P14348) are shown with amino acids defining the core epitopes of clusters A and B. Below the heatmap panels, the scale of relative alignment loads (0–35 000) is shown in colour code. The acronyms of the discovery and validation cohorts are shown in the left. *Average_rnd* – average median values of scrambled motifs derived of clusters A and B motifs. Representative images from validation studies of antigenic epitope predictions using western blot analysis are shown in the lower part of the figure (see full blots on Figure S5). Western blot analysis of recombinant phages containing the epitope gB CMV or VCA EBV was performed using recombinant phages encoding Y..TL.Y-pIII or P..T.PR-pIII (1) and flag-pIII fusion proteins (2) with primary antibodies: i) pre-cleared serum/plasma (dilution 1:750), CSF (dilution 1:7,5); ii) mouse anti-FLAG antibody (Sigma Aldrich, No. 287) and secondary antibodies: i) rabbit anti-human-HRP (Abcam), ii) rabbit anti-mouse-HRP (Abcam). Protein molecular weight markers (kDa) are shown in the left. *Gel staining* – protein loading control with Coomassie blue staining, *pIII* – pIII protein of M13. Source of primary antibodies is indicated on top of the blot.

CI 0.743 to 0.842, $p<0.0001$; Fig. 4**c).** The ROC curve analysis is shown in **Fig S4c,** and the calculated prediction values for each patient are shown in **Table S4**. Moreover, when the independent external cohort of subjects with non-demyelinating disease (n = 192; **Table 2**) was used as a control group, the prediction values for clusters A and B epitope biomarkers for blood-based detection of MS with ON onset were even higher with balanced accuracy of 76.62 at 60% sensitivity and 93.23% specificity (AUC 0.82, CI 0.761 to 0.869, $p<0.0001$; Fig. 4**c** and **S4c, Table S4**).

4. Discussion

In this study, we present an unbiased antibody epitope discovery strategy that resulted in delineation of two epitope biomarkers stratifying subjects with ON and those with MS after ON from healthy controls and patients without any demyelinating disease. Using hypothesis-free MVA immunoprofiling approach we observed highly individual immune response profiles in the paired peripheral blood and CSF samples of the study subjects (**Fig. 1**). Group comparative



c)

Study cohort	AUC (p<0.0001)	Sensitivity	Specificity	Accuracy
ON^{MS}/MS^{ON} (n=20) vs healthy (n=256)	**0.796*** **	75	74.22	**74.61**
ON^{MS}/MS^{ON} (n=20) vs neurologically healthy (n=192)	**0.82*** **	60	93.23	**76.615**
neurologically healthy (n=192) vs healthy (n256)	0.532	21.35	87.89	54.62

Fig. 4. Epitope clusters A and B predict MS with ON onset at high diagnostic accuracy in validation cohorts. a) Validation of epitopes of clusters A and B by MVA confirmed the diagnostic power of these as two blood-based biomarkers to detect MS with ON onset. Box plot analysis of immunoreactivity values of clusters A and B epitopes as obtained by MVA in sera samples of MS with ON onset (samples of ON^{MS} and MS^{ON}, n = 20) and controls. *y-axes* - peptide abundance (in $\log_{10}$); *x-axes* –study cohorts; *p*- ANOVA on log-transformed data p value, *- $p<0.05$, ***- $p<0.001$. b) High immunoreactivity to epitopes of clusters A and B in sera was always co-detected with high immunoreactivity in CSF. Pearson correlation analysis of ELISPOT values of gB CMV (cluster A) and VCA p18 of EBV (cluster B) in the CSF samples of MS^{ON} and MS^{other} patients (n = 20) upon normalisation to total IgG amount. *y-axes* - ELISPOT values in CSF; *x-axes* – ELISPOT values in serum; *r* - correlation coefficient; *p* - correlation significance level (t-Test). **c)** ROC analysis data of the predictive value of clusters A and B epitope biomarkers in different validation groups – MS with ON onset (samples of ON^{MS} and MS^{ON}, n = 20; **Table 1-2**), healthy (CTRL samples from discovery cohort (n = 27) and samples from external cohorts of healthy individuals (n = 256) and subjects with non-demyelinating diseases (n = 192; **Table 2**). *Accuracy* – balanced accuracy of sensitivity and specificity; *area under the curve* (AUC).

clustering analysis of peptide epitope profiles identified case-discriminating antigenic repertoires forming two major clusters A and B (**Fig. 2**). Our further analysis determined these epitopes A and B as mimics of highly antigenic epitopes of CMV and EBV (**Fig. 3**). In validation analyses, we confirmed these two epitopes (A and B) as highly potential serologic biomarkers of MS (**Fig. 4**).

Antibody epitope repertoire shared similarity in the CSF and serum. Our study demonstrated the power of next generation phage display MVA method for high-throughput profiling of antibody response at the epitope precision of the clinical samples from the early phase of MS with ON onset. Despite the highly individual top immune response profiles, we observed that paired sera and CSF samples showed highly similar antigenic features (**Fig. 1**), arguing for shared robust antibody responses both in the periphery and in the CNS. Although findings of similar patterns of immunoreactivity to single antigens in the peripheral blood and CSF have been reported (29–31), our current data extended these findings to the depths of millions of peptide antigens. Furthermore, amongst these peptide antigens we describe repertoires (epitopes forming clusters A and B) that can distinguish different demyelinating inflammatory conditions (ON^{ON}, ON^{MS}, MS^{ON} and MS^{other}) and controls (**Figs. 2** and **4**). The emerging questions still remain of the origin and also of role of these loads of antigenic epitopes in the physiology and pathology of the brain. Although our data provided early answers to these inquiries, future studies would be needed to elucidate the mechanisms leading to the peptide antigen signatures described here.

Two highly antigenic epitopes mimicking herpesviruses behind pathophysiology of MS after ON. We show here that the highly antigenic epitopes of CMV gB and EBV VCA p18 (**Fig. 3**) could be true antigens underlying epitope clusters A and B, respectively. These specific epitopes have not, to our knowledge, been previously reported in the neurological disease literature. Previous studies about the associations between viral, bacterial, and helminthic infections and the prevalence of MS contributed towards creation of the "hygiene hypothesis" [19]. Despite plethora of conflicting reports including also those on herpesviruses [20-22], the mechanisms behind pathogens in the incidence of MS have remained unclear. This is the first study describing associations between the highly antigenic epitopes of gB CMV and VCA p18 EBV with MS **(Figs. 2** and **3)** suggesting direct involvement of these two herpesviruses in the pathophysiology of MS. Thus, why and how the humoral response against these two viral epitope mimics is associated with CNS antigens remains an open question.

Epitope-specific serologic biomarkers of ON prognosis and MS disease. We found that different demyelinating inflammatory conditions (ON^{ON}, ON^{MS}, MS^{ON} and MS^{other}) were associated with different antibody epitopes (**Figs. 2** and **4**). Based on these promising data, one would argue that epitopes including the two A and B clusters could detect MS with different onsets. Biomarker risk score model with 74.61% accuracy established epitopes of A and B as blood-based biomarkers of MS with ON onset. For stratifying MS from other non-myelinating disease, the clinical accuracy of these two biomarkers reached a value as high as 76.62% (**Fig. 4c**). To date, there are no biomarkers that predict ON progression to MS . Most recent findings indicated that serum neurofilament light chain (sNfL) might serve as a biomarker from very early stages of MS (see also ref in [23]). It will be important to investigate the additional diagnostic value of epitopes A and B would carry for patients with first MS symptoms and high initial sNfL levels all combined as serologic biomarkers for the disease course prognosis.

Strengths of this study include the unbiased nature of the discovery phase to define epitopes that predictthe risk of developing MS after ON. Further, we highlight the study cohort of native Finnish (59 participants out of 62), residents of Southern Finland, providing a fair homogeneity of the studied population. In addition, this is the first study in MS clinical research to characterise antibody epitope repertoires of this magnitude from CSF and serum accenting on their potential clinical value. It should be mentioned that application of methods like MVA with high epitope resolution and links to novel antigens and biological pathways clearly facilitate the discovery of immune response specifics. Agreeably, mechanisms of these immune features in underlying pathophysiologies reflecting neurodegenerative processes associated with MS still remain to be elucidated.

Our study was a pilot-scale small sample size study and limited at variable degrees of metadata available for subjects of different cohort. Hence the findings may limit generalisable extrapolation and may include biases of factors such as genetic and environmental factors (smoking, infection history, etc.) that might contribute to distinct MS immunological signatures in the blood. Further longitudinal studies including different pre-symptomatic stages would strengthen our findings and should be able to establish the role of the specific epitopes in MS pathogenesis. Another limitation of this study is that only the two major and highly antigenic epitopes could be thoroughly studied. It also remains unclear how these associations between sera and CSF profiles of epitopes A and B relate to MS pathogenesis and whether these associations are cause or consequence of the disease. Further delineation of detected antibody epitopes is desirable as these might provide further understanding of the pathophysiological context behind MS progression.

Our findings indicate that specific antibody epitope biomarkers of MS exist in the blood of patients at different stages of disease. These epitope biomarkers can be combined into a multivariate model with high discriminatory potential. Further validation of the selected two-epitope biomarker analysis from this initial study are warranted to assess their exact value in MS development. Overall our results stress the importance of dissecting global antibody immunoreactivity patterns at the epitope level towards the personalised care of MS.

Contributors

HS - conceptualisation, data processing, formal analysis, investigation, methodology, software, supervision, validation, data visualisation, writing original draft, reviewing and editing; **AP** – data processing, formal analysis, investigation, methodology, software conceptualisation, formal analysis, visualisation; **MJ** – data processing, investigation, methodology, software conceptualisation, data visualisation, writing, reviewing and editing; **NP** – writing, reviewing and editing; **AR** – writing, reviewing and editing; **MT** – writing, reviewing and editing; **AV** – conceptualisation, writing, reviewing and editing**; JN** – clinical data analysis, writing, reviewing and editing**; MS** – funding acquisition, clinical data analysis, writing, reviewing and editing**; PT** – conceptualisation, patient recruitment, funding acquisition, clinical data analysis, writing, reviewing and editing, and **KP** – conceptualisation, formal analysis, investigation, methodology, visualisation, project administration, supervision, funding acquisition, writing original draft, reviewing and editing. All authors had full access to all underlying data. All authors read and approved the final version of the manuscript.

Data sharing statemant

Data on annotation are freely available from the database of IEDB.org. Any data not published within the article or supplementary materials will be shared in anonymised format by request from any qualified investigator. If desired, please contact the corresponding author of this article.

Declaration of Competing Interests

AP and KP are inventors of the patent application (PCT Application No. US/14079626) filed by Protobios that covers the use of phage display method to manipulate and monitor humoral immunity. PT

reports reimbursement for activities not related to the present article. All other authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103211.

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Identification of two highly antigenic epitope markers predicting multiple sclerosis in optic neuritis patients

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Figures

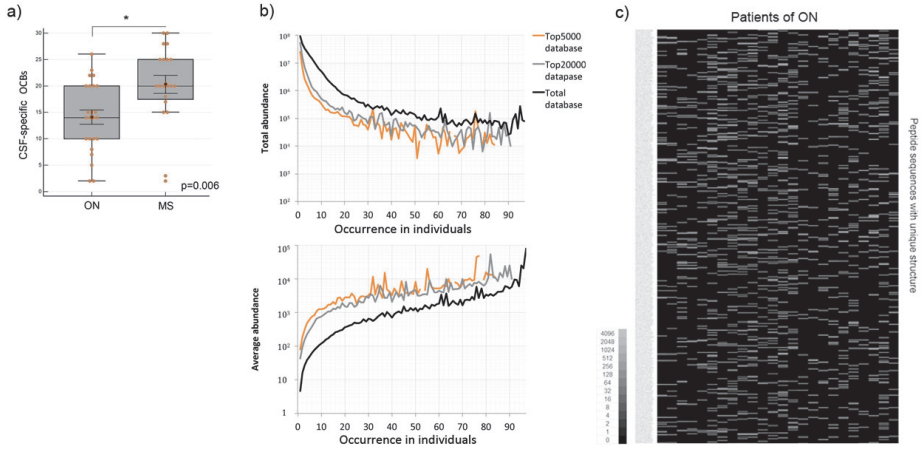

Figure S1. Peptide epitope immunoreactivity profiles in study cohorts. a) The number of CSF-specific oligoclonal bands (OCBs) observed in MS patients (MS^{ON} and MS^{other}, combined) was significantly higher as compared to patients with ON (ON^{ON} and ON^{MS}, combined). *y-axis* – number of CSF-specific IgG fractions (minimum number of IgG fractions was 2 whereas maximum number was 30); *x-axis* – study groups (see Table 1-2); * ANOVA p value <0.05. **b)** Data structure analysis plots of unique peptides across all samples studied by MVA shown as total (*top*) and average abundance (*bottom*) distribution values (range – number of peptides described in 1 to 97 (all) samples including the discovery cohort (CSF, n=24; BL, n=15), validation cohort (BL, n=20) and control cohorts (CSF, n=11; BL, n=27)). Three different data fractions were analysed: Top5000, Top20000 and the entire peptide dataset. *y-axes* – total or average abundance values of peptides; *x-axes* – samples from study cohorts (see Table 1); *abundance* – frequency of peptides. **c)** Peptide profiles were individual-specific with highly varying abundance. Heatmap image of a random fraction of MVA profile of ON patients (n=24) in CSF samples. Each column represents the peptide profile from individual sample. Each line represents a single peptide with a unique primary structure. *scale* - white represents peptides captured at high levels (high abundance) whereas black represents peptides captured at low levels (low abundance).

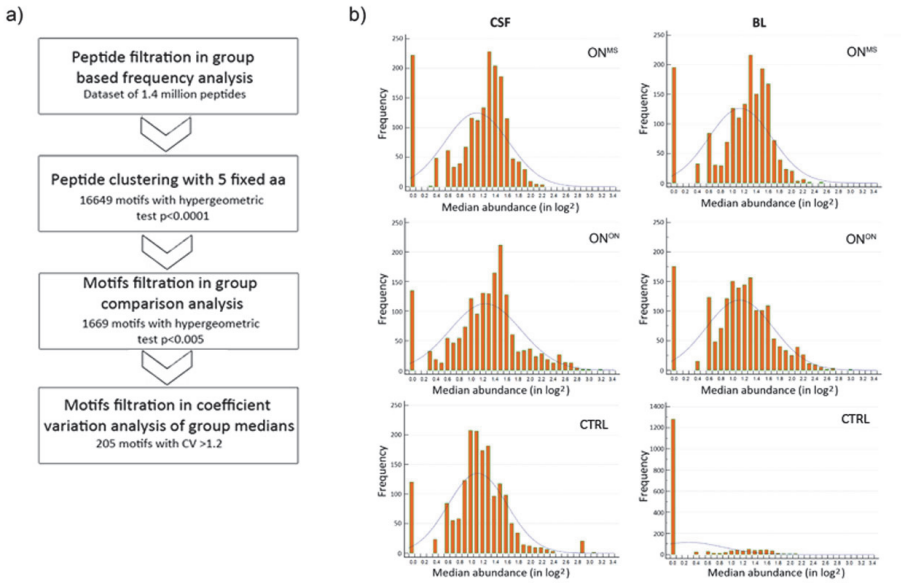

Figure S2. Determination of epitopes with differential immunoreactivity profiles across study groups. a) Overview of the MVA data analysis process. For clustering analysis and hypergeometric test (SPEXS2 software (16)), peptides were selected according to the criteria that a peptide was to be shared by some individuals in a study cohort (at least two in cohorts 1 to 5, at least three in cohort 6, see Table 1) and was to be detected by at least 10 times in a sample. With this selection 4,210,739 peptides were included to analysis. Clustering of peptides with random or group specific references resulted in 16,649 epitope motifs with patterns of 5 fixed amino acids ($p<0.0001$ (hypergeometric test)). Abundance of peptides contributing to motifs was calculated across the entire data set and 1699 motifs ($p<0.05$; (hypergeometric test)) were selected for further group-comparison analysis studies. The median abundance values in each group were calculated for each motif. Altogether 205 motifs from the 1699 were selected that displayed coefficient of variation CV >1.2 of medians over all 6 study groups. **b)** Distribution profiles of median abundance of 1699 motifs in different study cohorts (cohorts 1-6, see Table 1), *y axis* –frequency of median abundance values of 1699 mimotope motifs as counts, *x-axis* - $\log_2$ of median abundance values.

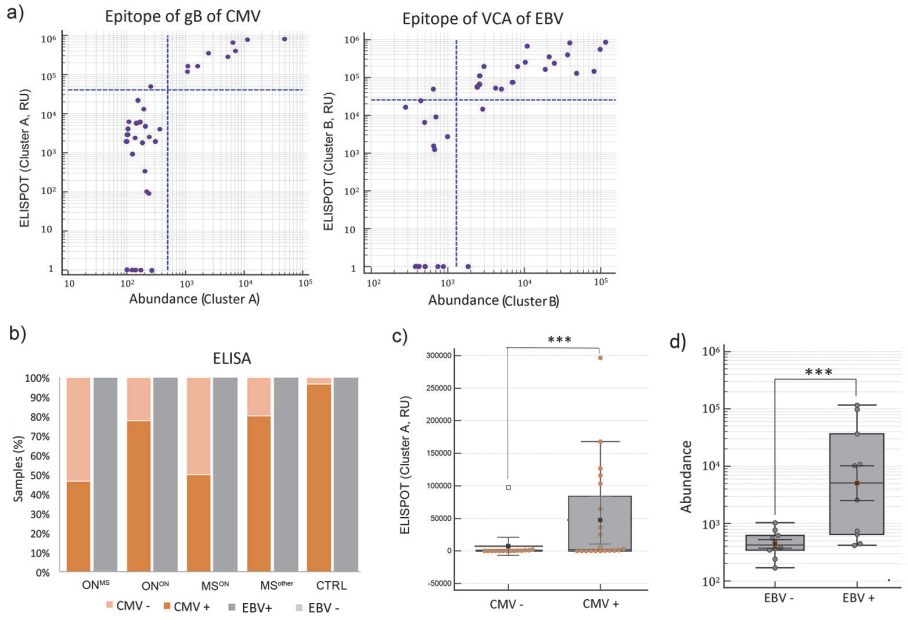

Figure S3. Analysis of VCA p18 EBV and gB CMV seropositivity of samples using ELISPOT and ELISA methods. a) Comparative seropositivity measurements with MVA and ELISPOT. Scatter plot images of abundance of peptides containing cluster A and B epitopes (in $\log_{10}$) and respective ELISPOT values (in $\log_{10}$) of all studied sera/plasma samples. The signal/noise threshold was identified by frequency distribution analysis of epitope-containing peptides (blue lines). ROC analysis determined 95% accuracy for VCA p18 EBV and 98% accuracy for gB CMV. ELISPOT analysis of recombinant phages displaying peptides containing epitopes A or B or biotin-conjugated relevant peptides were printed onto nitrocellulose coated slides (Whatman) by SpotBot® 4 (Arrayit). Human CSF was used as source of primary antibodies and rabbit anti-human-HRP (Abcam) as secondary antibodies. Images were scanned using Ettan DigeImager (GE Healthcare Life Sciences) and the signal intensities were calculated as Signal/Background ratio (RU). **b).** CMV and EBV seronegativity and seropositivity of study patients were determined by anti-CMV IgG by ELISA using EUROIMMUN diagnostic kit (EI 2570 9601 G) and EBV IgG by ELISA using EUROIMMUN diagnostic kit (EI 2731 9601). *y-axis* – percentage of CMV and EBV seropositive (CMV+/EBV+) and –negative (CMV-/EBV-) in study group; *x-axis*- study groups (Table 1-2); *color code* for CMV+, CMV- , EBV+ and EBV- is shown at the bottom of the graph. **c)** Peptides of Cluster A with Y..TL.Y core sequence were used in ELISPOT analysis to assess their power in discriminating between CMV seropositive (CMV+) and –negative (CMV -) samples over study cohort as in **Figure S3b** (ON^{MS}; ON^{ON}; MS^{ON}; MS^{other}, CTRL). *y-axis* - ELISPOT values in RU; *x-axis* - study groups by CMV serology; ***- t-test p value < 0.001. **d)** All patients were EBV seropositive as concluded by IgG titers from anti-EBV VCA IgG ELISA analyses (data not shown), independent samples from Estonian donors (5 EBV negative and 5 EBV positive samples) were used for confirming correct cluster B annotation as only EBV seropositive samples were anti-VCA p18 positive. EBV positive peptides with P..T.PR core sequence were used in analysis to assess their power in discriminating between EBV seropositive and –negative samples. Serological analysis performed by anti-EBV IgG ELISA method using EUROIMMUN diagnostic kit (EI 2731 9601). 10 EBV seronegative samples were added into analysis (EBV-) in comparison with 10 EBV seropositive samples (sera/plasma samples of ON^{MS}). *y-axis* - ELISPOT values; *x-axis* - study groups by EBV serology; ***- t-test p value < 0.001. *Abundance* – frequency of peptides containing motifs (P..T.PR) sequences.

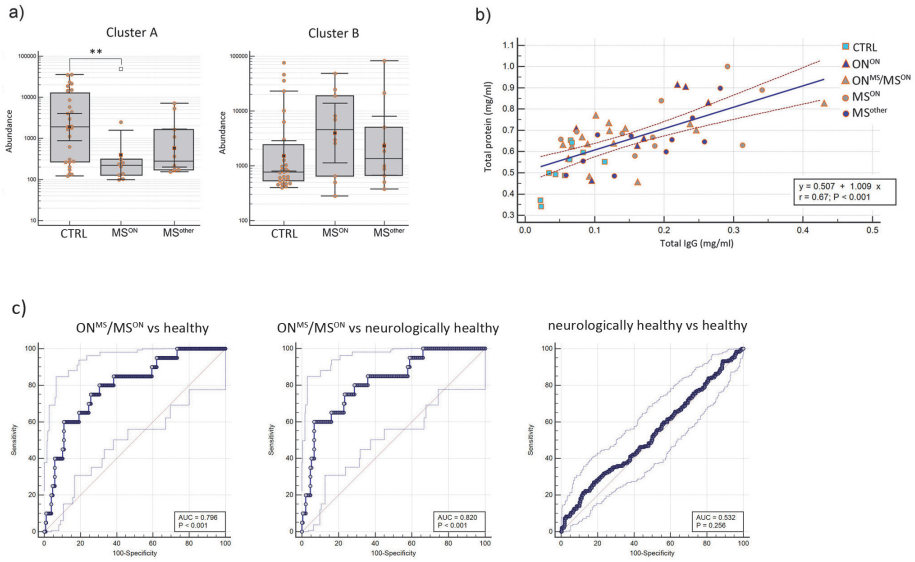

Figure S4. Epitopes A and B as true biomarkers of MS. a) Box plot analysis of immunoreactivity to peptides containing epitopes A and B from MVA analysis of sera/plasma samples of MS and control cohorts. *y- axes* - peptide abundance (in $\log_{10}$); *x-axes* – validation group (**Tables 1 and 2**); *p*- ANOVA on log-transformed data p value, **- p<0.01. **b)** In CSF, linear regression analysis revealed good correlation between the levels of total IgG and total protein. Total amount of IgGs (mg/ml) in CSF samples was measured using ELISPOT method and the total amount of protein (mg/ml) in CSF samples was measured using Pierce BCA Protein Assay kit (Thermo Scientific) according to the original protocol. *y-axis* – total amount of protein (mg/ml); *x-axis* – total amount of IgG (mg/ml); *colour code* – study groups (See Table 1-2); *y=0.507+1.009x* - equation of the regression line; *r* - correlation coefficient; *p* - correlation significance level. In scatter plots - *blue line* marks the regression line; *red curves* mark the 95% confidence intervals for the regression line. **c)** ROC analysis of the predictive value of epitopes A and B as biomarkers in different validation groups – MS with ON onset (samples of ON^{MS} and MS^{ON}, n=20; **Table 1-2**), healthy (CTRL samples from discovery cohort (n=27) and samples from external cohorts of healthy individuals (n=256) and subjects with non-demyelinating diseases (n=192; **Table 2**) ; *x-axis* –100-specificity, *y-axis* – sensitivity; *area under the curve* (AUC).

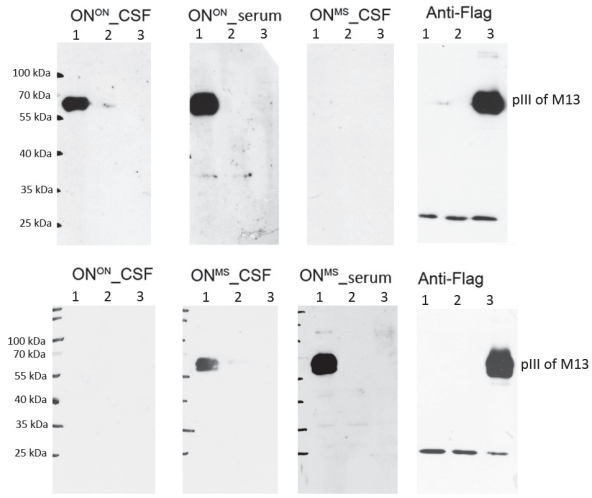

Figure S5. Validation of antigenic epitope predictions using Western blot analysis. Western blot analysis of recombinant phages containing the epitope gB CMV or VCA EBV was performed using recombinant phages encoding Y..TL.Y-pIII (*top*) or P..T.PR-pIII (*bottom*) (1), phages encoding mutant peptides (2) and phages encoding flag-pIII fusion proteins (3) with primary antibodies: i) pre-cleared serum/plasma (dilution 1:750), CSF (dilution 1:7,5); ii) mouse anti-FLAG antibody (Sigma Aldrich, No. 287) and secondary antibodies: i) rabbit anti-human-HRP (Abcam), ii) rabbit anti-mouse-HRP (Abcam). Protein molecular weight markers (kDa) are shown in the left**.** *pIII* – pIII protein of M13. Source of primary antibodies is indicated on top of the blot.

Tables

Table S1. Clinical characteristics of ON and MS samples.

Sample name	First diagnosis	Second diagnosis	ON-MRI	Visual FS	Baseline EDSS	T2 lesion count	Gd+ lesion count
ON^{MS}-1	ON	MS	Yes	5	4.0	4	2
ON^{MS} -2	ON	MS	Yes	2	2.0	>9	≥5
ON^{MS} -3	ON	MS	No	NA	2.0	≥5	3
ON^{MS} -4	ON	MS	Yes	1	1.0	≥4	1
ON^{MS} -5	ON	MS	Yes	1	1.0	>9	2
ON^{MS} -6	ON	MS	Yes	2	2.0	>9	0
ON^{MS} -7	ON	MS	Yes	NA	5.5	>9	≥3
ON^{MS} -8	ON	MS	Yes	2	2.0	>9	0
ON^{MS} -9	ON	MS	Yes	2	2.0	>9	>9
ON^{MS} -10	ON	MS	Yes	3	3.0	>9	0
ON^{MS} -11	ON	MS	Yes	5	4.0	>9	1
ON^{MS} -12	ON	MS	Yes	3	3.0	≥4	NA
ON^{MS} -13	ON	MS	Yes	0	0.0	>9	0
ON^{MS} -14	ON	MS	Yes	2	2.0	>9	0
ON^{MS} -15	ON	MS	Yes	3	3.0	>9	2
ON^{ON} -16	ON	ON	Yes	5	4.0	>9	0
ON^{ON} -17	ON	ON	Yes	3	3.0	4	0
ON^{ON} -18	ON	ON	Yes	1	1.0	≥4	0
ON^{ON} -19	ON	ON	No	3	3.0	2	0
ON^{ON} -20	ON	ON	Yes	2	2.0	>9	0
ON^{ON} -21	ON	ON	No	1	1.0	4	0
ON^{ON} -22	ON	ON	Yes	2	2.0	1	0
ON^{ON} -23	ON	ON	Yes	1	1.0	1	0

ON^{ON} -24	ON	ON	Yes	5	4.0	≥9	0
MS^{ON} -1	ON	MS	-	2	2	≥5	0
MS^{ON} -2	ON	MS	-	1	1	>9	3
MS^{ON} -3	ON	MS	-	2	2	>9	0
MS^{ON} -4	ON	MS	-	5	4	>9	1
MS^{ON} -5	ON	MS	-	1	1	1	1
MS^{ON} -6	ON	MS	-	2	2	≥7	0
MS^{ON} -7	ON	MS	-	4	4	>9	≥5
MS^{ON} -8	ON	MS	-	2	2	≥9	0
MS^{ON} -9	ON	MS	-	0	0	>9	0
MS^{ON} -10	ON	MS	-	1	2	>9	0
MS^{other} -11	other	MS	-	NA	2.5	>9	0
MS^{other} -12	other	MS	-	NA	2.5	>9	0
MS^{other} -13	other	MS	-	NA	0	>9	0
MS^{other} -14	other	MS	-	NA	2.5	>9	2
MS^{other} -15	other	MS	-	NA	2	>9	(1-)2
MS^{other} -16	other	MS	-	NA	2	>9	1
MS^{other} -17	other	MS	-	NA	1.5	≥7	1
MS^{other} -18	other	MS	-	NA	4.5	>9	1
MS^{other} -19	other	MS	-	NA	2.5	>9	NA
MS^{other} -20	other	MS	-	NA	3.5	>9	0

Table S2. Top epitopes of cluster A and B with coefficient of variation (CV) >1.2 for medians over 6 study groups (Groups: CSF and sera/plasma samples of ON^{ON}, ON^{MS} and CTRL; see Figure 2).

Cluster A			Cluster B		
Nr	**Motif sequence**	**CV**	**Nr**	**Motif sequence**	**CV**
1	YQxTLxY	2.127874717	1	PWxxTxPR	1.766773218
2	NxxxYxxTLxY	2.029954016	2	PPxxTxPR	1.419558923
3	DxYxxTLxY	1.927365855	3	PxQxTxPR	1.333628066
4	VxxYxxTLxY	1.915920144	4	PPQxTxP	1.241571767
5	YxATLxY	1.851462177	5	VxxPxxTxPR	1.588938576
6	AxxYxxTLxY	1.850831965	6	PxxTxPRF	1.577245443
7	YxxYxxTLxY	1.850718698	7	PFxTxPR	1.534746225
8	DxxxYxxTLxY	1.836829951	8	SxxPxxTxPR	1.470307307
9	GxxxYxxTLxY	1.806849351	9	PxxTTPR	1.46126723
10	YxxTLVY	1.792122896	10	RxPxxTxPR	1.44596043
11	YxxTLxYT	1.773704407	11	PxNTxPR	1.445089863
12	MYxxTLxY	1.768358136	12	PxxTxPRG	1.414176552
13	YxxTLxYxQ	1.763920037	13	PxxTEPR	1.335615052
14	NYxxTLxY	1.721322619	14	SxxxxPxxTxPR	1.330690391
15	YAxTLxY	1.675259778	15	MxPxxTxPR	1.305992759
16	YDxTLxY	1.668808935	16	QxPxxTxPR	1.281911301
17	YxxTLAY	1.663593187	17	PxxTHPR	1.280974126
18	TxxYxxTLxY	1.662305034	18	PxxTxPRxxxT	1.278193578
19	RxYxxTLxY	1.652330879	19	MPxxTxPR	1.246142689
20	LxYxxTLxY	1.649680295	20	PLxTxPR	1.234214183

Table S3. Results of MVA epitope alignments with known epitopes of the IEDB database. The criterion for homology searches was set to 5 amino acid similarity matches**.** The signal/noise threshold was identified by frequency distribution analysis of peptides containing motif sequences and following ROC analysis was used to define seropositivity.

Motif sequence	Organism	Antigen	IEDB ID	Average abundance	Seropositive in ON^{MS} (BL)(%)	Seropositive in ON^{ON} (BL) (%)	Seropositive in CTRL (BL) (%)
P.DT.PR	EBV	VCA p18	5303	**2480**	60	63	25
CLGCK	EBV	EBNA1	55299	55	30	0	7
R.PFFH	EBV	EBNA1	431702	23	30	0	11
Y.TTL.Y	CMV	gB	57359	**1763**	0	60	67
M.HSHH	CMV	gB	768673	13	1	40	30

BL- sera/plasma samples
Abundance - frequency of peptides containing motifs sequences.

Table S4. The two epitope biomarker prediction values used for ROC analysis across the study cohort. Cohorts: Patients with ON, who were later diagnosed with MS (samples of ON^{MS} (n=10) and MS^{ON} (n=10); **Table 1-2**); Subjects with non-demyelinating diseases (samples from external cohort (n=194); **Table 3**); healthy (CTRL samples from the discovery cohort (n=27)) and healthy individuals (from external cohort (n=229); **Tables 1** and **3**).

#	Sample	Prediction value	#	Sample	Prediction value	#	Sample	Prediction value
	Patient with ON, who were later diagnosed with MS		156	Subject_136	0,240180296	312	Subject_100	0,082173241
1	ON^{MS}_1	671,6011561	157	Subject_137	159,7022901	313	Subject_101	0,075436515
2	ON^{MS}_2	371,2651515	158	Subject_138	140,6908213	314	Subject_102	0,072953319
3	ON^{MS}_3	187,2233503	159	Subject_139	0,075791978	315	Subject_103	0,051204648
4	ON^{MS}_4	73,96376812	160	Subject_140	1,355670103	316	Subject_104	0,025926136
5	ON^{MS}_5	58,39247312	161	Subject_141	0,16818527	317	Subject_105	0,022561044
6	ON^{MS}_6	20,81451613	162	Subject_142	1,614243323	318	Subject_106	0,020765229
7	ON^{MS}_7	6,180952381	163	Subject_143	0,016818964	319	Subject_107	0,019964349
8	ON^{MS}_8	3,8	164	Subject_144	0,019699538	320	Subject_108	922,7154472
9	ON^{MS}_9	3,609756098	165	Subject_145	3,064356436	321	Subject_109	364,627551
10	ON^{MS}_10	3	166	Subject_146	0,076169874	322	Subject_110	99,88888889
11	MS^{ON}_1	190,5656566	167	Subject_147	0,068275054	323	Subject_111	23,46707161
12	MS^{ON}_2	103,394958	168	Subject_148	41,3490566	324	Subject_112	23,00773667
13	MS^{ON}_3	78,94174757	169	Subject_149	1,525735294	325	Subject_113	18,28
14	MS^{ON}_4	22,47909968	170	Subject_150	2,189349112	326	Subject_114	12,22003035
15	MS^{ON}_5	21,3	171	Subject_151	0,042834359	327	Subject_115	11,23076923
16	MS^{ON}_6	20,03100775	172	Subject_152	0,004685924	328	Subject_116	10,7619532
17	MS^{ON}_7	2,549019608	173	Subject_153	0,056379037	329	Subject_117	10,49548427
18	MS^{ON}_8	1,350961538	174	Subject_154	0,584929757	330	Subject_118	9,596201486
19	MS^{ON}_9	0,990497405	175	Subject_155	1,460526316	331	Subject_119	9,360208062
20	MS^{ON}_10	0,199919549	176	Subject_156	0,120561283	332	Subject_120	9,234513274
	Subjects with non-demyelinating diseases		177	Subject_157	2,941176471	333	Subject_121	7,267175573
21	Subject_1	46,89825119	178	Subject_158	1,947598253	334	Subject_122	6,723662807
22	Subject_2	33,86138614	179	Subject_159	2,175324675	335	Subject_123	6,555555556
23	Subject_3	18,40740741	180	Subject_160	1,879120879	336	Subject_124	6,406593407
24	Subject_4	12,27918782	181	Subject_161	0,291807718	337	Subject_125	6,280487805
25	Subject_5	10,55023923	182	Subject_162	0,354630294	338	Subject_126	6,208530806

26	Subject_6	7,289256198	183	Subject_163	2,198019802	339	Subject_127	6,038961039
27	Subject_7	6,622377622	184	Subject_164	0,116449086	340	Subject_128	5,401785714
28	Subject_8	6,120992761	185	Subject_165	1,752808989	341	Subject_129	5,278350515
29	Subject_9	5,72972973	186	Subject_166	1,172351885	342	Subject_130	5,184466019
30	Subject_10	4,754901961	187	Subject_167	2,642045455	343	Subject_131	4,730994152
31	Subject_11	4,748571429	188	Subject_168	0,022619904	344	Subject_132	4,690140845
32	Subject_12	4,514124294	189	Subject_169	0,016176761	345	Subject_133	4,681383285
33	Subject_13	3,501616628	190	Subject_170	2,825581395	346	Subject_134	4,348993289
34	Subject_14	2,757142857	191	Subject_171	3,483516484	347	Subject_135	4,275
35	Subject_15	2,675496689	192	Subject_172	0,036929498	348	Subject_136	3,952182217
36	Subject_16	2,260416667	193	Subject_173	6,052083333	349	Subject_137	3,764705882
37	Subject_17	2,084158416	194	Subject_174	5,093959732	350	Subject_138	3,421965318
38	Subject_18	2,06547619	195	Subject_175	3,222772277	351	Subject_139	3,387587822
39	Subject_19	1,896103896	196	Subject_176	11,1	352	Subject_140	3,223021583
40	Subject_20	1,580645161	197	Subject_177	0,082259663	353	Subject_141	3,164772727
41	Subject_21	0,948562784	198	Subject_178	0,056894365	354	Subject_142	2,988571429
42	Subject_22	0,808340728	199	Subject_179	1,814393766	355	Subject_143	2,974358974
43	Subject_23	0,511627907	200	Subject_180	0,009715211	356	Subject_144	2,965317919
44	Subject_24	0,218377976	201	Subject_181	2,422110553	357	Subject_145	2,927083333
45	Subject_25	0,165373894	202	Subject_182	1,82173913	358	Subject_146	2,890829694
46	Subject_26	0,157579817	203	Subject_183	2,339622642	359	Subject_147	2,825471698
47	Subject_27	0,145096731	204	Subject_184	2,993464052	360	Subject_148	2,683823529
48	Subject_28	0,134583194	205	Subject_185	0,01919297	361	Subject_149	2,612244898
49	Subject_29	0,04639086	206	Subject_186	3,162790698	362	Subject_150	2,619926199
50	Subject_30	0,027483853	207	Subject_187	1,826771654	363	Subject_151	2,584
51	Subject_31	0,022517998	208	Subject_188	0,043158949	364	Subject_152	2,526315789
52	Subject_32	0,03290569	209	Subject_189	7,890243902	365	Subject_153	2,352739726
53	Subject_33	10,79213483	210	Subject_190	2,09947644	366	Subject_154	2,296116505
54	Subject_34	0,022412444	211	Subject_191	0,391412056	367	Subject_155	2,284046693
55	Subject_35	0,049234902	212	Subject_192	18,88311688	368	Subject_156	2,194174757
56	Subject_36	2,544444444		Healthy		369	Subject_157	2,175925926
57	Subject_37	2,19205298	213	Subject_1	293,942623	370	Subject_158	2,115789474
58	Subject_38	18,29012346	214	Subject_2	150,286645	371	Subject_159	2,114649682
59	Subject_39	0,046844124	215	Subject_3	58,28323699	372	Subject_160	2,055384615
60	Subject_40	1,67357513	216	Subject_4	39,72372529	373	Subject_161	1,981651376
61	Subject_41	0,00829652	217	Subject_5	37,18608414	374	Subject_162	1,935643564
62	Subject_42	0,048703017	218	Subject_6	24,01515152	375	Subject_163	1,675276753
63	Subject_43	0,033081933	219	Subject_7	6,021582734	376	Subject_164	1,625714286
64	Subject_44	0,015812728	220	Subject_8	4,544776119	377	Subject_165	1,493087558
65	Subject_45	0,088204297	221	Subject_9	3,111913357	378	Subject_166	1,473514106
66	Subject_46	340,3561644	222	Subject_10	2,266009852	379	Subject_167	1,370253165
67	Subject_47	0,197854589	223	Subject_11	1,563432836	380	Subject_168	1,288209607
68	Subject_48	2,342857143	224	Subject_12	0,425359712	381	Subject_169	1,186695279
69	Subject_49	11,37931034	225	Subject_13	0,332931969	382	Subject_170	1,012084592
70	Subject_50	0,024736536	226	Subject_14	0,325378151	383	Subject_171	0,537934492
71	Subject_51	139,1710526	227	Subject_15	0,294052251	384	Subject_172	0,49776709
72	Subject_52	2,184210526	228	Subject_16	0,289781393	385	Subject_173	0,490886051
73	Subject_53	1,925925926	229	Subject_17	0,143541762	386	Subject_174	0,425908667

74	Subject_54	2,5125	230	Subject_18	0,14010138	387	Subject_175	0,405853659
75	Subject_55	3,418604651	231	Subject_19	0,11191246	388	Subject_176	0,386371011
76	Subject_56	4,083333333	232	Subject_20	0,108706852	389	Subject_177	0,358457436
77	Subject_57	0,024971215	233	Subject_21	0,08708256	390	Subject_178	0,295339697
78	Subject_58	147,86	234	Subject_22	0,069013896	391	Subject_179	0,288712422
79	Subject_59	0,010972831	235	Subject_23	0,067192629	392	Subject_180	0,222246456
80	Subject_60	3,409395973	236	Subject_24	0,032371618	393	Subject_181	0,215320122
81	Subject_61	0,186350888	237	Subject_25	0,022897063	394	Subject_182	0,211580087
82	Subject_62	0,066681242	238	Subject_26	0,021476435	395	Subject_183	0,186348123
83	Subject_63	3,09375	239	Subject_27	0,01722203	396	Subject_184	0,18601626
84	Subject_64	10,15178571	240	Subject_28	1143,275	397	Subject_185	0,17965587
85	Subject_65	1,983695652	241	Subject_29	326,9944751	398	Subject_186	0,175624207
86	Subject_66	1,772972973	242	Subject_30	248,3708609	399	Subject_187	0,173232596
87	Subject_67	0,068096782	243	Subject_31	210,543956	400	Subject_188	0,170698925
88	Subject_68	0,033201765	244	Subject_32	146,4818182	401	Subject_189	0,166340509
89	Subject_69	14,67241379	245	Subject_33	41,09558824	402	Subject_190	0,154444069
90	Subject_70	320,4890511	246	Subject_34	36,19186047	403	Subject_191	0,153225806
91	Subject_71	2,963099631	247	Subject_35	32,62837838	404	Subject_192	0,148565904
92	Subject_72	0,015719255	248	Subject_36	29,02508961	405	Subject_193	0,132329635
93	Subject_73	0,178536103	249	Subject_37	22,45318352	406	Subject_194	0,125063036
94	Subject_74	0,004299707	250	Subject_38	21,91509434	407	Subject_195	0,079293994
95	Subject_75	4,616071429	251	Subject_39	19,70862471	408	Subject_196	0,076142132
96	Subject_76	3,310344828	252	Subject_40	12,00729927	409	Subject_197	0,067695427
97	Subject_77	0,021945256	253	Subject_41	8,825095057	410	Subject_198	0,061087908
98	Subject_78	1,512658228	254	Subject_42	7,535564854	411	Subject_199	0,053151896
99	Subject_79	0,134864547	255	Subject_43	7,413461538	412	Subject_200	0,046506574
100	Subject_80	0,09313268	256	Subject_44	6,745945946	413	Subject_201	0,035196816
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102	Subject_82	0,215909253	258	Subject_46	4,392523364	415	Subject_203	0,033380884
103	Subject_83	0,142999667	259	Subject_47	3,757142857	416	Subject_204	0,032497876
104	Subject_84	0,015650108	260	Subject_48	3,66	417	Subject_205	0,024131842
105	Subject_85	7,398648649	261	Subject_49	3,545023697	418	Subject_206	0,02238292
106	Subject_86	0,690697674	262	Subject_50	3,396103896	419	Subject_207	0,020806877
107	Subject_87	0,049131724	263	Subject_51	3,242424242	420	Subject_208	0,009594298
108	Subject_88	2,255319149	264	Subject_52	2,692857143	421	Subject_209	0,007677378
109	Subject_89	2,608247423	265	Subject_53	2,641255605	422	Subject_210	0,005676737
110	Subject_90	0,0598351	266	Subject_54	2,647058824	423	Subject_211	0,003222492
111	Subject_91	1,604519774	267	Subject_55	2,623188406	424	Subject_212	401,4352941
112	Subject_92	61,40939597	268	Subject_56	2,56768559	425	Subject_213	316,5112782
113	Subject_93	128,1818182	269	Subject_57	2,537313433	426	Subject_214	297,4538462
114	Subject_94	1,930232558	270	Subject_58	2,432835821	427	Subject_215	94,79545455
115	Subject_95	15,83030303	271	Subject_59	2,405555556	428	Subject_216	83,68229167
116	Subject_96	0,02645095	272	Subject_60	2,291005291	429	Subject_217	53,29864253
117	Subject_97	3,347826087	273	Subject_61	2,277310924	430	Subject_218	6,742138365
118	Subject_98	0,076462939	274	Subject_62	2,177083333	431	Subject_219	3,421686747
119	Subject_99	0,063696087	275	Subject_63	2,110599078	432	Subject_220	3,42411948
120	Subject_100	0,009314445	276	Subject_64	2,090517241	433	Subject_221	3,208070618
121	Subject_101	2,20729632	277	Subject_65	1,931914894	434	Subject_222	3,066666667

122	Subject_102	0,086661643	278	Subject_66	1,840707965	435	Subject_223	2,965517241
123	Subject_103	4,716666667	279	Subject_67	1,805309735	436	Subject_224	2,92893401
124	Subject_104	8,225165563	280	Subject_68	1,814749862	437	Subject_225	2,848648649
125	Subject_105	2,054054054	281	Subject_69	1,791471531	438	Subject_226	2,712643678
126	Subject_106	0,066849101	282	Subject_70	1,780701754	439	Subject_227	2,642857143
127	Subject_107	12,4138796	283	Subject_71	1,694779116	440	Subject_228	2,524475524
128	Subject_108	1,594027441	284	Subject_72	1,688596491	441	Subject_229	2,473684211
129	Subject_109	0,153892577	285	Subject_73	1,686520376	442	Subject_230	2,463768116
130	Subject_110	2,807909605	286	Subject_74	1,676724138	443	Subject_231	2,368055556
131	Subject_111	1,085034014	287	Subject_75	1,650537634	444	Subject_232	2,238636364
132	Subject_112	0,010667195	288	Subject_76	1,648241206	445	Subject_233	2,216666667
133	Subject_113	0,004154072	289	Subject_77	1,647302905	446	Subject_234	2,165803109
134	Subject_114	2,990990991	290	Subject_78	1,630841121	447	Subject_235	2,048611111
135	Subject_115	0,097014925	291	Subject_79	1,625984252	448	Subject_236	1,962790698
136	Subject_116	5,301075269	292	Subject_80	1,611940299	449	Subject_237	1,708487085
137	Subject_117	2,513888889	293	Subject_81	1,582750583	450	Subject_238	0,630801688
138	Subject_118	6,156626506	294	Subject_82	1,543845895	451	Subject_239	0,274518389
139	Subject_119	209,8651685	295	Subject_83	1,279661017	452	Subject_240	0,272376046
140	Subject_120	0,492131617	296	Subject_84	1,171364311	453	Subject_241	0,209817894
141	Subject_121	2,306306306	297	Subject_85	1,128205128	454	Subject_242	0,167274622
142	Subject_122	2,90990991	298	Subject_86	1,033033033	455	Subject_243	0,12543554
143	Subject_123	12,98373984	299	Subject_87	0,534801136	456	Subject_244	0,08880623
144	Subject_124	0,005657367	300	Subject_88	0,441510988	457	Subject_245	0,073221423
145	Subject_125	587,8601399	301	Subject_89	0,340506329	458	Subject_246	0,070551271
146	Subject_126	0,881072027	302	Subject_90	0,322759315	459	Subject_247	0,069212106
147	Subject_127	5,662162162	303	Subject_91	0,308166863	460	Subject_248	0,067910735
148	Subject_128	2,532319392	304	Subject_92	0,240924092	461	Subject_249	0,065555417
149	Subject_129	2,943548387	305	Subject_93	0,222655079	462	Subject_250	0,033428272
150	Subject_130	3,714285714	306	Subject_94	0,206271777	463	Subject_251	0,032690247
151	Subject_131	1,962616822	307	Subject_95	0,159738214	464	Subject_252	0,030364187
152	Subject_132	1,723004695	308	Subject_96	0,137865311	465	Subject_253	0,024454949
153	Subject_133	0,104849727	309	Subject_97	0,109368951	466	Subject_254	0,021407584
154	Subject_134	0,377610372	310	Subject_98	0,105960265	467	Subject_255	0,018799647
155	Subject_135	2,575129534	311	Subject_99	0,089904273	468	Subject_256	0,007985314

Appendix 2

PUBLICATION II

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Research Paper

Prostaglandin D2 Receptor DP1 Antibodies Predict Vaccine-induced and Spontaneous Narcolepsy Type 1: Large-scale Study of Antibody Profiling

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ABSTRACT

Background: Neuropathological findings support an autoimmune etiology as an underlying factor for loss of orexin-producing neurons in spontaneous narcolepsy type 1 (narcolepsy with cataplexy; sNT1) as well as in Pandemrix influenza vaccine-induced narcolepsy type 1 (Pdmx-NT1). The precise molecular target or antigens for the immune response have, however, remained elusive.

Methods: Here we have performed a comprehensive antigenic repertoire analysis of sera using the next-generation phage display method - mimotope variation analysis (MVA). Samples from 64 children and adolescents were analyzed: 10 with Pdmx-NT1, 6 with sNT1, 16 Pandemrix-vaccinated, 16 H1N1 infected, and 16 unvaccinated healthy individuals. The diagnosis of NT1 was defined by the American Academy of Sleep Medicine international criteria of sleep disorders v3.

Findings: Our data showed that although the immunoprofiles toward vaccination were generally similar in study groups, there were also striking differences in immunoprofiles between sNT1 and Pdmx-NT1 groups as compared with controls. Prominent immune response was observed to a peptide epitope derived from prostaglandin D2 receptor (DP1), as well as peptides homologous to B cell lymphoma 6 protein. Further validation confirmed that these can act as true antigenic targets in discriminating NT1 diseased along with a novel epitope of hemagglutinin of H1N1 to delineate exposure to H1N1.

Interpretation: We propose that DP1 is a novel molecular target of autoimmune response and presents a potential diagnostic biomarker for NT1. DP1 is involved in the regulation of non-rapid eye movement (NREM) sleep and thus alterations in its functions could contribute to the disturbed sleep regulation in NT1 that warrants further studies. Together our results also show that MVA is a helpful method for finding novel peptide antigens to classify human autoimmune diseases, possibly facilitating the design of better therapies.

1. Introduction

Narcolepsy type 1 (NT1) is a chronic neurological disease characterized by irresistible daytime sleepiness, disturbed nocturnal sleep, and cataplexy associated with the inadequate function of the hypothalamus (Peyron et al., 2000; Thannickal et al., 2000; Partinen et al., 2014). The major neuropathological features of NT1 are loss of orexinergic neurons and an increased gliosis in the posterior hypothalamic nuclei (Partinen et al., 2014). Increased levels of pro-inflammatory cytokines have been associated with (spontaneously occurring) idiopathic (sNT1) and Pandemrix vaccine-induced narcolepsy (Pdmx-NT1) close to disease onset (Lecendreux et al., 2015). Pandemrix (Pdmx) is an influenza vaccine used during the H1N1 2009 swine influenza A(H1N1) pandemic and was distributed to over 30 million people in EU/EEA countries

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during the A(H1N1) outbreak. As of January 2015, >1300 cases of vaccine-associated NT1 had been reported to the European Medicines Agency. Epidemiologic and clinical studies conducted in different countries including Finland, Sweden, Ireland, England, Norway, and France have confirmed the association of NT1 in children and adolescents with the AS03-adjuvanted Pdmx (Partinen et al., 2014; Sarkanen et al., 2017). Subsequently, wild-type influenza A(H1N1) infections in China were associated with narcolepsy (Han et al., 2013, 2011). Along with the pandemic A(H1N1) infection, seasonality and post-infectious priming by upper respiratory tract viruses and streptococci have been suggested as triggers of autoimmune response that leads to NT1 in genetically susceptible individuals (Aran et al., 2009; Longstreth Jr et al., 2009).

Genome-wide association studies have revealed a strong association of narcolepsy with the T-cell receptor alpha locus (Hallmayer et al., 2009) and especially with Major Histocompatibility Complex (MHC) class II *DQB1*06:02* alleles (Bonvalet et al., 2017; Tafti et al., 2014). *DQB1*06:02* is present in approximately 30% of Finnish and Swedish populations (Bomfim et al., 2017). In Finland, all patients with Pdmx-NT1 have been positive for *DQB1*06:02* (Partinen et al., 2014). The latter immune haplotype is also strongly associated with the Pdmx-NT1 in Sweden (Bomfim et al., 2017). In another series of 522 patients with narcolepsy and cataplexy from different countries, only 9 patients (1.7%) with low levels of orexin (OX) in cerebrospinal fluid (CSF) were *DQB1*06:02* negative (Han et al., 2014). It was also suggested that cross-reactive epitopes to Pdmx vaccine antigens may exist in NT1 diseased as a significant proportion of HLA-*DQB1*0602*–positive Finns diagnosed with NT1 and with a history of H1N1 vaccination were immunoreactive to OX receptors (Ahmed et al., 2015). However, it still is unclear whether OX-positive neurons and/or their neighboring cells express OX receptors that could be targets for the immune response in NT1 (Valko et al., 2013; Vassalli et al., 2015). The antibody levels to viral nucleoprotein (NP), a Pdmx vaccine antigen, were increased in NT1-diseased carrying the HLA *DQB1*06:02* allele (Vaarala et al., 2014), whereas the role of this and other circulating (including intrathecal) autoantibodies in NT1 pathogenesis is not fully understood (see list of previously identified antigens in Table S1). Although NT1-related autoantibodies are found in some patients, the clinical response to intravascular immunoglobulin (IVIG) has been hard to predict (Knudsen et al., 2012). Likewise, use of the drug rituximab might have only short-lasting beneficial effects in NT1 (Sarkanen et al., 2016).

Recent advances in proteomics (immunomics) have made it possible to study the adaptive immune response in various diseases in great detail and at a high resolution (lately reviewed in: (Ayoglu et al., 2016, Wu et al., 2016). We and others have suggested a strategy of high-throughput sequencing-assisted epitope mapping directly on serum for biomarker discovery and disease detection based on the idea that self- and environmental (exposome) antigens are reflected in the immune response profiles (immunoprofiles) (Anastasina et al., 2017; Christiansen et al., 2015; Ionov, 2010; Xu et al., 2015). Hence, the profiling of antibody response repertoire with high-density random peptide/polypeptide display methods could be a novel mean to characterize and classify human diseases in an unbiased manner according to the molecular/cellular targets relevant for the disease.

In the present study, we have used the mimotope-variation analysis (MVA) method to immunoprofile autoantibody repertoires in patients afflicted by NT1 and in controls. We had access to the clinical cohorts composed of 16 NT1 (sNT1 ($n = 6$) and Pdmx-NT1 ($n = 10$)) cases, where all NT1-diseased subjects carried the HLA *DQB1*06:02* allele, and apart from 2 sNT1 patients, all had been vaccinated with Pdmx. For reference, we used three well-defined control groups: 16 Pandemrix-vaccinated healthy controls (Pdmx-HC), 16 H1N1-infected Finnish subjects (H1N1-HC), and 16 healthy Estonian donors (HC – healthy controls) (Table 1). Our data revealed complex patterns of immune response in all patient groups including novel epitope sequences present in sera of Pdmx-NT1 and H1N1-HC. One such peptide epitope was identified as belonging to the prostaglandin D2 receptor (DP1) that together with its ligand prostaglandin D2 (PGD2) is involved in sleep regulation in humans and experimental animal models (see ref. in Urade and Hayaishi (2011)).

2. Materials and Methods

2.1. Vaccines

Pandemrix vaccine is derived from X-179A, a reassortant of hemagglutinin (HA), neuraminidase (NA) and polymerase acidic protein (PA) of A/California/07/2009 and X-157 H3N2 in a PR8 backbone (Jacob et al., 2015; Nicolson et al., 2012; Robertson et al., 2011). The vaccine composition can be found summarized by European Medicines Agency and GlaxoSmithKline plc (European Medicines Agency, 2009).

2.2. Study Population

The present study comprises a total of 64 individuals (Table 1). Altogether, 16 serum samples of H1N1-infected military servicemen (H1N1-HC), 16 serum samples of age/sex-matched Pandemrix-vaccinated healthy controls (Pdmx-HC) were kindly provided by National Institute of Health and Welfare, Finland. 16 serum samples were collected from patients with H1N1-induced (Pdmx-NT1) and sporadic narcolepsy (sNT1). Four out of 6 sNT1 patients were vaccinated with Pdmx after they had been diagnosed with NT1. Narcolepsy patients were diagnosed at the

Table 1
Description of samples studied.

Characteristics	Narcolepsy (NT1) patients		Healthy controls (HC)		
	Pdmx-NT1	sNT1	Pdmx-HC	H1N1-HC	Other HC
Group size (*n*)	10	6	16	16	16
Gender (female/male)	5/5	5/1	12/2[a]	0/16	10/6
Pandemrix vaccination	11/2009–1/2010	11/2009-1/2010[b]	11/2009-1/2010	–	–
Sample collection	2011	2011	2011	2011	2009
Median age at onset (y)	13	18	–	–	–
Median age at sampling (y)	14	22	NA	21	34.5
Unambiguous cataplexy	10/10 (100%)	6/6 (100%)	–	–	–
MSLT mean SL (range)	2.0 (0.4–4.3)	2.6 (0–7.5)	NA	NA	NA
SOREMPS mean (range)	3.7 (2–5)	2.7 (2–4)	NA	NA	NA
HLA *DQB1*0602* (%)	10/10 (100%)	6/6 (100%)	NA	NA	NA
CSF-orexin < 150 pg/mL (lower 1/3 limit in Finland)	7/7 (100%)	5/5 (100%)	NA	NA	NA

HC- healthy control, *H1N1-HC* – H1N1 infected, *Pdmx-HC*– Pandemrix-vaccinated, *NT1*- narcolepsy type 1 (including 10 Pdmx-induced NT1 samples (Pdmx-NT1) and 6 sporadic NT1 (sNT1) samples), *NA* – not available, *SL* – sleep latency, *MSLT* – Multiple sleep latency test, *SOREMPS* – Sleep onset REM periods as defined by the American Academy of Sleep Medicine.

[a] Gender of two Pdmx-HC is unknown.

[b] Four out of 6 sNT1 patients were vaccinated after they had been diagnosed with NT1.

Finnish Narcolepsy Research Center (Helsinki Sleep Clinic, Vitalmed Research Center) by experienced neurologists with a special competence in sleep medicine. All narcolepsy patients had NT1 as defined by the American Academy of Sleep Medicine international criteria of sleep disorders version 3. All excessively sleepy patients with NT1 had unambiguous cataplexy and an abnormal MSLT (sleep latency <8 min and at least 2 sleep onset REM periods; Multiple Sleep Latency Test) recording after a polysomnography. Twelve of the 16 patients had their CSF-orexin levels measured using the standardized Phoenix RIA method with Stanford reference. All except one had CSF-orexin levels <110 pg/mL (one woman with clearly abnormal MSLT and unambiguous cataplexy had CSF-orexin level of 127 pg/mL).

Control serum specimen for the study included 16 serum samples from Blood Centre, North Estonian Regional Hospital, Estonia, collected in 2009 prior to the swine influenza pandemic (other HC). Sera were stored at −135 °C until use.

2.3. Ethical Permissions

The patients have participated in the NARPANord narcolepsy study (Academy of Finland, grant nr. 260603), and they have given a written informed consent. The serum samples of H1N1-infected military servicemen and serum samples of the Pdmx-vaccinated healthy controls were provided by the National Institute of Health and Welfare, Finland. The ethical permissions were approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa, Finland.

2.4. Mimotope-Variation Analysis

For qualitative and quantitative characterization of humoral immune response from sera samples, we used an in-house developed mimotope-variation analysis (MVA) method. Fig. 1A provides an overview of the process. In brief, a random 12-mer peptide phage library (Ph.D.-12, NEB, UK) was used according to the manufacturer's protocol. 2 μL of serum sample was incubated with 2.5 μL library (~5×10^{11}) and immunoglubulin G (IgG) fraction was recovered using protein G-coated magnetic beads (Thermo Fisher Scientific). The unbound phage particles were removed by extensive washes with TBS-T (TBS + 0.1% [v/v] Tween-20). Selectively captured phage DNA was analyzed by using next generation (Illumina) sequencing with barcoding primers (Islam et al., 2014). For that, DNA was extracted by using standard NaI/EtOH precipitation method and enriched by PCR amplification using primers enriched with adapters for the sequencing reaction that flank the variable region at the end of pIII in M13KE vector (Fw: 5′-AATGATACGGCGACCACCGAGATCTACACTGATCTAGTGGTACCTTTCTATTCTCA*C*T*C*T-3′ Rv: 5′-CAAGCAGAAGACGGCATACGAGAT**NNNN(NN)**CCCTCATAGTTAGCGTAACG-3′). PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN), and the concentration of DNA with Illumina adapters was estimated by Qubit Fluorimeter (Invitrogen) according to the manufacturer's protocol. Sequencing was performed using Illumina HiSeq and 50-bp single end reads. Samples were analyzed at least in duplicates to ensure reproducibility. To evaluate the data reproducibility, we compared peptide abundance in two replicates using Pearson's correlation coefficient test (r value higher than 0.90, $p < .0001$).

2.5. Sequence Data Analysis

Every read that was considered valid by the Illumina HiSeq control software was prepared for further analysis by trimming. In brief, sequence reads of 50 bp were accompanied with a 4-bp tag to assign each read uniquely to one out of 48 multiplexed samples. After demultiplexing there were millions of sequence reads for each sample. Each read consisted of a 36-bp random insert region and a constant region. We discarded the reads with mismatches in flanking 4 bases of the constant region. Sequences from wild-type phages with no random insert were excluded. Next, we translated all the random insert regions in reads into peptide sequences of length 12aa. All non-translatable sequences were discarded. To reduce the effect of amplification and sequencing errors, only those peptide sequences were kept that had at least two copies sequenced per sample. In order to compensate for the different numbers of reads per sample normalization of read counts was performed. All samples were trimmed to 3 million reads (RPM units). The resulting data was represented as a cross-table where each row corresponded to a different 12mer peptide, each column corresponded to a different sample, and each cell showed the read count of the peptide in the respective sample measured in RPM-units. According to the manufacturer (NEB), naïve library contained up to 10^9 different sequences. For reasoned cost purposes, the estimated outcome of sequence data represented 0.1% of the initial library input containing up to 2.8×10^6 different peptide sequences per sample. Complete analysis of sequence diversities obtained by MVA remains out of the scope of the current study.

2.6. Clustering Workflow

The main assumption was that every obtained peptide sequence mimics the target of an antibody. The sequence reads of one sample often included many copies of the same peptide sequence. The read counts of a peptide could range from 1 to thousands. To reveal recognition patterns (epitope motifs) which were enriched in the cases compared to controls, we used SPEXS2 software (https://github.com/egonelbre/spexs2; (Vilo, 2002, Brazma et al., 1998)). For clustering the peptides with motifs and generating mimotope regular expression and sequence logos, the "motifTree" tool was used (Kruup, 2013). The Multiple EM for Motif Elicitation (MEME-MAST) algorithm (Bailey and Elkan, 1994; Bailey and Gribskov, 1998) was used to align peptides to proteins. For B cell epitope mapping IEDB 3.0 database was used (Vita et al., 2015).

2.7. Statistical Analysis

All statistical analyses (ANOVA, *t*-Test, correlation analyses, Chi-square test) were done using MedCalc software (MedCalc Statistical Software version 17.0.4 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2017)). For visualization of peptide abundance across samples, peptide frequency values were converted to heatmap images (Tagged Image File) with Excel Visual Basic for Applications (VBA) scripts. For visualization of selected peptide set alignment profile on proteins of interest Excel VBA script was used. The protein sequence was scanned with every peptide and at every position where the peptide aligned with it in at least four perfectly matching positions, one was added with its frequency. For random reference profile, amino acid sequence of each peptide was randomized and scanned using the same rules over the target sequence.

2.8. Influenza Virus Serology

Levels of influenza-specific IgG antibodies were determined by the enzyme-linked immunosorbent Vir-ELISA anti-H1N1/H3N2 IgG assay (Influenza virus type A IgG ELISA test system, Euroimmun), carried out in accordance with the manufacturer's specifications. Absorbance was measured at 450 nm with SpectraMax Paradigm.

2.9. Peptide ELISPOT

For peptide ELISPOT the following peptides were designed:
peptide #1 - RVLAPALDSWGTGGGDYKDDD{LYS(BIOTIN)} (Genescript)
peptide #2 - LPKFSAPSASGPGGGDYKDDD{LYS(BIOTIN)} (Genescript)
peptide #3 - ESTRYQLWLPHQGGGDYKDDD{LYS(BIOTIN)} (Genescript)
control peptide - AVLAAALASWGTGGGDYKDDD{LYS(BIOTIN)} (Genescript)

In brief, 110 pg biotin-conjugated peptides were printed on nitrocelluose coated slides (10485323, Whatman) by SpotBot® 4 (Arrayit). For primary antibody human precleared serum (1:100) was used, for secondary antibody rabbit anti-human IgG (H&L) (HRP) (Abcam) was used. All incubations were done for 1 h at room temperature. Results were scanned using Ettan DigeImager (GE Healthcare Life Sciences) and images calculated using ImageQuant software version 8.1 (GE Healthcare Life Sciences).

2.10. Cancer Cells, Human Mesenchymal Stem Cells (hMSC) and Post-Mortem Tissues

Immortalized glioblastoma multiforme cells (human glioma cells - hGC) (kind gift of Prof. Aavo-Valdur Mikelsaar, Estonia), human neuroblastoma cell line Kelly (ATCC) and human mesenchymal stem cells (hMSC) (isolated from human subcutaneous adipose tissue as described (Jaager and Neuman, 2011)) were grown in Dulbecco's modified Eagle's medium (DMEM (PAA)) containing 10% fetal bovine serum (PAA), 1 mg mL^{-1} penicillin (PAA) and 0.1 mg mL^{-1} streptomycin (PAA). All cells were cultured at 37 °C in 5% CO_2. The identity of hMSC was confirmed by using cell morphology and flow cytometry methods for analysis of cell surface markers: CD73+/CD90+/CD105+/CD45−/CD34− (Kauts et al., 2013). For treatments, hMSCs were grown with media containing IL-1β (1 ng/mL), IFNγ (2 ng/mL) for 8 h, or PGD2 (10 μM) for 1 h.

Human post-mortem tissues were procured from the North-Estonian Regional Hospital, Tallinn, Estonia. All experiments with human tissues were done with the approval of the local ethical committee (license no. 2234, date of issue 09.12.2010).

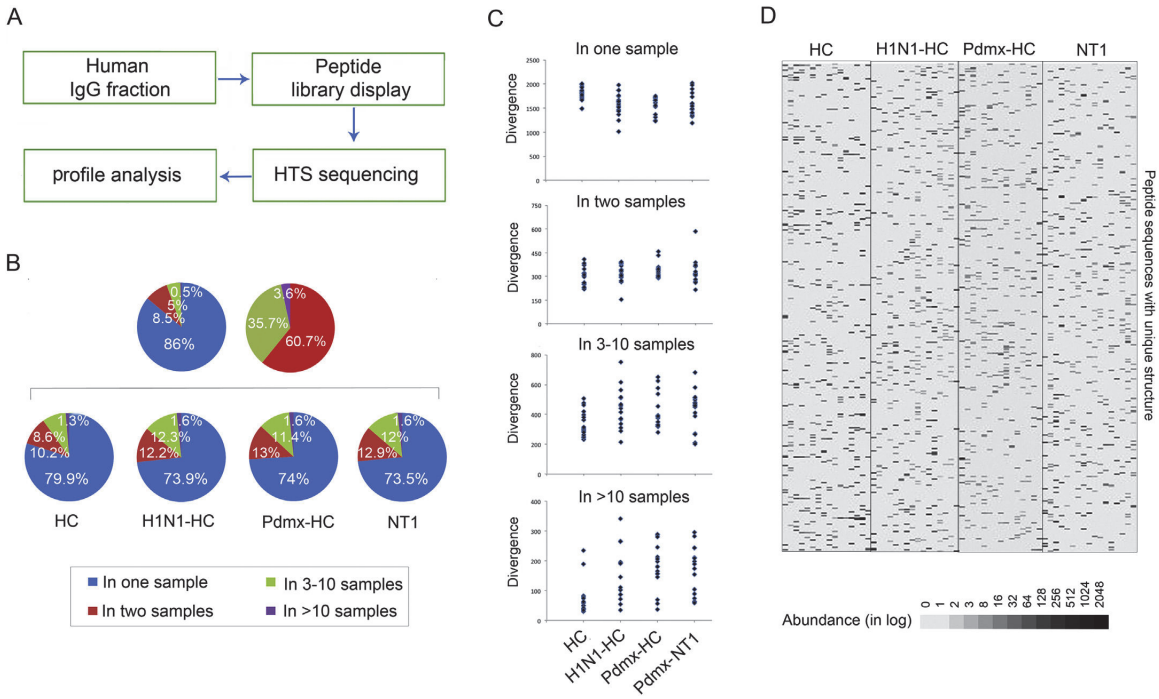


Fig. 1. Humoral immune response studied using the mimotope-variation analysis (MVA) method. A. Schematic drawing of the workflow in MVA. MVA is a high-throughput random peptide phage display analysis. A random peptide display library (PhD12) was used which contained 10^9 different 12-mer peptide sequences introduced to the N-terminus of the phage major coat protein pIII (NEB). For MVA, sample-specific IgG proteins (antibodies, *Human IgG fraction*) present in human sera of interest are allowed to interact with the phage-displayed peptides and the IgG-phage complexes were captured to protein G magnetic beads, while the unbound phages were washed away (*Peptide library display*). Captured phages were lysed and DNA amplified with primer sequences containing a tag with a unique barcode sequence and the final amplicons were pooled for NGS analysis (*HTS sequencing*). The primer set homologous to the M13KE vector sequences that flank the random peptide coding sequence was used to amplify a 50-bp fragment. Data analysis to classify peptides that were specific to Pmdx-infected, -vaccinated and NT1-diseased individuals was carried out by comparing the profiles of peptides (mimotopes) from diseased to those from non-diseased (*Peptide profile analysis*). On average, MVA generated 1.8 million peptide sequences with unique structure (divergence) totaling 2.8 million peptide sequences in abundance (total abundance; number of reads) per sample. Altogether, a peptide data set with >16 million sequences (Totpep) with unique structure was generated. B. Analysis of peptides revealed highly divergent patterns (immunoprofiles) across study cohorts. The fraction of top 2500 peptides with unique structure and highest values of abundance - reflecting the peaking immune reactivity of each sample - was analyzed for variance. Top2500 peptide dataset contained altogether 160,000 sequences out of which 121,142 were unique. Pie charts display the sequence distribution of unique peptides across all samples analyzed. The left pie (*blue*) displays the proportion of shared vs. unique peptides: ~86% were unique to one individual whereas ~14% of the peptide sequences were shared between samples, out of these ~8.5% were common to 2 samples, 5% to 3–10 samples and 0.5% were detected in >10 samples. The right pie (*red*) displays the distribution of shared 16,844 peptide sequences out of which ~60.7% were common to 2 samples, 35.7% to 3–10 samples and 3.6% were seen in >10 samples. The four pie charts (below) exemplify the peptide profile structures in different clinical cohorts. The size of each pie piece is proportional to the number of unique peptides common to one or more samples of a clinical cohort. *Blue* - represents unique peptides, *red* - the most shared. C. Individual variation in peptide divergence is characteristic to all immunoprofiles. Top 2500 peptides were analyzed to assess the range of individual peptide variation across study cohorts. Blue dots mark peptide divergence in a single sample. As indicated, between one to two thousand peptides were individual-specific, whilst the most common peptides (shared by >10 individuals) ranged in divergence from tens to 350 across samples. Range of unique peptide variations was similar across all study samples. D. Heat map image of a random fragment of MVA profile encompassing 400 peptides across study samples. Peptide profiles were individual-specific with a highly varying abundance. Each column represents the peptide profile of a single individual, and each line represents a peptide with a unique primary structure. Abundance is presented as counts in logarithmic scale (*in log*); black colour depicts peptides captured at higher abundance, and white those at lower abundance. Shown are peptide profiles that were common to 3–10 individuals. Abbreviations: *Abundance* – peptide frequency; *Divergence* – all unique peptides; *HC*- healthy control; *H1N1-HC* – H1N1 infected; *Pdmx-HC*- Pandemrix-vaccinated; *NT1*- narcolepsy type 1 (including 10 Pandemrix-induced NT1 samples).

2.11. Immunofluorescence and Western Blot Analysis

For immunofluorescence analysis, cells grown on glass inserts were fixed using 4% PFA (Scharlau) for 15 min and blocking of the unspecific reactivity was done with 5% BSA. The antibodies used included: anti-DP1 (Abnova; 1:500), precleared human sera (1:400), and the secondary Alexa Fluor 488 and 647 (Invitrogen, 1:2000) antibodies. For epitope blocking peptide #1 was used in final concentration 6.6 μg/mL. Hoechst 33342 (Invitrogen) was used to detect cell nuclei. Imaging was done using Nikon Eclipse 80i microscope.

Sequences (RVLAPALDSWGT and DYKDDDDK (flag)) were inserted at the N-terminus of the pIII of the M13KE phage by in vitro mutagenesis PCR using primers s1 5′GCTGGATAGTTGGGGAACCGGTGGAGGTTCG GCCGAAAC3′, as1 5′GCCGGAGCTAGTACACGAGAGTGGGAGTAAAACG GTACC3′,

s2 5′GCTGGATAGTTGGGGAACC3′, as2 5`GCCGGAGCTAGTACACG3′; s3 5′GATGATGATAAAGGTGGAGGTTCGGCCGAAAC3′, as3 5′ATCTTTATAAT CAGAGTGGGAGTAAAACGGTACC3′; s4 5′GATGATGATAAAGGTGG3′, as4 5′ATCTTTATAATCAGAGTGG3′. PCR reactions were carried out with phusion Hot Start II High-Fidelity DNA Polymerase (ThermoScientific). Constructs were verified by sequencing. For Western blot analysis, 30 μg of protein lysate or 1×10^{13} phage particles were resolved on 10% SDS–polyacrylamide gels and transferred onto PVDF membranes (Amersham) for 1.5 h using BioRad wet blotter in standard Towbin buffer. The membrane was blocked with 5% nonfat milk (AppliChem), incubated overnight with the following primary antibodies: anti-DP1 (St. John Laboratory, 1:1000), anti-GAPDH (Sigma, 1:10,000), precleared human sera (1:500). The epitope blocking peptide #1 was used in final concentration 6.6 μg/mL. The membrane was incubated for 1 h at room temperature with the secondary anti-mouse, anti-rabbit, or anti-human IgG antibodies (Abcam; dilution 1:10,000). The ECLfemto kit (Amersham) was used for detection of immunoblotted target proteins.

2.12. RNA Extraction, RT-PCR and qRT-PCR

Total RNA from human brain parts was extracted using RNAWiz (Ambion) as recommended by the manufacturer. Total RNA from cells was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed into cDNA using SuperScript III first strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The resulting cDNAs were used as templates for subsequent RT-PCR reactions. RT-PCR was carried out using FIREPol® DNA polymerase (Solis Biodyne), 40 amplification cycles and an annealing temperature of 58 °C. Amplification of the housekeeping gene *GAPDH* was performed for 25 cycles using FIREPol® DNA polymerase (Solis Biodyne) and used as an internal control. Used primer sequences: *PTGDR* sense 5′ATGAAGTCGCCGTTCTAC C3′, *PTGDR* antisense 5′CATGAAGAAGGCGAAGGCTTG3′, *GAPDH* sense 5′ GAAGGTGAAGGTCGGAGT3′, *GAPDH* antisense 5′GCATGGACTGTGGTCA TGAG3′. *IL-1β* sense 5′GGGCCTCAAGGAAAAGAATC3′: *IL-1β* antisense 5′ TTCTGCTTGAGAGGTGCTGA3′, *IFNγ* sense 5′CTGTTACTGCCAGGACCCA T3′, *IFNγ* antisense 5′TTTCTGTCACTCTCCTCTTTCCA3′.

3. Results

3.1. Autoimmune Response Profiles Across Cohorts are Highly Heterogeneous

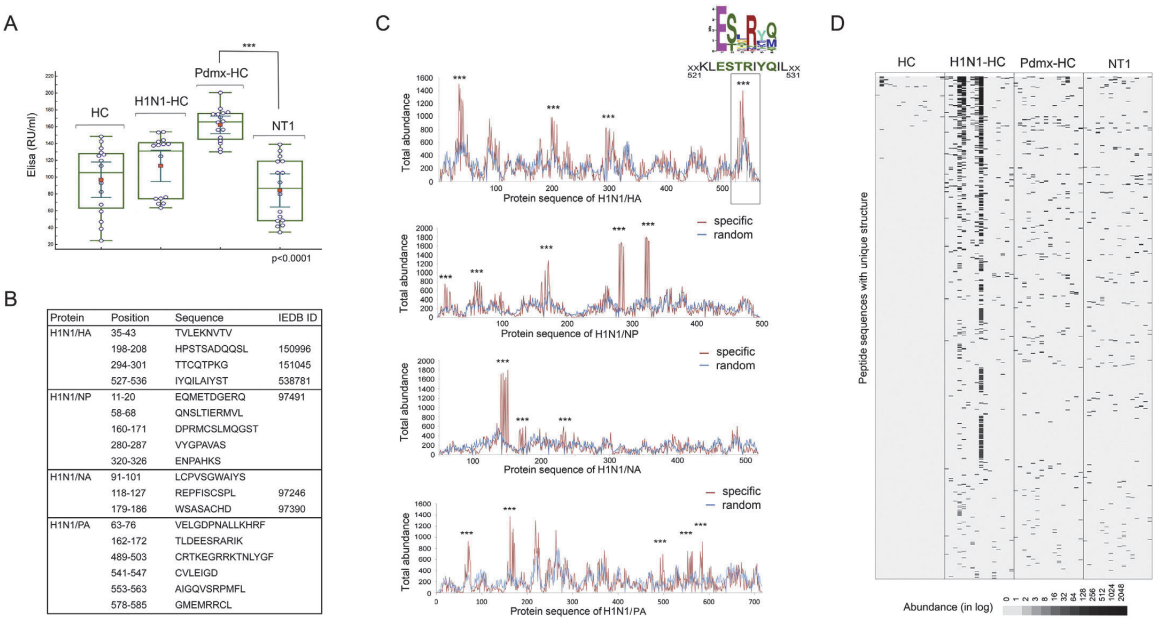
We performed MVA by selecting peptide antigens from random phage library (PhD12, NEB) with 10^9 different 12-mer peptide sequences based on their high avidity of interaction to antibodies in sera (Fig. 1A). A total dataset of 16 million peptides with unique sequences was generated. The data structure analysis of Top2500 peptide dataset (the most abundant peptides across individual samples) revealed that although these peptides were largely individual-specific (Fig. 1B and D), the study cohorts shared a fraction of common characteristics across Top2500 features (Fig. 1B). The remarkable heterogeneity of antigenic reactivity between individuals has also been noted previously (Zandian et al., 2017). However, the distribution of peptides according to the frequency was found to be similar in the different clinical subsets (Fig. 1C).

3.2. H1N1-specific Immunoprofiles are Largely Shared Between Pdmx-Vaccinated and Subjects Infected With H1N1

To evaluate the extent to which the presence of H1N1-specific peptides was restricted to specific clinical subsets, we assayed responses to H1N1 infection and Pdmx-vaccination using type A influenza ELISA (Quantum) diagnostic tests. High-titer responses to influenza A virus major antigens (including H1N1) were evident for both Pdmx-vaccinated and H1N1 naturally infected individuals (Fig. 2A). The humoral response to seasonal flu (A/H1N1 and A/H3N2) was relatively weaker in NT1-diseased as compared with Pdmx-HC individuals as determined by using a commercial ELISA test ($p < .001$). This was in slight contrast to earlier findings reporting that Pdmx-NT1 patients had higher median levels of anti-H1N1 antibodies than controls (Lind et al., 2014), and may reflect the characteristics of the samples collected (see Table 1, Materials and methods). Next, we assessed the reactivity of the sera to protein fragments representing the four major antigens of H1N1 virus proteome (strain A/California/7/2009). MVA data analyses of Top2500 peptide data set revealed 4 antigenic regions for hemagglutinin (H1N1/HA, C4RUW8), 5 for neuraminidase (H1N1/NA, C3W6G3), 3 for nucleoprotein (H1N1/NP, B4URE0), and 6 for polymerase acidic protein (H1N1/PA, I6THC5), some of which corresponded to known immunogenic epitopes from IEDB (http://www.iedb.org/; Fig. 2B). Statistically distinct coverage profiles with different peaks on H1N1 HA, NA, NP, and PA antigens were obtained from analysis of Top2500 peptide data sets of H1N1-HC, Pdmx-HC and NT1 samples (Fig. 2C). Data showed that the most commonly shared epitopes raised by the anti-Pdmx/anti-H1N1 immune response were found in the C-terminal region of H1N1/HA (C4RUW8) locating between amino acids 521 to 531 (Fig. 2B–C), directly before a proven T cell-antigenic region in HA between amino acids 527–541 of A/California/04/2009 (H1N1, (Schanen et al., 2011)). About 700 peptides from the total peptide dataset clustering to motif with sequence consensus E[ST].R.[QM] were highly abundant in H1N1-HC, and relatively infrequent in Pdmx-HC and NT1 samples as compared with HCs (Fig. 2D).

3.3. Examination of Identified NT1-specific Autoantigens in MVA Dataset

Next we determined peptides that were different between the clinical study groups to examine whether they were consistent with the prior knowledge of Pdmx-NT1-specific immunogenic epitopes. For the study, we used an exhaustive sequence pattern search (SPEXS - https://github.com/egonelbre/spexs2; (Vilo, 2002, Brazma et al., 1998)) gene ontology analysis, combined with the interrogation of the presence of known autoantigens previously identified in Pdmx-NT1 disease (Table S1(Ahmed et al., 2015, Bergman et al., 2014, Cvetkovic-Lopes et al., 2010, De La Herran-Arita et al., 2013, Haggmark-Manberg et al., 2016, Katzav et al., 2013, Zandian et al., 2017)). Thus, we were able to confirm statistically significant patterns of epitope recognition in the samples. Particularly, we identified epitopes resembling those in the N-termini of OX (4/16) and OX1R/2R (2/16; 4/16), in mitogen-activated protein kinase 7 (MAP3K7) (amino acids 318–328; 3/16) and in 5′-nucleotidase cytosolic IA (NT5C1A) (amino acids 35–48; 2/16), as well as in B-cell lymphoma 6 protein (BCL6), encompassing amino acids 279–288 in 6 out of 16 sera samples of NT1 diseased (Fig. 3, Table S1). According to MVA data, none of the previously identified antigens was prominently detected across NT1 diseased and were also common also to HC if less stringent statistical power criteria were used (Fig. 3). In contrast, we found no evidence of stratifying peptides with consensus sequences mimicking tribbles pseudokinase 2 (TRIB2), neuropeptide glutamic acid- isoleucine/α-melanocyte-stimulating hormone (NEI/aMSH), or others that were reported by earlier studies



Protein	Position	Sequence	IEDB ID
H1N1/HA	35-43	TVLEKNVTV	
	198-208	HPSTSADQQSL	150996
	294-301	TTCQTPKG	151045
	527-536	IYQILAIYST	538781
H1N1/NP	11-20	EQMETDGERQ	97491
	58-68	QNSLTIERMVL	
	160-171	DPRMCSLMQGST	
	280-287	VYGPAVAS	
	320-326	ENPAHKS	
H1N1/NA	91-101	LCPVSGWAIYS	
	118-127	REPFISCSPL	97246
	179-186	WSASACHD	97390
H1N1/PA	63-76	VELGDPNALLKHRF	
	162-172	TLDEESRARIK	
	489-503	CRTKEGRRKTNLYGF	
	541-547	CVLEIGD	
	553-563	AIGQVSRPMFL	
	578-585	GMEMRRCL	

Fig. 2. A novel common epitope of HA antigen of A/H1N1 in seasonal infection carriers and Pdmx-vaccinated individuals, encompassing a proven T cell- antigenic region. A. The humoral response to seasonal flu (A/H1N1 and A/H3N2) was relatively weaker in NT1-diseased as compared with Pdmx-HC individuals as determined by using a commercial ELISA test ($p < .001$). The presence of IgG antibodies against HA and H3N2 was assessed in sera samples of *HC*, *H1N1-HC*, *Pdmx-HC* and *Pdmx-NT1* by ELISA (Influenza virus type A IgG ELISA, Euroimmun). The HC samples were collected prior to A/H1N1 outburst in Estonia, before the fall 2009. *Blue circles* mark individuals of the study cohorts; *red dots* mark the mean values; *lines* depict median values; *inner whiskers* mark confidence interval for the mean; *boxes* mark upper and lower quartiles; *outer whiskers* mark the maximum and minimum values (excluding the outliers). *P*-values were calculated by ANOVA and are marked with asterisks. The cut-off value for ELISA was 16 RU/mL. Labels at the top of box plots demark the clinical origin of the sample. B. MVA predicted H1N1 epitopes partially overlapped with previously described H1N1 (A/California/08/2009(H1N1)) B cell specific epitopes from IEDB (http://www.iedb.org/). Top2500 peptide dataset containing 121,142 unique sequences was used to delineate the predominant epitopes of H1N1/HA (GI: 238,623,304), H1N1/NA (NA, GI:758899360), H1N1/NP (NP, GI:229891180) and H1N1/PA proteins in study samples. 9657 peptides from the studied dataset satisfied the selection criteria that these were not present in the HC samples. Specific alignment profiles for each of the A/H1N1 protein antigens were calculated with the criterion that the abundance of a peptide was to be 2-fold higher over random. C. MVA immunoprofiles predicted a novel epitope in the C-terminal region of HA encompassing amino acids 521–531 and with the sequence ESxRxQ that was common to both seasonal infection carriers and Pdmx-vaccinated individuals. The graphs show antigen-specific profiles of overall peptide abundance where the number of peptides were counted for each amino acid position for the following proteins: hemagglutinin (H1N1/HA, C4RUW8), neuraminidase (H1N1/NA, C3W6G3), nucleoprotein (H1N1/NP, B4URE0) and polymerase acidic protein (H1N1/PA_I6THC5). Amino acid sequence of the proteins is depicted on the x-axis. Marked with asterisks are regions where set calculation criteria were satisfied. Detailed analysis of immunoprofiles of H1N1 antigens revealed a novel immunogenic region of HA encompassing amino acids 521–531 that corresponds to the earlier experimentally determined A/Puerto Rico/8/1934 (H1N1) HA520–530 CTL epitope (Gianfrani et al., 2000) and is partially overlapping with broadly reactive CD4+ T cell epitope: HA527–541 of A/California/04/2009(H1N1) (Schanen et al., 2011). Peptides aligning to 521–531 of HA cluster to a minimal consensus sequence E[ST].R.[QM] by sequence homology alignment. D. Heat map image of immunoprofiles of peptides with consensus E[ST].R.[QM] across study samples. The total peptide data set was examined for the peptides with unique structure clustering to E[ST].R.[QM] motif. About 700 peptides with enriched abundance in H1N1-HC, Pdmx-HC or Pdmx-NT1 samples were found to cluster to the motif. The data of 700 peptides is presented on the heat map image. Each line represents peptides with unique sequence structure. The colour intensity of each cell corresponds to the peptide abundance (presented in log value). Black represents peptides captured at higher abundance whereas white represents peptides captured at lower abundance. Each column represents a peptide profile from a single sample. Labels at the top of the panels indicate the clinical origin of the sample. Abbreviations: *Random alignment* – amino acid sequences of peptides under analysis were randomized and aligned to respective protein coding sequence; *Total abundance* - the number of peptides counted for defined amino acid positions; *HC*– healthy control; *H1N1-HC* – H1N1 infected; *Pdmx-HC*- Pandemrix-vaccinated; *NT1*- narcolepsy type 1 (including 10 Pandemrix-induced NT1 samples).

(Table S1). These data allowed concluding that apart from the BCL6 related subset, peptides corresponding to previously identified autoantigens had relatively little discriminative power, suggesting also that these antigens were either rare or recognized promiscuously in patient groups with a clinical and ethnical heterogeneous background.

3.4. A Defined Set of Peptides Derived From DP1 Acts as Antigenic Epitopes in NT1

In analyses of the peptides that were unique among the disease groups, we observed that the Top2500 dataset contained >1300 peptides with a high enrichment in NT1 (Fig. S1A–B). The most abundant peptide having the sequence RVLAPALDSWGT showed a high sequence homology within the second extracellular loop region in the human prostaglandin D2 receptor DP1 (Q13258). This region in DP1 is predicted to function in ligand recognition (Avlani et al., 2007; Nagata et al., 2017) and is not conserved in mouse and rat (Fig. 4A). Extraction of all peptides from the total dataset having the highest homology to RVLAPALDSWGT and to DP1 revealed a set of 4428 unique peptides containing the RxxxPxxD (RPD) consensus sequence that discriminated the NT1 samples from controls ($p < .0001$, ANOVA, Fig. 4B–C). We then also determined that the 2157 RPD-containing peptides out of 4428 (Fig. 4B) had a high sequence homology to DP1 protein where the bona fide immunodominant epitope with sequence RVLAPALD encompassed amino acids 94 to 101 in DP1 (Fig. 4D). Interestingly, according to the IEBD database (www.iedb.org/), four MHC-I binding epitope regions of DP1 encompassing amino acids 132–140 (ID: 716767), 145–156 (ID: 637966), 195–203 (ID: 727099) and 303–311 (ID: 697995) have been defined. The latter (303—311) encoded another extracellular domain of DP1 that was also defined by us a potential target of B cell response (Fig. 4D).

To validate the data, we employed different methods and measured the serologic response to peptides carrying the RPD consensus sequence using sera samples of the study (Fig. 5–6). In line with previous reports

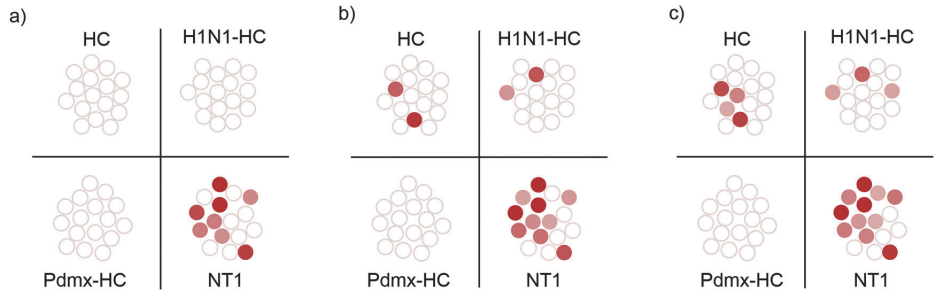


Fig. 3. Heterogeneity of the immune response to delineated antigens is apparent at the individual level. The total peptide data set was examined for peptides with homology to different antigens (see Table S1). Homology alignment analysis resulted in delineating six minimal consensus motifs with homologies to epitopes defined by previous studies. Namely, LPxTNxS (HCRT, O43612), RDxxYP (HCRTR1, O43613), SxLNxTxN (HCRTR2, O43614), KxxxPSAS (BCL6, P41182), STNxS (MAP3K7, O43318), LAxSxKP (NT5C1A, Q9BXI3), (Table S1). Datasets from different study cohorts were analyzed for the presence of peptides containing the 6 motifs and ROC analyze was performed at threshold values: a) specificity 100% (sensitivity 50%), b) specificity 90%, (sensitivity 63%), c) specificity 85% (sensitivity 69%). Note that the ensemble snapshots from MVA data did not distinguish between the possible scenarios of each individual antigen motif in single individuals. Individual samples are colour coded. White circles indicate that peptides with abundance values clustering to one or more aforementioned motifs were below threshold. Colored circles indicate peptides with abundance values clustering to one or more aforementioned motifs were above threshold. None of the motifs displayed a statistically significant association with many other known antigens (see the list of antigens in Table S1). This negative result may be due to a limited statistical power of delineated antigens, but also due to the heterogeneity of autoantibody repertoires in different individuals. Abbreviations: *HC*- healthy control; *H1N1-HC* – H1N1 infected; *Pdmx-HC*- Pandemrix-vaccinated; *NT1*- narcolepsy type 1 (including 10 Pandemrix-induced NT1 samples).

(Urade and Hayaishi, 2011), we observed a broad expression of *DP1* mRNA across different regions of human brain, in human glioma (hGC), and normal mesenchymal stem cells (hMSCs) (Fig. 5A). Treatments of hMSCs with the ligand prostaglandin D2 increased *DP1* expression, whilst the pro-inflammatory cytokines interleukin 1β and interferon γ (IL-1β and IFN-γ) either slightly increased or decreased its expression, respectively (Fig. 5B). In contrast, PGD2 strongly reduced *IL-1β* and *IFN-γ* expression suggesting that these cells recapitulate the intact PGD2-DP1 signaling pathway by inducing anti-inflammatory responses in the studied cells (Fig. 5B). Performing phage Western blot assay we confirmed that MVA predicted DP1-positive Pdmx-NT1 sera showed IgG reactivity to phages that displayed RVLAPALDSWGT peptides (RVLAPALD-pIII, Fig. 5C). No specific reactivity was detected using DP1-negative sera (Fig. 5C). This peptide target specificity was further confirmed by Western blot analysis using Pdmx-NT1 sera where the interactions between human IgGs and antigen expressing phages were blocked by **RVLAPALD**SWGT synthetic peptides (Fig. 5C, Fig. S2). Immunoblot analysis using commercial anti-DP1 polyclonal sera and clinical sera of Pdmx-NT1, confirmed the presence of DP1 expression in hGC_1 and not in hGC_2 glial cells and also here specific blocking effects to the seroreactivity in the presence of **RVLAPALD**SWGT peptides were confirmed (Fig. 5D), but not in case of control peptides or irrelevant sera (Fig. 5C, Fig. S2). Immunocytochemical analyses showed that DP1 was predominantly localized on the cell surface of hMSC and hGC cells, and more importantly, was equally well-detected by immunocytochemistry using commercial anti-DP1 polyclonal sera and Pdmx-NT1 clinical sera (Fig. 5E). Furthermore, the synthetic peptide **RVLAPALD**SWGT competed for the binding of anti-DP1 antibodies present in sera of Pdmx-NT1 diseased (Fig. 5E). Data combined from Western blot and immunocytochemistry analysis suggested that peptide **RVLAPALD**SWGT could embed a structural as well as a linear epitope given that upon competition it interfered with DP1-specific serorecognition of globular as well as denatured epitopes (Fig. 5D and E).

We next studied whether the peptides identified here could be developed to an ELISPOT assay to discriminate sera in different disease groups. ELISPOT analysis data showed that peptides containing H1N1/HA-specific sequence ESTRYQL (peptide_3) discriminated between naturally H1N1 infected and healthy samples with no earlier H1N1 infection (ANOVA $p < .001$, Fig. 6A). RVLAPALD (epitope on DP1) and KAPSAS (epitope on BCL6) (peptide_1 and _2) peptides that were selected upon MVA data, correctly assigned upon ELISPOT analysis the NT1 group from HC samples (ANOVA $p < .001$, Fig. 6B). Combined ELISPOT analysis using all 3 peptides, could correctly classify 11 out of 16 NT1 (specifically - 7 Pdmx-NT1 and 3 NT1) samples across all controls ($p < .001$ Chi-squared test, Fig. 6C). Notably, majority of the DP1 and BCL6-peptide-positive NT1 samples had undetectable OX findings from the related CSFs (with average values of 6.1 pg/mL), whereas those 4 that were negative by our ELISPOT measurements, had OX levels in respective CSF samples still low but in detectable range (with average values of 77 pg/mL (Fig. 6C and see Materials and methods). Unlike the IgG response, the IgM levels in response to the tested peptides were low or absent in all studied individuals (data not shown). These findings confirmed that peptides carrying the epitope motifs identified in the study could be used in ELISPOT analysis to develop a novel multi-biomarker diagnostic assay for NT1.

4. Discussion

Despite extensive research using biomarker and neurophysiological approaches, known heterogeneity among NT1 diseased is not always consistent with serologic marker-based subtype classification schemes. Using an unbiased analysis of serum samples from single individuals, we detected a high variance in humoral immune response profiles, both in healthy and diseased people. We found that variance in immunoprofiles representing multifactorial heterogeneity of NT1 clearly determined distinct disease-specific serological profiles. We focused our analysis on peptides specific to Pdmx-immunized and -NT1 diseased subjects, which encompassed vaccine antigens and autoantigens in order to have a full coverage of potential triggers of the disease. Our results show that patients with NT1 exhibit a specific immune response to epitopes of receptor DP1. This finding highlights the importance of the PGD2-DP1 pathway in the functioning of sleep-wake homeostasis as suggested by the role of DP1 in slow-wave sleep (Terao et al., 1998). However, the precise mechanism by which PGD2-DP1 signaling may influence orexinergic neurons and immune regulation in NT1 requires further studies. In addition, using MVA-based immunoprofiling, we discovered epitopes, such as those of the protein BCL6 specific for Pdmx-NT1 and sNT1 patient group. This underscores the complexity of NT1 with different molecular targets and pathways involved and contributing to the immune response. Enhanced inflammation due to immune system malfunction has been detected in human narcoleptics in the regions of OX cell loss (Bassetti et al., 2010; John et al., 2013; Nishino, 2011; Thannickal et al., 2000, 2003,

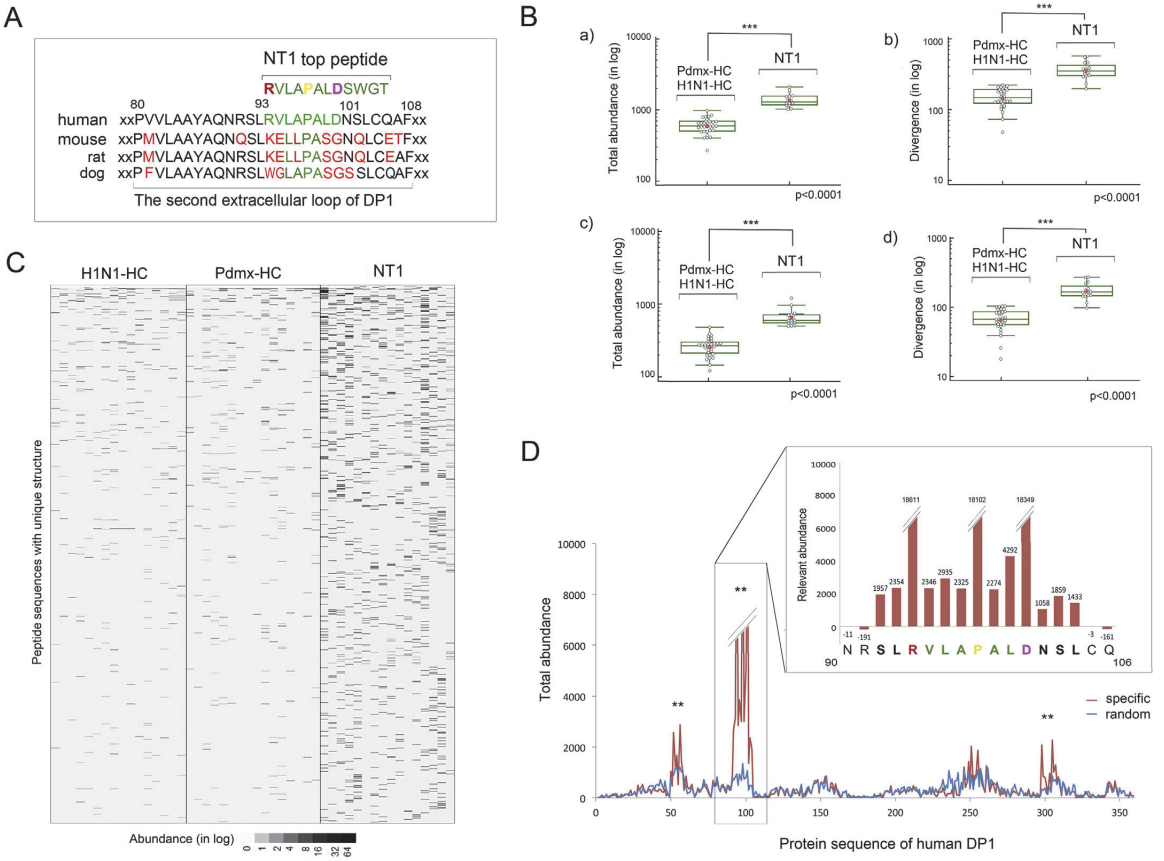


Fig. 4. Peptides with minimal epitope motif RxxxPxxD (RPD) are enriched in samples of NT1 and align to the extracellular loop of human DP1. A. Peptide RVLAPALDSWGT found as the most abundant in Pdmx-NT1 was highly homologous to the second extracellular loop region of DP1 protein. More than 1,300 peptides from the selection of the Top2500 peptide dataset were enriched in NT1 samples (Fig. S1A–B) where the peptide RVLAPALDSWGT had the highest values of abundance across the Pdmx-NT1 cohort. BLAST analysis revealed that RVLAPALDSWGT region of DP1 is highly dissimilar in mammals. To identify peptides from Totpep dataset with the highest homology to DP1 and to RVLAPALDSWGT, SPEXS2 software (https://github.com/egonelbre/spexs2.) was used and the top motif that fulfilled both criteria was found to be RxxxPxxD (RPD, a motif defined by three shared amino acids). B. Peptide sequences containing RPD motif were enriched in immunoprofiles of NT1 samples. Analyzing the Totpep library, 4428 peptides were identified from NT1 (including 10 Pdmx-induced NT1 samples and 6 sNT1 samples) data sets that contained the RPD motif. Box plot depicting that these 4428 peptides (with RVLAPALDSWGT eliminated beforehand as dominant) contained sufficient information to discriminate between HC and NT1 samples in a statistically significant manner a) by their abundance (in log, student *t*-test *p*-value < .0001) and b) divergence (in log, student *t*-test *p*-value < .0001). SPEXS2 analysis resulted in 2157 peptides that out of 4428 were highly homologous (with at least 4 consecutive amino acid matches) to RVLAPALD of human DP1 and discriminated NT1 samples (including 10 Pdmx-induced NT1 samples and 6 sNT1 samples) in a statistically significant manner c) by their abundance (in log, student *t*-test *p*-value < .0001) and d) divergence (in log, student *t*-test *p*-value < .0001). Six NT1 samples that were not Pdmx-induced were similarly to Pdmx-NT1 samples discriminated by the set of 4428 peptides from HC. In box plots - *blue circles* mark the single individuals of study cohorts; *red dots* mark the mean values; *line* marks the median values; *inner whiskers* mark confidence intervals for the mean; *boxes* mark the upper and lower quartiles; *outer whiskers* mark the maximum and the minimum values (excluding outliers). C. Heatmap images depicting the immunoprofiles of the top one thousand NT1-specific peptides out of 4428 across the clinical study-groups. The data are presented as heat map image generated via conditional formatting in MS Excel. Each column represents a peptide profile from a single individual. Each line represents peptides with unique sequence structure. The colour intensity of each cell corresponds to the peptide abundance (counts of sequences in log). Black represents peptides captured at higher abundance whereas white represents peptides captured at lower abundance. D. Epitope mapping of anti-peptide response to human DP1 in sera of Pdmx-NT1 diseased. SPEXS2 analysis resulted in 2157 peptides carrying RxxPxxxxD motif that were aligned to DP1 protein sequence (Q13258) with a 2-fold higher abundance over random as a chosen criterion. In addition, MEME-MAST algorithm (Bailey and Elkan, 1994) aligned these 2157 peptides to the region encompassing 94-101aa of DP1, E-value = 0.0078. Each bar on the x-axis corresponds to one of the overlapping peptides required to cover the antigen, and the height of the profiles shows the relative abundance. Three potentially immunogenic regions were described with the predominant alignment containing the sequence RVLAPALD and encompassing amino acids 94 to 101. Zoomed in the box is an extract of the immunoprofile of DP1 in positions 90–106. Calculated relative abundance values are marked above each amino acid position. Note that the weaker immunogenic regions of DP1 are expanded toward both N- and C-termini. Abbreviations: *Abundance* – peptide frequency; *Divergence* – all unique peptides; *HC*- healthy control; *H1N1-HC* – H1N1 infected; *Pdmx-HC*- Pandemrix-vaccinated; *NT1*- narcolepsy type 1 (including 10 Pandemrix-induced NT1 samples).

2009), but the exact factors or mediators leading to the ultimate death of OX neurons are yet unknown.

Here we show that the DP1 receptor is linked to NT1 by acting as a possible antigen in the disease process. Prostaglandins play a key role in the inflammatory response and their synthesis is significantly increased after tissue injury and cell stress (Ricciotti and Fitzgerald, 2011). PGD2 is a major eicosanoid both in the Central Nervous System (CNS) and peripheral tissues with a role in inflammation as well as homeostasis (Jowsey et al., 2001; Vijay et al., 2017). PGD2 is abundantly produced by mast cells and Th2 cells, and among a wide range of

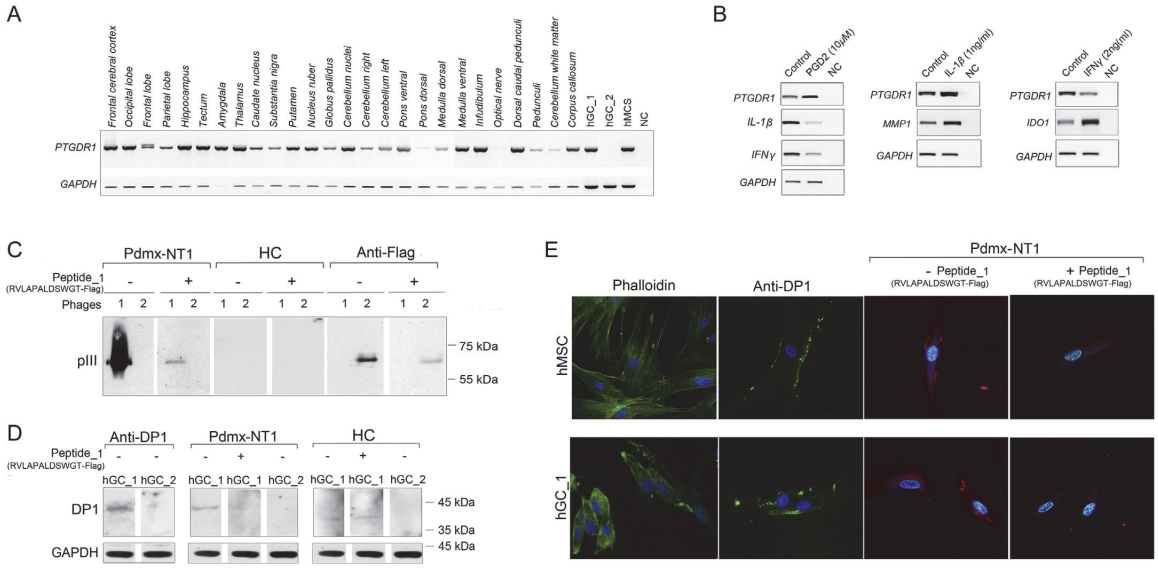


Fig. 5. Validation of DP1 as a true antigenic target in NT1 disease. A. Human *DP1* is expressed widely in different brain regions, and by mesenchymal and cancer stem cells. Cerebral cortex: *Frontal cerebral cortex*, *Occipital lobe*, *Frontal lobe*, *Parietal lobe*, *Hippocampus*; brain nuclei: *Tectum*, *Amygdala*, *Thalamus*, *Caudate nucleus*, *Substantia nigra*, *Putamen*, *Nucleus ruber*, *Globus pallidus*; *Cerebellum*: *Cerebellar nuclei*, *Cerebellum right*, *Cerebellum left*; Brainstem: *Ventral pons*, *Dorsal pons*, *Dorsal medulla*, *Ventral medulla*; Axonal tracts: *Infundibulum*, *Optical nerve*, *Dorsal caudal pedunculi*, *Pedunculi*, *Cerebellum white matter*, *Corpus callosum*; human glioma cells (hGC_1, hGC_2); human mesenchymal stem cells (hMSC); NC – negative control. PCR analyses were done using specific primers for human *DP1*. *GAPDH* mRNA expression was used to normalize the data across samples. B. PGD2/DP1 signaling is associated with inflammation regulation. Expressions of human *DP1*, *IL-1β*, *MMP1*, *IFNγ* and *IDO-1* were analyzed by PCR in human mesenhymal stem cells treated with PGD2 (10 μM) or cytokines IL-1β (1 ng/mL) and IFNγ (2 ng/mL). NC – negative control. *GAPDH* mRNA expression was used to normalize the data across samples. C. RVLAPALD was identified as a target antigen sequence for NT1-specific polyclonal IgG response. Western blot analysis of phage particles containing the RVLAPALD-pIII (phage 1) or FLAG-pIII fusion proteins (phage 2) show that human Pdmx-NT1 serum (dilution 1:500) reacted specifically with the pIII protein containing the peptide RVLAPALDSWGT sequence, but not with the phage backbone or FLAG-pIII fusion protein. Duplicate membranes were incubated with Pdmx-NT1 sera treated with the synthetic peptide (RVLAPALDSWGTGGGDYKDDD: final conc 6.6 μg/mL) that significantly blocked the interaction between phage #1 and human IgG similarly to anti-FLAG antibody (dilution 1:2000) and phage #2. RVLAPALDSWGT-pIII fusion protein was not detected by HC sera (1: 500). Protein size markers are indicated at the right side of blot. D. NT1-specific seroreactivity to DP1 protein is specifically blocked by RVLAPALD peptide. Western blot analysis of endogenous levels of DP1 protein (MW 40 kDa) in human glioma hGC_1 and hGC_2 cells using anti-PTGDR1 (DP1) polyclonal antibodies (1:500) (*left*, first panels). Note that, hGC_2 cells were negative of DP1 expression. The use of the Pdmx-NT1 serum (1:500) showed similar pattern of DP1 reactivity in hGC_1 and hGC_2 cells, and the DP1-specific signal was attenuated by pre-treatments of Pdmx-NT1 sera with a synthetic peptide #1 (final conc 6.6 μg/mL). Anti-GAPDH monoclonal antibody (1:10,000) was used as a control for immunoblots. E. DP1 expressed by hMSCs and cancer was specifically blocked by RVLAPALD peptide. IF analysis of DP1 in hMSC and glioma cells. The antibodies used included: anti-PTGDR1 (1:500; green), Pdmx-NT1 serum (1:400; red) and the secondary Alexa Flour 488 and 647 (Invitrogen, 1:2000) antibodies. For antibody-blocking, Pdmx-NT1 sera (1:400) and synthetic peptide #1 (final conc 6.6 μg/mL) were used. Cells were analyzed for phalloidin-labelled cytoskeleton proteins (green, left) and nuclear structures (Hoechst 33342, blue). Abbreviations: *Pdmx-NT1*- Pandermix-induced narcolepsy type 1; *HC* – healthy control.

other body cells (see ref. in Farhat et al. (2011)). PGD2 elicits its downstream effects by activating DP1 and DP2 receptors with opposing effects on cyclic AMP (cAMP) production, and/or phosphoinositol turnover and intracellular Ca2+ mobilization (Liang et al., 2005). In the brain, PGD2 regulates sleep, body temperature, and nociception and its levels exhibit marked changes in different neuropathologies (reviewed in (Liang et al., 2005, Mohri et al., 2006, Urade and Hayaishi, 2011)). The microglial PGD2-DP1 pathway is also known to mediate neuronal damage through microglial activation (Bate et al., 2006; Vijay et al., 2017).

Among cells expressing DP1, mast cells (MCs) can release histamine and other factors that affect sleep and the immune response in the brain. Accumulating evidence shows that MCs play a role in the regulation of sleep and behavior (Chikahisa et al., 2013). MCs are most abundant in young individuals under the age of 19, after which their counts decline with age (Porzionato et al., 2004; Turygin et al., 2005). Most significantly, the maturation of MCs is influenced by PGD2 and the receptor DP1 (Taketomi et al., 2013). In addition to histamine, DP1 signaling may also influence the levels of adenosine that is known to regulate sleep (Urade and Hayaishi, 2011). The precise role of MCs and their released factors such as histamine in NT1 warrants further studies.

In conclusion, the present study shows that anti-DP1 antibodies are autoimmune agents in the course of NT1 prompting more studies on the role of PDG2-DP1 signaling in OX-signaling and in the disease. Currently DP1-selective agonist/antagonist therapies are considered in treating autoimmune disorders such as asthma (Maicas et al., 2012; Santini et al., 2016; Santus and Radovanovic, 2016). Our data also indicate that, depending on the antibody concentrations and affinities, anti-DP1 antibodies may modify the function of pharmaceutical compounds targeting PGD2-DP1–signaling pathways (Narumiya and Fitzgerald, 2001) that need to be taken into account in clinical studies.

Supporting evidence that the humoral response in the CNS is derived from different peripheral tissue antigens is provided by the findings that sera from NT1 diseased can bind brain and muscle structures (Ahmed et al., 2014; Smith et al., 2004). There is a plethora of data that genetic or experimental alterations of the OX system are associated with NT1, however, OXs are not restricted to the CNS and together with their receptors OX1R and OX2R are widely expressed in peripheral tissues (see ref. in (Voisin et al., 2003)). BCL6, another NT1 antigen, is a master regulator required in mature B-cells during germinal center (GC) reaction (Ref in Pei et al. (2017)). NRXN1-α has been isolated from brain and heart tissues suggesting a role also in heart development (Nagase et al., 1998). TRIB2 is present in many

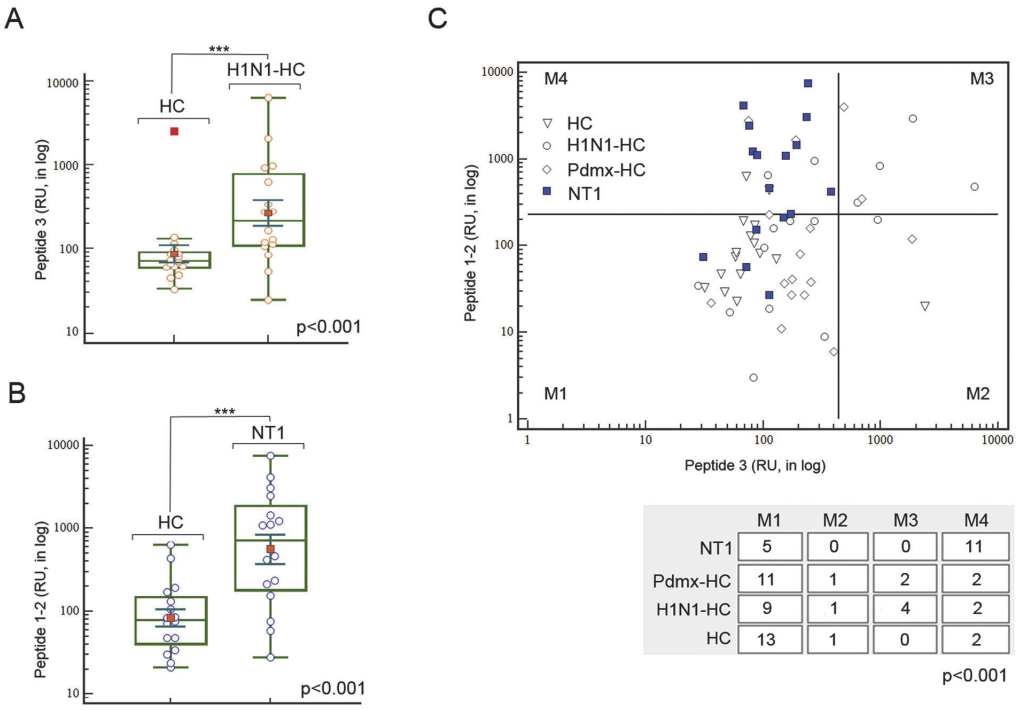


Fig. 6. Use of MVA-defined peptides for immunodiagnostic applications of NT1. A. ELISPOT analysis of peptides containing H1N1/HA-specific sequence ESTRYQL (peptide_3) confirmed the power of the peptide to discriminate samples of natural H1N1 infection from HC (ANOVA $p < .001$). In box plots - *yellow circles* mark the single individuals of study cohorts; *red dots* mark the mean values; *line* marks the median values; *inner whiskers* mark confidence intervals for the mean; *boxes* mark the upper and lower quartiles; *outer whiskers* mark the max and the min values (excluding outliers). B. ELISPOT analysis of peptides containing RVLAPALD (epitope of DP1) and KAPSAS (epitope of BCL6) (peptide_1 and _2, respectively) resulted in correct assignment of 10 Pdmx-NT1 samples and 6 sNT1 samples to NT1 group (ANOVA $p < .001$). On box plots - *blue circles* mark the single individuals of study cohorts; *red dots* mark the mean values; *line* marks the median values; *inner whiskers* mark confidence intervals for the mean; *boxes* mark the upper and lower quartiles; *outer whiskers* mark the max and the min values (excluding outliers). C. ELISPOT analysis data combined revealed the power of peptides 1, 2, and 3 to discriminate 11 (7 Pdmx-NT1 and 4 sNT1) out of 16 NT1 samples across all samples ($p < .001$ Chi-squared test). Thresholds were calculated by using ROC curve analysis and results were visualised using scatter plot analysis with divided threshold values (*M1-M4*). Statistical significance of differences was calculated by two-way classification Chi-square test (Chi-squared *p*-value > .001). Abbreviations: *HC*– healthy control; *H1N1-HC* – H1N1 infected; *Pdmx-HC*- Pandemrix-vaccinated; *NT1*- narcolepsy type 1 (including 10 Pandemrix-induced NT1 samples).

cell populations both in and outside the nervous system, including the immune cells (Eder et al., 2008; Sung et al., 2006). Gangliosides (anti-GM3) are abundant in the brain, but in extraneural tissues, relatively high concentrations of ganglio-series GMs were found in bone marrow, erythrocytes, intestine, liver, spleen, testis, kidney, and in embryonic stem cells (Kolter, 2012). NEI-MCH has mostly been detected in peripheral organs (Viale et al., 1997). NT5C1A is highly abundant in skeletal muscle tissue (Hunsucker et al., 2001). GLS2 is expressed specifically in the liver, but also in extrahepatic tissues, like the brain, pancreas, cells of the immune system (ref in Martin-Rufian et al. (2012)). However, it remains elusive what pathogenic roles these antibodies against the above-mentioned proteins may exert within the periphery.

Our data of immunoprofiling support the existence of immune defects in multiple pathways associating NT1 to a) DP1 and PDG2/histamine associated disorders, b) BCL6 and the chronic status of latent herpesviruses (such as EBV), c) orexin/OX1/2R–related dysfunctions, d) stress and inflammation-associated mitogen-activated pathways (such as MAP3K7, also known as transforming growth factor (TGF)-β-activated kinase 1 (TAK1)), and e) adenosine-deficiency linked dysfunctions (involving NT5C1A) (Table S1, Fig. 7). Together these results provide a comprehensive map of potential molecular targets contributing to NT1 that can be of help in designing future strategies for the diagnostics and treatment of the disease. More broadly, our study demonstrates the usefulness of MVA as a method for disease classification and for the discovery of novel biomarkers that can be applicable to any human disease.

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Conflicts of Interest

AV, OV, MP, KP, TN, and KP are co-applicants on a pending patent application related to diagnosing of narcolepsy (WO 2017/203106). All other authors declare no competing interests.

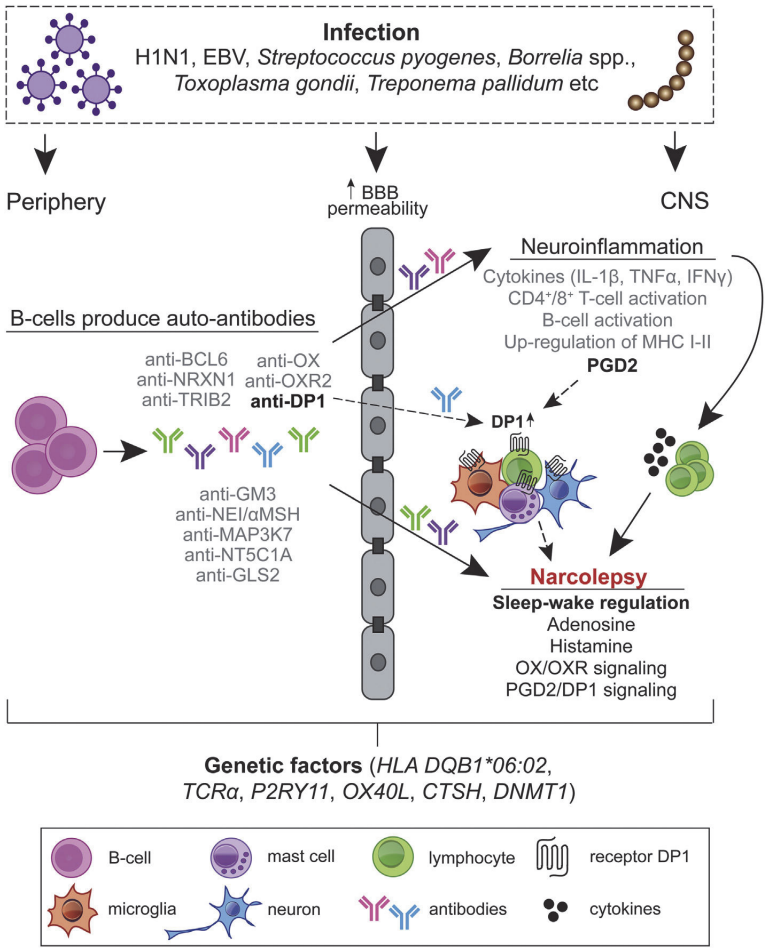

Fig. 7. Hypothetical model for the aggravation of autoimmune response in Pdmx-associated and spontaneous NT1. The immune response in NT1 is highly heterogenic with different pathways affected during the disease progression. We favor the idea that the lifelong risk for NT1 or for disease aggravation in pre-disposed individuals is increased following inflammatory triggers upon breaching of the blood–brain barrier (BBB) and with activation of preexisting auto-reactive antibodies (Ab) and cells reaching brain. Consequently, an immune response to A/H1N1 (and subsequent molecular mimicry) or a generalized stimulation of the immune system mediated by the Pdmx vaccine as AS03-adjuvanted vaccine can act as the inflammatory trigger (Morel et al., 2011; Carmona et al., 2010; Meyer et al., 2011). The inflammation triggers include i) *infections* (examples of pathogens are shown), ii) *genetic factors*, or iii) chronic *inflammation* (Kornum et al., 2011). The polyclonal Ab response from peripheral tissue may initiate disease by concentrating antigens in the brain to presentation-competent cells (Getahun et al., 2004). Recent data show further that peripherally produced human anti-CNS reactive antibodies are capable of opsonizing human CNS antigens (Kinzel et al., 2016). The entry of immune cells (T cells, B cells, macrophages, microglia and mast cells) cause *neuroinflammation* with the release of cytokines that damage neurons including HCRT+ neurons involved in sleep/wake regulation. Production of auto-reactive antibodies as a secondary response to cell death of HCRT-or other brain-resident cells can occur via antigen presenting cells. Prostaglandins are part of the inflammatory response in the brain acting via specific receptors. In particular, DP1 is produced by astrocytes, oligodendrocytes, neurons, microglia and meningeal cells (Liang et al., 2005; Mohri et al., 2007; Beuckmann et al., 2000). *PGD2* signaling is known to prevent excessive inflammasome activation and may act as an anti-inflammatory pathway in the brain. Additionally, in brain residing mast cells, DP1 activity promotes maturation and histamine release (Taketomi et al., 2013). The latter is of particular interest given that histamine levels in the CSF of NT1-diseased are reduced (Nishino et al., 2009). Thus, our findings suggest that the anti-DP1 immune response, whether causal or sequel, can interfere with PGD2 signaling in the brain. The results provide also evidence that the dysfunctional DP1 network can be a target for diagnosis and intervention of NT1, a conclusion that warrants further investigations.

Author Contributions

HS, AP, TN, MP, AV, and KP contributed to the design of the study. MP, OV, and AV were members of NARPANord consortium, MP was the chairman. HS, AP, AK, SP, MJ, MP, and AV contributed to data collection. HS, AP, AK, SP, MJ, DL, and KP contributed to the development and implementation of the data analysis plan. JV and PA were responsible for data management and pattern recognition analysis of the MVA data. All authors were involved in data interpretation, drafting, review, and approval of the report, and the decision to submit for publication.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.01.043.

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Supplementary Material

Prostaglandin D2 receptor DP1 antibodies predict vaccine-induced and spontaneous narcolepsy type 1: large-scale study of antibody profiling

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Table S1. Examination of previously identified NT1-specific autoantigens in MVA dataset

Motif identified in MVA	Pdmx-NC	Antigen	ID	Immunogenic region	Position	References
		TRIB2	Q92519	NSAYGAKEVSDQLVPDVNMEE NLDPFFN	316-343	Cvetkovic-Lopes *et al* 2010
LPxTNxS	**4/16**	**HCRT**	**O43612**	**MNLPSTKVSWAAV**	1–13	De la Herrán-Arita *et al* 2014
		HCRT		ALLSSGAAAQPLPDC	25–39	-"-
		HCRT		RQKTCSCRLYELLHG	41–55	-"-
		HCRT		LHGAGNHAAGILTLG	53–67	-"-
		HCRT		AGNHAAGILTLGK	56–68	-"-
		HCRT		QASGNHAAGILTMGR	85–99	-"-
		HCRT		SGNHAAGILTMGR	87–99	-"-
		HCRT		RRCSAPAAASVAPGG	113–127	-"-
RDxxYP	**2/16**	**HCRTR1**	**O43613**	**ATPGAQMGVPPGSREPSPVPP DYEDEFLRYLWRDYLYPKQY EWVLIAAYV**	5-54	Ahmed *et al* 2015
SxLNxTxN	**4/16**	**HCRTR2**	**O43614**	**MSGTKLEDSPPCRNWSSASEL NETQEPPLNPTDYDDEEFLRY LWREYLHPKEYE**	1-55	**-"-**
		NEI	P20382	EIGDEENSAKFPI	131-143	Bergman *et al* 2014
		aMSH	P01189	SYSMEMFRWGKPV	138-150	-"-
KxxxPSAS	**6/16**	**BCL6**	**P41182**	**EGLKPAAPSA**	279-288	Zandian *et al* 2017
		CNTN2	Q02246	RDATKITLAPSS	506-517	-"-
		DYSF	O75923	FPDPYTELNTGK	536-547	-"-
		NRXN1	Q9ULB1	LHTGKSADYVNL	322-333	-"-
		RXRG	P48443	SMSPSAALSTGK	26-37	-"-
		SCN5A	Q14524	AMKKLGSKKPQK	1497-1508	-"-
		ABL2	P42684	TQEGGKKAALGA	1018-1029	-"-
		RTN4	Q9NQC3	MEAPLNSAVPSA	616-627	-"-
		SOCS7	O14512	SSPGRGGGGGGR	138-149	-"-
		SOX11	P35716	GGSAGGGAGGA	146-157	-"-
		GOLGA6L10	A6NI86	VALDSSSAIISQ	96-107	-"-
STNTS	**3/16**	**MAP3K7**	**O43318**	**SFMDIASTNTSN**	318-329	-"-
		PDE8B	O95263	FNRRFMENSSII	234-245	-"-
		PLCG2	P16885	IIIKHKKLGPRG	451-462	-"-
		SETX	Q7Z333	DEEMSNSTSVI	924-934	-"-
LAxSxKP	**2/16**	**NT5C1A**	**Q9BXI3**	**PVWEEAKIFYDNLAPKKKPK SPKPQNAVTIAVSSRALFRMD EEQQIYTEQGVEEYVRYQLE HENEPFSPGP**	25-95	Häggmark-Månberg *et al* 2016
		GLS2	Q9UI32	EVVKLLQDYQDSYTLSETQAE AAAEALSKENLESMV	567-602	-"-

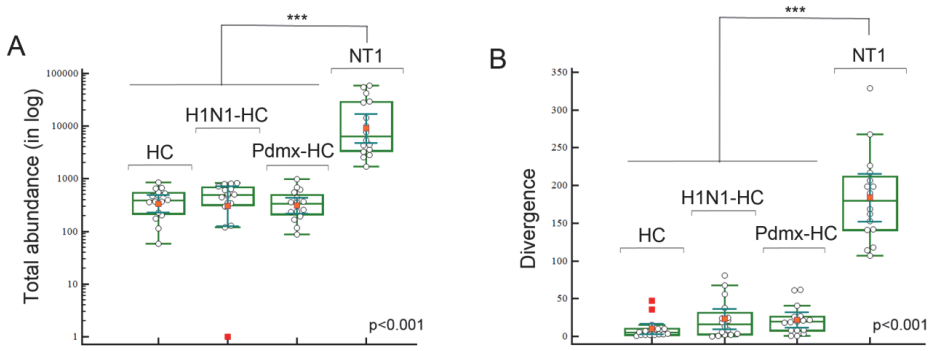

Figure S1. 1,300 peptides from Top2500 peptide dataset were enriched in NT1 samples. > 1,300 peptides from Top2500 peptide set were enriched in NT1 samples (including 10 Pdmx- NT1 and 6 sNT1 samples) by abundance ($p<0.0001$) and by divergence ($p<0.0001$) and the peptide with sequence RVLAPALDSWGT displayed the highest abundance value across study cohorts. In box plot, grey circles mark individual samples; red dots mark the mean; line marks the median; inner whiskers mark confidence intervals for the mean; box marks the upper and lower quartile; outer whiskers mark the max and min values (excluding outliers); red dots out of whiskers mark outliers. P-values were calculated by ANOVA. Abbreviations: *Abundance* – peptide frequency; *Divergence* – all unique peptides; *HC*- healthy control; *H1N1-HC* – H1N1 infected; *Pdmx-HC*- Pandemrix-vaccinated; *NT1*- narcolepsy type 1 (including 10 Pandemrix-induced NT1 samples).

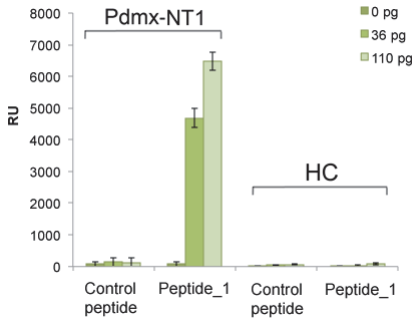

Figure S2. Peptide RVLAPALD reacts with Pdmx-NT1 sera in a concentration-dependent manner. In ELISPOT analysis, Peptide_1 (RVLAPALDSWGTGGGDYKDDD) and control peptide (AVLAAALASWGTGGGDYKDDD) were tested in three conditions: 0 pg; 36 pg and 110 pg per spot and using *Pdmx-NT1* and *HC* sera in 1:100 dilution. Abbreviations: *HC*- healthy control; *Pdmx-NT1*- Pandemrix-induced NT1 narcolepsy type 1.

Curriculum vitae

Personal data

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Education

2006–	Tallinn University of Technology, doctoral studies
2004–2006	Tallinn University of Technology, *MSc* in gene technology
2003–2004	University of Tartu, Diploma in biology teaching
2000–2004	University of Tartu, *BSc* in biology

Work experience

2006–	OÜ Protobios
2005–2006	The Competence Centre for Cancer Research
2005–2006	Tallinn University of Technology

Trainings

2019	Inter-sectorial mobility, prof. Makeyev's lab, Centre for Developmental Neurobiology, King's College London, UK
2019	4th SZTest week: Development of molecular tools for early detection of schizophrenia. Tallin-Tartu, Estonia
2018	EASME business innovation coaching, Dr. Stefan Blarer, DRIMARCH GmbH, Tallin, Estonia
2018	3rd SZTest mini-symposium: Gene expression in health and disease. Tallinn, Estonia, oral presentation
2018	International Conference: Microbiome Therapeutics. Amsterdam, Netherlands, poster presentation
2017	42nd FEBS Congress: From molecules to cells and back. Jerusalem, Israel, poster presentation
2017	2nd SZTest conference: Schisophrenia: Today's clinical need, emerging opportunities. Tallinn, Estonia
2017	H2020-MSCA-ITN PROFILE mini-symposium: From study design to technology marketing and large scale production of Biologicals. Tallinn, Estonia.
2015	International Conference: Cell therapy and tissue engineering: regenerative medicine - challenges and controversies. Tallinn, Estonia
2009	International Conference: Dermatological Advances 2009. London, UK
2009	EMBO bHLH transcription factors Workshop. London, UK, poster presentation

2008 The 18th annual biocity symposium: Genes, chromatin, and disease. Turku, Finland

2007 Summer school: Spetses Summer School: Nuclear receptor signalling: from molecular mechanisms to integrative physiology. Spetses, Greece, poster presentation

Academic thesis and industry training

Silvia D'Angelo, PhD student, industry training, 2017-2018, (sup) Helle Sadam, Kaia Palm, Immunoprofiling of anti-ADAMTS13 autoantibodies as a new type of biomarker in acquired TTP. "Profile" Marie Skłodowska-Curie, H2020-MSCA-ITN-2015, Protobios

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Mariann Moosar, Master's degree, 2011, (sup) Helle Sadam, Jekaterina Kazantseva, Studies of TFIIH basal transcription factor complex p34 subunit. The Institute of Gene Technology, Tallinn University of Technology

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* Equal contribution

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2004–2006	Tallinna Tehnikaülikool, magistri kraad (MSc, geenitehnoloogia)
2003–2004	Tartu Ülikool, bioloogia õpetaja diplom
2000–2004	Tartu Ülikool, bakalaureuse kraad (BSc, bioloogia)

Erialane töökogemus

2006–	OÜ Protobios
2005–2006	Vähiuuringute Tehnoloogia Arenduskeskus
2005–2006	Tallinna Tehnikaülikool

Kursused ja konverentsid

2019	Rahvusvaheline teadusvahetus prof. Makeyevi laboris, *Centre for Developmental Neurobiology, King's College London,* London, UK
2019	*4nd SZTest week: Development of molecular tools for early detection of schizophrenia*, Eesti
2018	EASME äriinnovatsiooni praktika, Dr. Stefan Blarer, DRIMARCH GmbH, Tallinn, Eesti
2018	*3nd SZTest mini-symposium: Gene expression in health and disease*. Tallinn, Eesti, suuline ettekanne
2018	Rahvusvaheline konverents: *Microbiome therapeutics*. Amsterdam, Netherlands, poster ettekanne
2017	*42nd FEBS Congress: From molecules to mells and back.* Jeruusalemm, Israel, poster ettekanne
2017	*2nd SZTest conference: Schisophrenia: Today's clinical need, emerging opportunities*. Tallinn, Eesti
2017	*H2020-MSCA-ITN PROFILE mini-symposium: From study sesign to sechnology sarketing and large-scale production of siologicals*. Tallinn, Eesti
2015	Rahvusvaheline konverents: *Cell therapy and tissue engineering: regenerative medicine - challenges and controversies*. Tallinn, Eesti
2009	Rahvusvaheline konverents: *Dermatological advances 2009*. London, UK

2009 *EMBO bHLH transcription factors workshop*. London, UK, poster ettekanne

2008 *The 18th annual biocity symposium*: Genes, chromatin, and disease. Turu, Soome

2007 Suvekool: *Spetses summer school on nuclear receptor rignalling: from molecular mechanisms to integrative physiology*. Spetses, Kreeka, poster ettekanne

Juhendatud lõputööd ja tööstuspraktikad

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