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The *p*HERV-W Envelope Protein and the Clinical Forms of Multiple Sclerosis

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Foreword

This thesis is based on manuscripts published during my Ph.D. and on unpublished data.

The results presented in this thesis have been obtained during my enrolment as a Ph.D. student at the Department of Biomedical Sciences, University of Sassari, Italy, in the period from December 1st, 2020, to February 29th, 2024.

The samples analyzed in the present study were collected from subjects referred to the Neurology Unit of the University of Cagliari, from the Blood Transfusion Center of Sassari, and from the Hospital Clinic San Carlos in Madrid, Spain.

Preface

The thesis submission is in line with the requirements for the PhD degree in Life Sciences and Biotechnologies. The research project work was carried out at Department of Biomedical Sciences, Viale San Pietro 43b, University of Sassari, Italy and at the "Laboratorio de Investigación de Esclerosis Múltiple" at the Hospital Clinico San Carlos sited in C./ Profesor Martín Lagos s/n, 28040 Madrid, Spain.

Abstract

The role of retroviral envelope proteins belonging to the Human Endogenous Retroviral family 'W' (HERV-W) as potential risk factors in multiple sclerosis (MS), has been establish. The transcripts of retroviral sequences that contain the envelope proteins from the HERV-W family, such as syncytin-1 and *p*HERV-W, were found in the MS patients. Studies have shown that the pathological variant of the HERV-W envelope (*p*HERV-W) is more strongly associated with MS compared to syncytin-1.

The first aim of this study was to evaluate the serological response to *p*HERV-W and syncytin-1-derived peptides in different clinical forms and inflammatory phases of MS, including remission, as well as progressive forms. In addition, the analysis included populations belonging to distinct geographic areas with different MS incidence rates and genome selective pressures. Subsequently, the incidence and the levels of humoral response against other viral factors already strongly associated with MS, as *Epstein-Barr virus* (EBV), *Human Herpesvirus* (HVV-6A/B) and *Cytomegalovirus* (CMV), were evaluated. Finally, the inflammatory response, through evaluation of cytokine production, was study in peripheral blood mononuclear cells (PBMCs) of MS patients and healthy controls following antigenic stimulation with *p*HERV-W and syncytin-1-derived epitopes.

The results showed significantly higher humoral response levels of anti-*p*HERV-W than anti-syncytin-1 in MS patients in both relapsed and progressive forms compared with HC. Individuals with acute and progressive forms of MS exhibited higher levels of antibodies to other viral infections, such as EBV and HVV-6A/B. Finally, *p*HERV-W exposure significantly increased the cytokine production response compared with syncytin-1 in MS PBMCs, supporting the idea of *p*HERV-W-involvement to the development of MS neuroinflammation. A strongly correlation was also found between the proinflammatory cytokine production from *p*HERV-W and other viral antibody titers in MS patients. Based on the results, both EBV, HHV and CMV could act in the pathogenesis of MS by regulating the proinflammatory action of *p*HERV-W.

In conclusion, the evaluation of the *p*HERV-W envelope protein could be a useful pathogenic biomarker for monitoring the development and progression of central nervous system inflammation.

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Abbreviations

AMS = Acute multiple sclerosis
AUC-ROC = “Area Under the Curve” of the “Receiver Operating Characteristic”
BBB = blood brain barrier
BFA = brefeldin A
CIS = clinically isolated syndrome
CMV = *Cytomegalovirus*
CNS = central nervous system
CSF = cerebrospinal fluid
CV = coefficient variation
DMSO= Dimethyl Sulfoxide
EBV = *Epstein-Barr virus*
EDSS = Expanded Disability Status Scale
ELISA = enzyme linked immune-sorbent assay
ERV = endogenous retrovirus
FBS = fetal bovine serum
FSC-A = forward scatter area
GM-CSF = Granulocyte-macrophage colony-stimulating factor
HC = healthy control
HERV = Human endogenous retrovirus
HLA = Human Leucocyte Antigen
HVV *= Human Herpesviruses*
ICAM = intracellular adhesion molecules
ICS = Intracellular Cytokine Staining
IEF = isoelectric focusing
IFN = Interfereon
IL-17 = Interleukin 17
IQR = Interquartile Range
LTR = long terminal repeat
MRI = Magnetic Resonance imaging
MS = Multiple sclerosis
MSRV = Multiple Sclerosis Associated Retrovirus
NFDM = Nonfat dry milk
O.R.= Odd Ratio
OCB = oligoclonal band
OPC = oligodendroglial cells
PBMC = peripheral blood mononuclear cell
PBS = Phosphate buffered saline
PC = Principal component
PCA = principal component analysis
PP = primary progressive
PR = progressive relapsing

RR = relapsing remitting
SCC-A = Side Scatter Area
SCC-H = Side Scatter height
SMS = stable multiple sclerosis
SP = secondary progressive
SU = surface subunit
TBS = Tris-Buffered Saline
TLR = toll-like receptor
TM = transmembrane subunit
TNF = Tumor necrosis factor
VEP = visual evoked potentials

INTRODUCTION

Multiple Sclerosis

Pathophysiology

Multiple sclerosis (MS) is an inflammatory chronic condition that affects the central nervous system (CNS), leading to inflammation and demyelination, ultimately resulting in damage to the neurons[1]. This chronic Inflammation can affect various parts of the CNS, including the *corpus callosum*, the optic nerve, and the brainstem, and can also damage axons responsible for carrying action potentials[1]. Therefore, MS can manifest in different ways depending on the affected neuronal population. This condition is regarded as a disease of a thousand faces due to its heterogeneity. In the initial stages of MS, an abnormal immune response occurs in the periphery, targeting the CNS. Flare-ups, characterized by episodes of new or worsening symptoms, include focal demyelination and inflammation. The interaction between the immune system, glia (astrocytes, microglia and oligodendrocytes) and neurons contribute to the disease's two primary pathological components: neurodegeneration and inflammation[2]. During the flare-up the neuroaxonal deterioration usually occurs in proportion to the severity of the inflammation. Following the resolution of the inflammation, factors such as neuronal plasticity and axon repair can help in restoring nerve conduction[3]. While remyelination and repair can occur, the extent of the repair process may be limited[4,5], with lesions potentially visible through magnetic resonance imaging (MRI).

Epidemiology and Prevalence

It is estimated that around 2.8 million people worldwide are affected by MS. Although it can manifest at any age, it is most commonly diagnosed in people between the ages of 20 and 50 [6,7]. The prevalence of MS is higher in women than in men (fig. 1). Usually, women with the disease tend to experience a more benign course, whereas men often experience more severe symptoms[8].

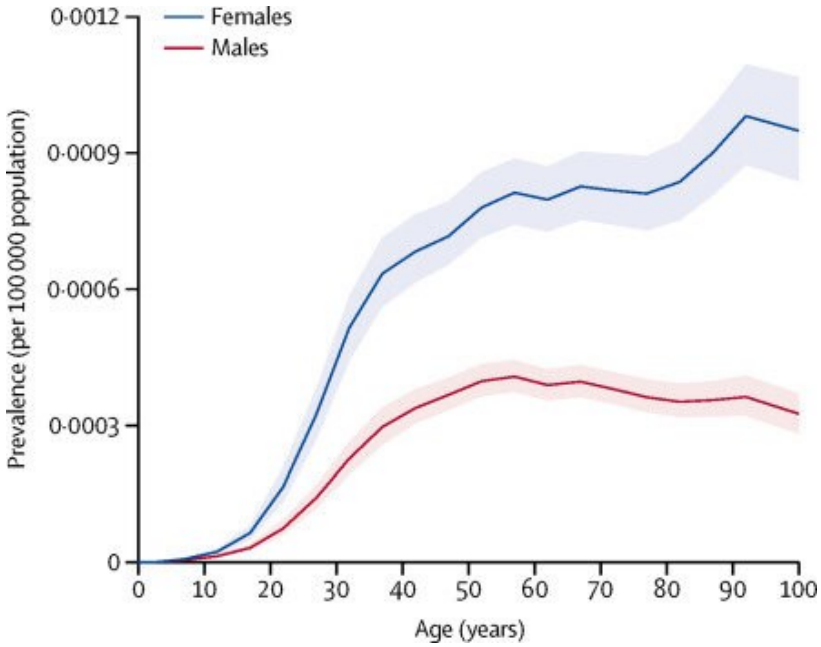


Figure 1.

Age-standardized prevalence of multiple sclerosis by age and sex[9].

Studies have shown a correlation between the prevalence of MS and geographical latitude (fig. 2). For instance, people living near the poles are at a higher risk of developing the disease. Countries with the highest prevalence include Australia, North America, and Western Europe, while those near the equator, such as countries in Africa, Asia and South America, have a lower frequency of MS[10]. This latitudinal distribution gradient is attributed to various factors, including migration, genetic insulation, and differences in life expectancy[11,12,13]. For instance, in Spain, the prevalence of MS ranges from 101 to 200 cases per 100.000 inhabitants. Placing it in the category of regions with medium to high prevalence. In Italy, the estimated number of cases of MS exceeds 200 per 100.000 residents, classifying it as a high prevalence area[6].

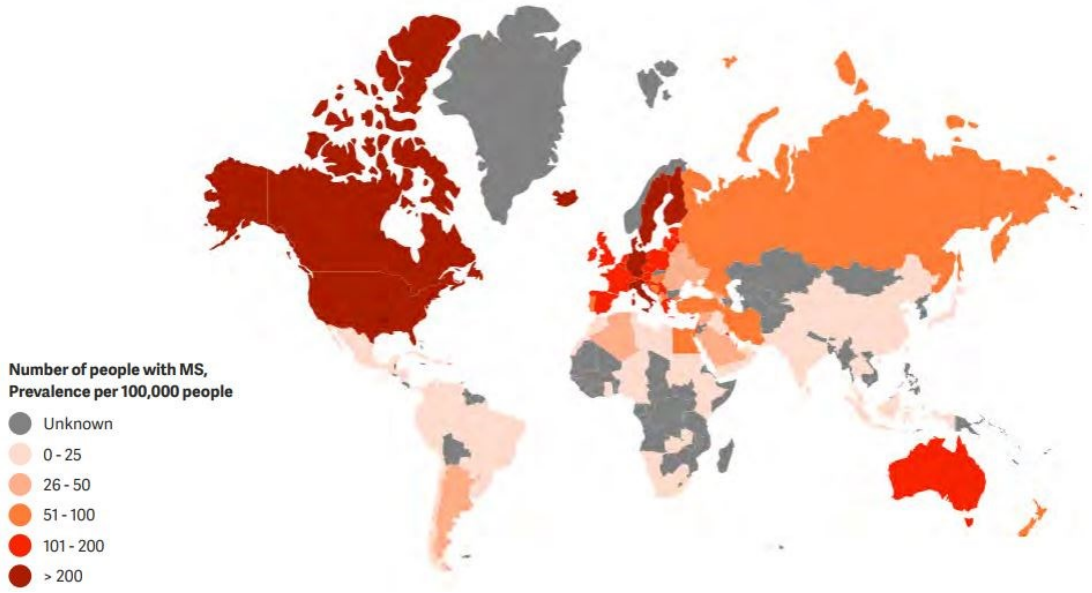


Figure 2.
Prevalence of MS cases per 100,000 people in the world[6]

Classification and Clinical Forms

MS is typically described as a type of disease which provides a comprehensive description of its course but unfortunately it is a complex disease that can't be easily categorized. In 2014, Lublin updated the classification of the clinical forms by grouping them based on their relapses and progression of disability over time (fig. 3A/B)[14]. About 85% of MS patients experience an acute flare-up of their neurological function, characterized by either complete recovery or residual sequelae that may recur at regular intervals. This phenotype includes clinically isolated syndrome (CIS)[15]. Depending on CIS activity (presence of clinical exacerbation or active lesions in the CNS), it can be classified as active or inactive. CIS can progress to relapsing remitting (RR) MS, with occurs in about 80% of cases. RRMS is characterized by relapses of symptoms (acute phase, AMS) followed by periods of remission (stable phase, SMS)[14].

Primary progressive (PP) MS is the least common form occurring in 10-15% of cases at onset) and involves a progressive accumulation of disability from the outset[14]. Secondary progressive (SP) MS occurs when disability worsen following a flare-up, with approximately 50% of individuals with RR-MS transitioning to SP-MS within a decade[16].

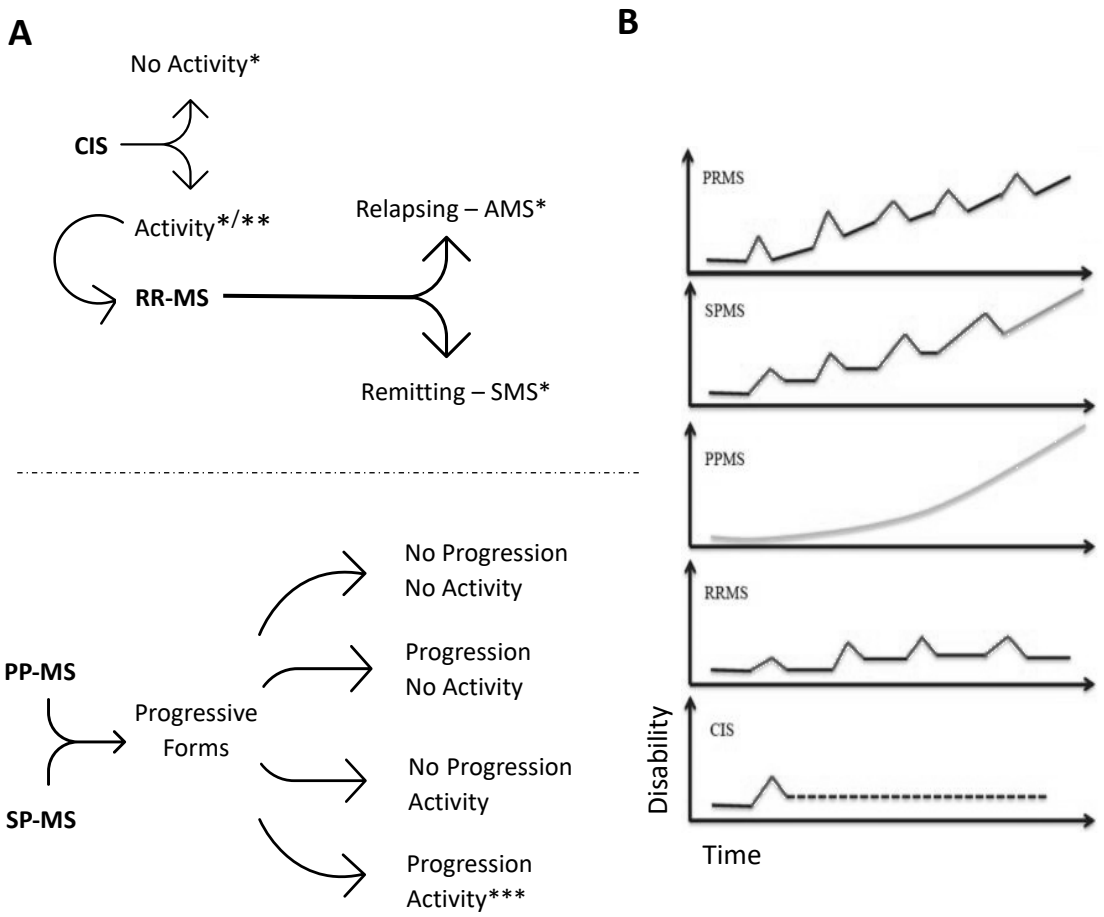


Figure 3.
(A) MS phenotypes according to the 2014 Lublin classification[14]. (*: activity determined annually by clinical flare-up or MRI; **: if clinically active and meets MS criteria, becomes RR-MS; ***: progression determined by annual clinical evaluation. (B) The different sub-types of MS characterized by their increasing severity as the disability progresses as time progresses.

About 5% of people with MS will develop a progressive-relapsing condition known as PR-MS, characterized by regular relapses and remissions, sometimes also referred to as PP-MS. Although there may be a recovery periods following a relapse, the symptoms of this condition continue to worsen, leading to increased disability over time due to incomplete recovery from the flare-ups[16].

In the progressive stages of the disease, the neurodegenerative component became predominant while the inflammatory component diminishes. Demyelination, atrophy, and axonal loss occur independent of inflammation. The volume of brain tissue decreases, and the progressive neurological deterioration leads to the destruction of axons. Although specific immune cells are not present in lesions, diffuse infiltrations of microglia, astrocytes, and lymphocytes can be seen in areas where immune cells are known to infiltrate. Myelin-specific T-cells, activated in the periphery, are attracted to the blood brain barrier (BBB) by a chemokine gradient[17]. In addition, secondary lymphoid structures located in the meninges are related to tissue damage and cortical demyelination. Failure to recover from the initial inflammation can result in progressive deterioration of the condition. Chronic inflammation may trigger different factors such as protein folding breakdown, oxidative stress, and fatigue[18].

Predicting prognosis and Diagnostic criteria

Currently, there is no pathognomonic diagnostic test or clinical feature for MS. Instead, a differential diagnosis can be made based on the results of paraclinical studies using Magnetic Resonance brain imaging (MRI)[19], cerebrospinal fluid (CSF) analysis, and visual evoked potentials (VEP)[20].

MRI allows researchers to identify demyelinated lesions and determine their spatial distribution and appearance over time (fig. 4A). In MS, MR images typically reveals hypointense lesions in T1 and hyperintense lesions in the white matter in T2, often located in the infratentorial, periventricular, and juxtacortical regions. Spinal cord lesions are also present around 80-90% of the subjects with MS[4].

An increase in the concentration of IgG and other immunoglobulins can be observed in the CSF (fig. 4B). The presence of isoelectrofocus oligoclonal bands suggests intrathecal immunoglobulin production, which persists throughout the course of the disease and serves as a biomarker for inflammatory disorders[21].

VEP is a neurophysiological test that assesses the electrical conductivity of the CNS after sensory organ stimulation (fig. 4C). Demyelination of fibers can result in delayed or blocked evoked potentials, providing additional evidence of CNS lesions[22].

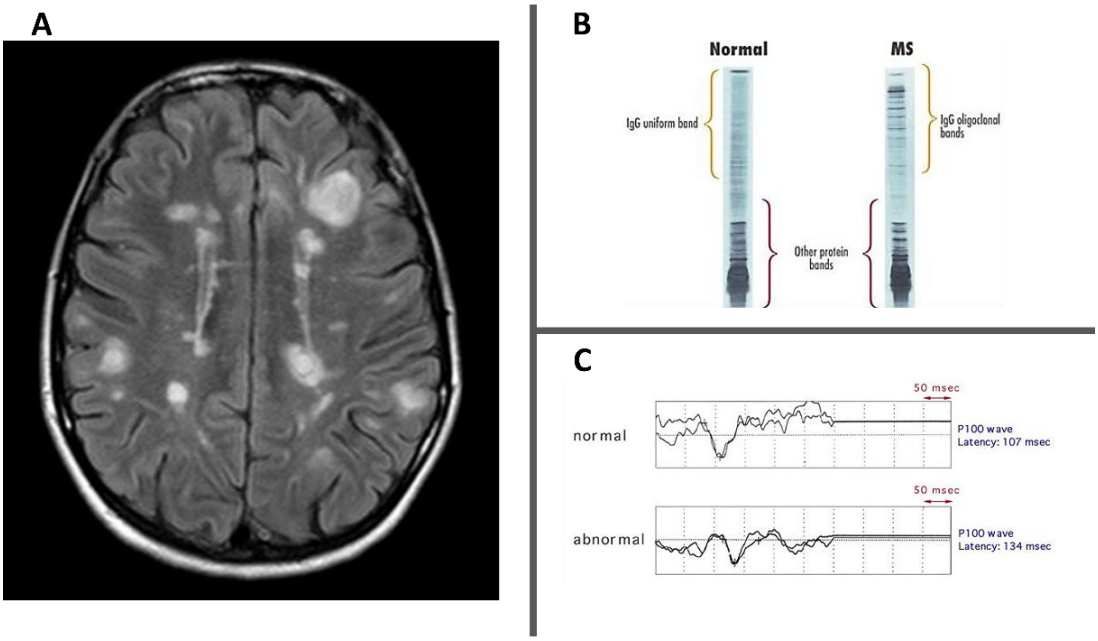


Figure 4.

Investigations for the diagnosis of MS by different paraclinical studies. (A) MRI scan of the brain in a patient with multiple sclerosis showing typical lesions. (B) IgG oligoclonal band (OCB) profiles obtained with isoelectric focusing (IEF) and antigen-specific immunoblotting, respectively, in paired CSF in healthy control (normal) and RR-MS patient (MS). (C) Response of the evoked potential by VEP in healthy control (normal) and patient with demyelination in CNS (abnormal).

The Expanded Disability Status Scale (EDSS) is a widely used scale that measures disability for people suffering from MS. It categorizes the various aspects of the condition by estimating the impairments in multiple functional systems. The scale measures the various types of impairments in the eight neurological functional systems of the CNS. These include the brainstem, pyramidal, visual, sensory, bowel and bladder and other. Mobility and ambulation abilities are also taken into account to calculate the disability level[23]. The EDSS scale ranges from 0 (Normal neurological exam) to 10 (death due to MS). Patients with a score below 3.5 typically experience mild symptoms, while those with a score of 4 or higher are considered to have a gait impairment[24]. As the condition progresses, the EDSS level increases, reflecting worsening disability.

Etiology

Although MS has been recognized for a hundred years, its exact etiology remains elusive. The varying frequency of MS across different populations caused by migration and geographic gradients, along with the lack of a monozygotic twin's concordance, suggests that environmental factors may play a role in its development. However, evidence also supports a significant role for the interplay of epigenetic and genetic factors in MS pathogenesis[25].

While MS is not considered a heritable condition, relatives of patients have a 2-4% higher chance of developing the disease than unrelated individuals. Concordance rates in both monozygotic and dizygotic twins indicate a genetic predisposition[26], with certain alleles of the HLA class II *loci,* particularly DQ and DR, being responsible for about 10-60% of the risk of MS[27].

HLA class II molecules are involved in antigens presentation to lymphocytes[28] and changes in these genes could induce an abnormal inflammatory response targeting CNS antigens, resulting in demyelination[29]. However, the DR-15 haplotype, while associated with increased MS risk, does not fully explain the genetic component of the disease[29], suggesting involvement of other genetic factors outside this region[30].

In addition to genetic predisposition, various environmental factors may trigger or contribute to MS development. These include exposure to ultraviolet light, vitamin D deficiency, viral infections, and gut microbiota exposure[31,32,33]. Certain diet-related factors and lifestyle

habits, such as tobacco use, could also influence disease risk[34,35]. Several studies have linked MS to bacterial such as *Chlamydia pneumoniae*[36], *Helicobacter pylori*[37] and *Mycobacterium avium subsp. paratuberculosis*[38].

Among the most common viral infectious agents that may be related to the MS onset and progression are the *Epstein-Barr virus* (EBV), *Human herpesviruses-6A* and *6B* (HHV-6A/B) and *Cytomegalovirus* (CMV)[39,40,41]. These viruses can establish persistent infections in the host, potentially triggering immune-mediated damage to the CNS.

- It has been established that people who have been infected with the EBV virus are more prone to developing MS than those who have not. Serum levels of neurofilaments, a biomarker of neuroaxonal damage, have been found to increase following EBV infection[42].
- Two types of human herpesvirus, HHV-6A and HHV-6B, have been identified. While they share homology, they have varying clinical, immunological, and epidemiological characteristics[43]. Studies have shown that HHV-6A may possess a higher necrotrophic aggressiveness than other types of HVV[44].
- The relationship between CMV and MS remains controversial[41]. Several studies have revealed that individuals with a positive CMV status may have a lower risk of developing MS and may exhibit fewer T2-lesions on diagnostic scans [45]. The protective role for CMV infection in MS etiology is further supported by interactions between CMV serum negativity and EBV and HHV-6A seropositivity [41,46,47].
- Recent findings propose that Human endogenous retrovirus (HERVs) may act as risk factor in MS progression[48].

Human Endogenous Retroviruses

Classification and Structure

About 8% of the human genome is made up of endogenous retroviruses, which are remnants of ancient viral infections[49]. Among these, Human Endogenous Retroviruses (HERVs) are a subset. While most HERVs are defective and unable to produce viral particles, some have evolved into functional and physiological retroviruses over time [50].

Retrovirus classification lacks uniform standards, but frequently used *criteria* include the amino acid sequence that carries the transfer RNA, which serves as a primer for the retro transcription process. This gives rise to classification such as HERV-H, -R, -W and -K[51].

HERVs have a typical proviral structure consisting of four basic structural genes (*gag*, *pro*, *pol* and *env*) flanked by two long terminal repeats (LTRs) regions of about 780 base pairs (bp) each[52]. While complete proviral sequence can be found in the genome, some HERVs may have partial or truncated proviral structures with missing LTR regions, often due to loss of internal regions during the homologous recombination [53,54].

Pathological condition

HERVs have been implicated in various pathological conditions, including autoimmune disorders, cancer, and neurological ailments. Reactivation of HERVs sequences can trigger targeted immunological responses implicated in cancer development, such as breast, ovarian, and prostate cancer[55,56]. Factors regulating HERVs levels in the CNS include insertional and sequence variations of retroviruses, environmental triggers, and epigenetic modifications [57].

Activation of the innate immune response by HERVs can contribute to the development of neuroinflammation and damage the nerve cells, while also enhancing the adaptive immune response. HERVs can trigger the development of neuropathogenic responses by interacting with viral elements, as EBV, HVV-6 and CMV[57,58].

In MS, several retroviral families, including HERV-W, HERV-Fc1, HERV-K18 and HERV-H, have been associated with the development and progression of the condition[59].

Among these, the HERV-W gene family has received significant attention due to its connection with MS.

The structure of HERV W is composed of the *gag* gene of 1,458 nt long and gives rise to a putative polyprotein of 486 aa with two glycosylation sites. The *pol* gene is 1,194 nt long and gives rise to a protein whose deduced sequence is 398 aa. Finally, the *env* gene is 1,626 nt long and its predicted protein has 542 aa[60]. The env protein is composed of a surface subunit (SU) and a transmembrane (TM) subunit, which are respectively 290 and 252 aa (fig. 5A). The former has a potential receptor function, and the latter has a fusogenic function[61]. The cleavage site between SU and TM subunits is characterized by a polybasic motif. Although both subunits are heavily exposed to the immune response, the TM is relatively inactive[61].

Two major members of the HERV-W family are ERVWE1 and MSRV. ERVWE1, located on chromosome 7.q21.2, carries mutations in the *pol* and *gag* genes, preventing viral particles production[57,62,63]. MSRV, on the other hand, can generate complete viral particles and possesses activity in retro-transcriptase, with a genome containing env, gag, pol, and LTR sequences[49,62,63]. The origin of MSRV in the human genome remains unclear.

Syncytin-1

Syncytin-1, encoded by the ERVWE1 gene, is the first protein identified from a HERV in humans that has physiological function. It plays a role in the development and formation of the placenta syncytiotrophoblast. A study have discovered complete env sections of HERV-W on 13 chromosomes, with 10 of them having complete env sequences: chromosomes 3, 4, 5, 7, 12, 14, 15, 17, 18 and X[64].Other proteins derived from the env sequences of the HERV-W retrovirus family are often highly defective, indicating mutations affecting their functional regions, likely due to the influence of syncytin-1 mutations[65].

Research has shown that the env protein sequence of syncytin-1 and MSRV share a 94% homology. Although several single nucleotide polymorphisms (SNPs) exist throughout their sequences, the major difference lies in MSRV having an additional 12 nucleotides than ERVWE1, that encoding the addition of four amino acids (His–Val–Leu–Gin) in the TM segment (fig. 5A/B).

Despite these differences, the high homology rate between syncytin-1 and MSRV explains their similar properties, including their ability to alter systems or activate neurotoxicity and immune responses[60,66].

The main difference between the two components is that the *p*HERV-W env is present extracellular, while the syncytin-1 component only appears in the plasma membrane or cytosol[60,66]. Extracellular sequences of the MSRV protein have been detected in the spinal fluid and blood of MS patients, suggesting the potential involvement in the progression of the disease[60].

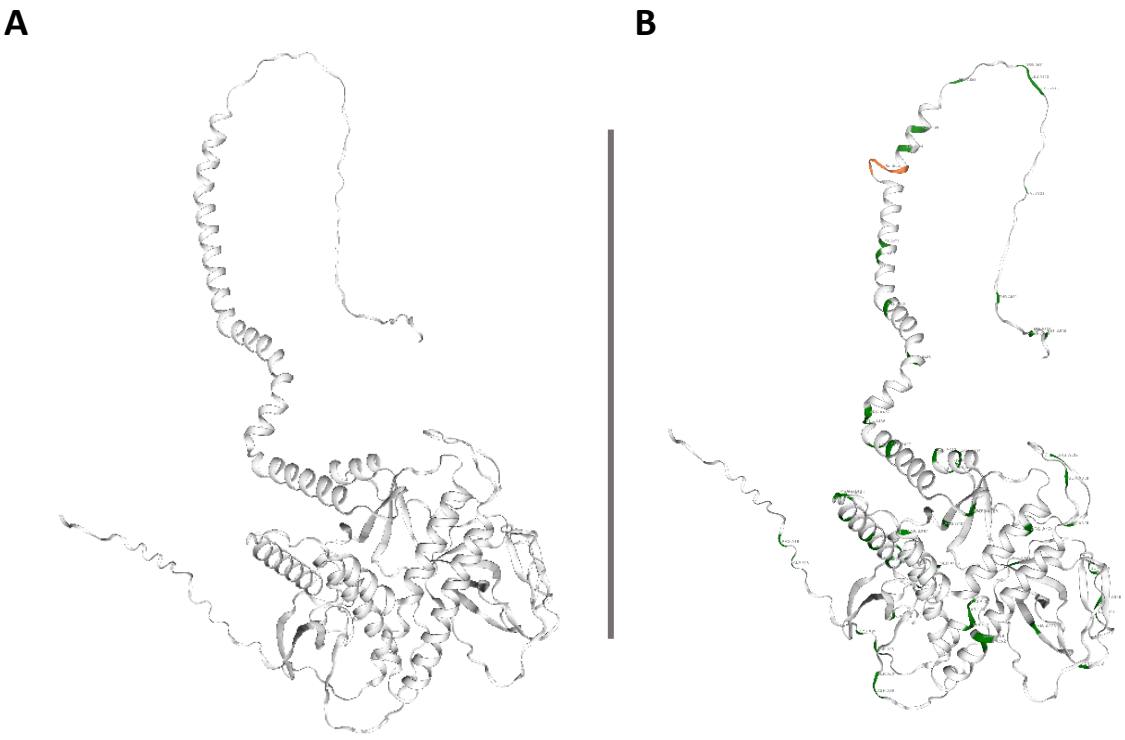


Figure 5.
Templates of AlphaFold DB protein model of (A) Syncytin-1 and (B) MSRV/*p*HERV-W. The orange color marks the location of the 4 additional amino acids (His-Val-Leu-Gin) in the *p*HERV-W while the green the amino acid substitutions than Syncytin-1.

MSRV/*p*HERV-W

Since the discovery of the retroviral particles of MSRV in 1989, various studies have suggested that the encoded env protein could play a role in the development of MS [67], hence the name MSRV, which stands for MS-associated retrovirus. Several studied reported the presence of the MSRV env protein in areas of the brain actively undergoing demyelination, suggesting a link between the disease and long-term systemic pathology. Additionally, studies have shown that MSRV can promote experimental autoimmune encephalomyelitis (EAE) in mouse and induce elevated autoimmune T cell reactivity[68,69].

Conserved pathogenic env sequences of the MSRV envelope are collectively referred to as *p*HERV-W env. The *p*HERV-W env component of MSRV can activate the body's innate immune system by interacting with toll-like receptor (TLR) 4 and CD14 coreceptors, both involved in the pathogenesis of MS (fig. 6A/B)[60,66,70,71]. This activation leads to the generation and release of proinflammatory cytokines such as TNF-α, IL-12 and IFN-γ, triggering a positive feedback that exacerbate the immune response (fig. 6B)[72]. Treatment of primary oligodendroglial cells (OPCs) with *p*HERV-W env protein significantly decreases their differentiation by interacting with TRL4, potentially contributing to the failure of the myelin repair process in adult CNS (fig. 6C). Furthermore, *p*HERV-W induces overexpression of intracellular adhesion molecules (ICAM) in endothelial cells, such as ICAM-1, leading to increased recruitment and extravasation of T lymphocytes into the CNS[72].

Studies have also noted higher expression levels of the MSRV and viral load of *p*HERV-W env in the brains and blood of MS patients. It has also been shown that the presence of these proteins can be used as a conversion markers and prognostic indicators for disease severity. Patients with higher expression of these proteins in the early stages of the disease may have a worse prognosis in term of disability, response to treatments and progression to progressive phases of the illness[48,60,64,66,74,75].

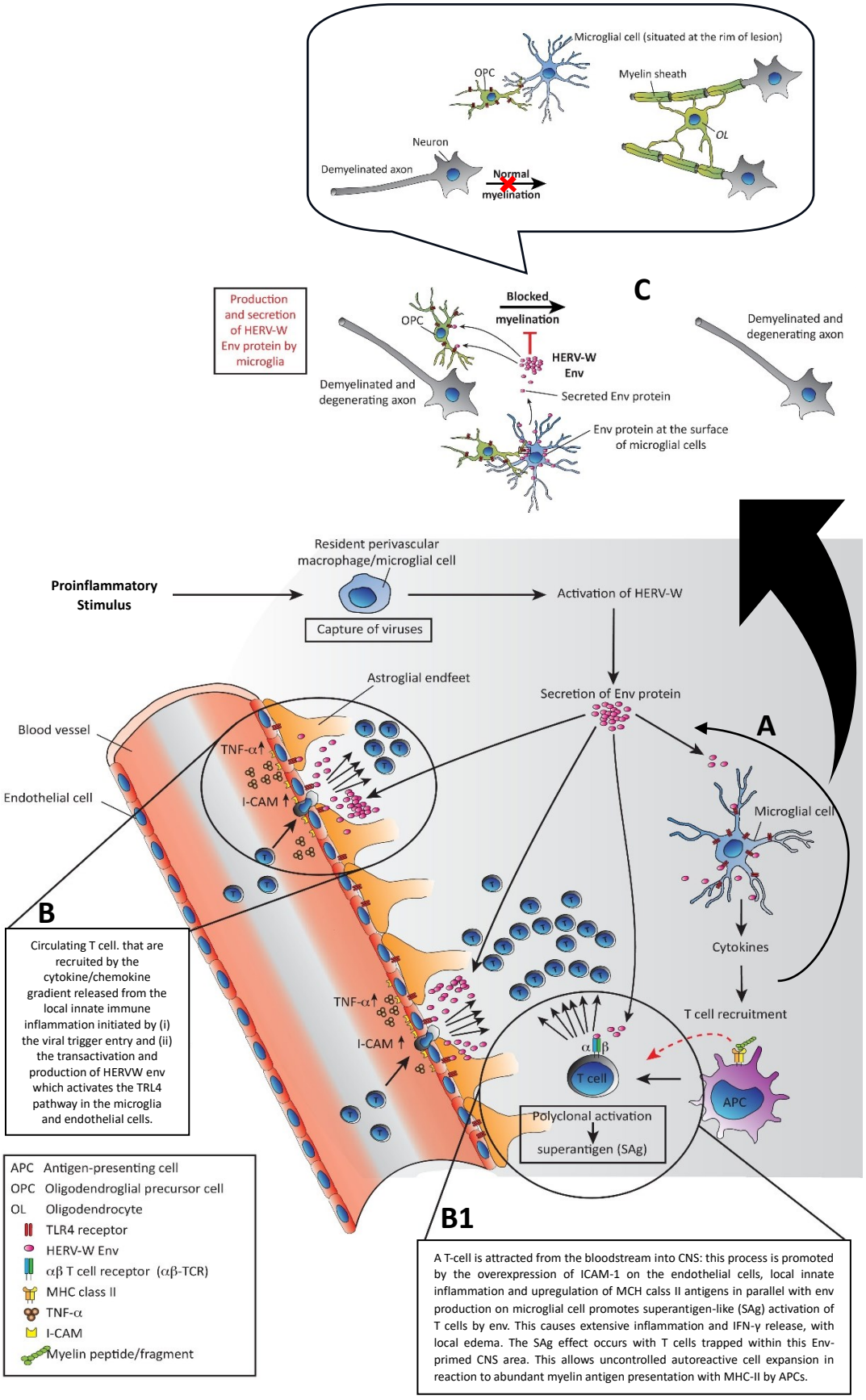

Microglial cell (situated at the rim of lesion)
OPC
Myelin sheath
OL
Neuron
Demyelinated axon
Normal myelination
Production and secretion of HERV-W Env protein by microglia
Blocked myelination
C
OPC
HERV-W Env
Demyelinated and degenerating axon
Secreted Env protein
Env protein at the surface of microglial cells
Demyelinated and degenerating axon
Proinflammatory Stimulus
Resident perivascular macrophage/microglial cell
Capture of viruses
Activation of HERV-W
Secretion of Env protein
Astroglial endfeet
Blood vessel
Endothelial cell
TNF-α
I-CAM
A
Microglial cell
Cytokines
T cell recruitment
B
Circulating T cell. that are recruited by the cytokine/chemokine gradient released from the local innate immune inflammation initiated by (i) the viral trigger entry and (ii) the transactivation and production of HERVW env which activates the TRL4 pathway in the microglia and endothelial cells.
TNF-α
I-CAM
αβ
T cell
APC
Polyclonal activation
superantigen (SAg)
APC Antigen-presenting cell
OPC Oligodendroglial precursor cell
OL Oligodendrocyte
TLR4 receptor
HERV-W Env
αβ T cell receptor (αβ-TCR)
MHC class II
TNF-α
I-CAM
Myelin peptide/fragment
B1
A T-cell is attracted from the bloodstream into CNS: this process is promoted by the overexpression of ICAM-1 on the endothelial cells, local innate inflammation and upregulation of MCH calss II antigens in parallel with env production on microglial cell promotes superantigen-like (SAg) activation of T cells by env. This causes extensive inflammation and IFN-γ release, with local edema. The SAg effect occurs with T cells trapped within this Env-primed CNS area. This allows uncontrolled autoreactive cell expansion in reaction to abundant myelin antigen presentation with MHC-II by APCs.

Figure 6.

The proposed pathways by which *p*HERV-W env can affect the MS development. (A) The release of pro-inflammatory cytokines triggered by a pro-inflammatory *stimulus* can generate a feedback loop between the activation, synthesis and release of *p*HERV-W. (B/B1) The cytotoxic damage to the epithelial cells could also contribute to the recruitment of T lymphocytes by overexpression of ICAM-1, as a response to the release of chemokines and cytokines following TRL4 activation by *p*HERV-W. (C) The presence of *p*HERV-W env proteins in the membranes of microglia cells can prevent the differentiation of these cells. This prevents the remyelination process, which usually happens under normal conditions[72].

HYPOTHESES

The presence of envelope proteins from the HERV-W gene has been implicated in the development of MS. Studies have shown that the pathological variant of the envelope (*p*HERV-W) is more strongly associated with MS compared to Syncytin-1. Furthermore, expression levels of the *p*HERV-W *env* gene (MSRV) are higher in patients with progressive MS than in those with relapsing MS[76].

The theory behind this study is that MS patients may exhibit higher serological titers for *p*HERV-W compared to syncytin-1. Consequently, the env antigen of *p*HERV-W may elicit a major immunological response than syncytin-1, potentially exacerbating the inflammatory reaction in MS patients.

One proposed mechanism for the role of certain viral components, such as CMV, EBV, and HHV-6A, in MS etiopathogenesis is their ability to transactivate HERV sequences. A further hypothesis suggested that the level of serological responses to EBV, CMV, and HHV could modulate the immune system's response to *p*HERV-W exposure, potentially influencing MS severity.

OBJECTIVES

The objectives of this investigation are outlined as follows:

- Analyze and compare the humoral response to *p*HERV-W $env_{486-504}$ and Syncytin-1 $env_{486-500}$ in a cohort of healthy controls and MS patients in different disease phases, including remission (RR), both stable and acute, as well as progressive phases (PP-MS and SP-MS). This analysis will include two distinct geographic areas with different MS incidence rates and genome selective pressures.

- Investigate, through a cross-sectional study, the potential correlation between serological viral titers of CMV, HHV-6A/B and EBV and the levels of autoantibody detected against the envelope protein portions of *p*HERV-W $env_{486-504}$ and Syncytin-1 $env_{486-500}$ in RR-, PP- and SP-MS patients.

- Examine the inflammatory response by evaluation of cytokine production, including tumor necrosis factor alpha (TNFα), interleukin-17 (IL-17), interferon-gamma (IFNγ), and granulocyte-macrophage colony-stimulating factor (GM-CSF), in peripheral blood mononuclear cells (PBMCs) of RR-MS, PP-MS, and healthy controls following antigenic stimulation with *p*HERV-W $env_{486-504}$ and Syncytin-1 $env_{486-500}$.

MATERIALS and METHODS

Population Study

Research was performed on two MS and healthy controls (HC) populations of different ethnicity. On the first study group was conducted a cross-sectional study including samples of patients diagnosed with RR, -SMS and -AMS[77], and HCs from Sardinia, a small geographically isolated community of Italy.

The second study group, which included individuals of Hispanic descent, is composed of HCs and MS patients in both relapsing and progressive forms, according to the Mc Donald criteria[77], recruited from the Hospital Clínico San Carlos in Madrid, Spain.

All the study's participants agreed to participate by filling out the required consent forms, previously approved by the Clinical Research Ethics Committee. Participants' demographic, clinical, and radiological data obtained from review of medical records and/or information collected at the time of accepting inclusion in the study were considered. The Table 1 summarizes the demographic and clinical characteristics of the study's participants.

Table 1.

Demographic and Clinical data of MS patients

Parameter	Population	Values			
		RR-SMS	RR-AMS	PP-MS	SP-MS
No.	Sardinia (Italy)	36	46	---	---
	Madrid (Spain)	66	62	61	52
Sex (no. of men/women)	Sardinia (Italy)	12/24	11/35	---	---
	Madrid (Spain)	20/46	15/47	31/30	26/26
Age (yr) [median ± SD]	Sardinia (Italy)	56 ± 13	49 ± 9	---	---
	Madrid (Spain)	48 ± 9	50 ± 8	57 ± 10	59 ± 8
EDSS † [median (range)]	Sardinia (Italy)	4.3 (1-7.5)	1.5(0-5.5)	---	---
	Madrid (Spain)	2.15 (0-7.9)	2.5 (0-5.5)	5 (0 ± 8.5)	5.3 (2.5-8.5)
Age (yr) at MS onset [median ± SD]	Sardinia (Italy)	38 ± 11	33 ± 8	---	---
	Madrid (Spain)	30 ± 9	28 ± 8	39 ±11	33 ± 10

Blood Processing

20 ml of peripheral blood was collected from each participant: 10 ml in dry tubes for serum collection, and another 10 ml in CPTTM tubes (Cell Preparation Tube; Becton Dickinson,

Franklin Lakes, NJ, USA) to obtain PBMC. Serum was used for the different determinations of antiviral antibody titer by ELISA assay (fig. 7A). Serum was isolated by centrifugation at 900 g for 15 min at room temperature (RT). It was stored at -80°C until analysis. Samples collected in CPTTM tubes, from which PBMC were isolated, were used to perform flow cytometry experiments for evaluation of cytokine production after viral epitope exposure (fig. 7B). CPTTM tubes allow density gradient separation by Ficoll-Paque™. The tubes were centrifuged (900 g, 30 min, RT) to induce differential migration that yielded different layers corresponding to the distinct cell types, enabling the PBMC isolation. After PBMCs isolation, they were washed with saline buffer and then subjected to differential centrifugation (400 g, 10 min, RT), discarding the supernatant.

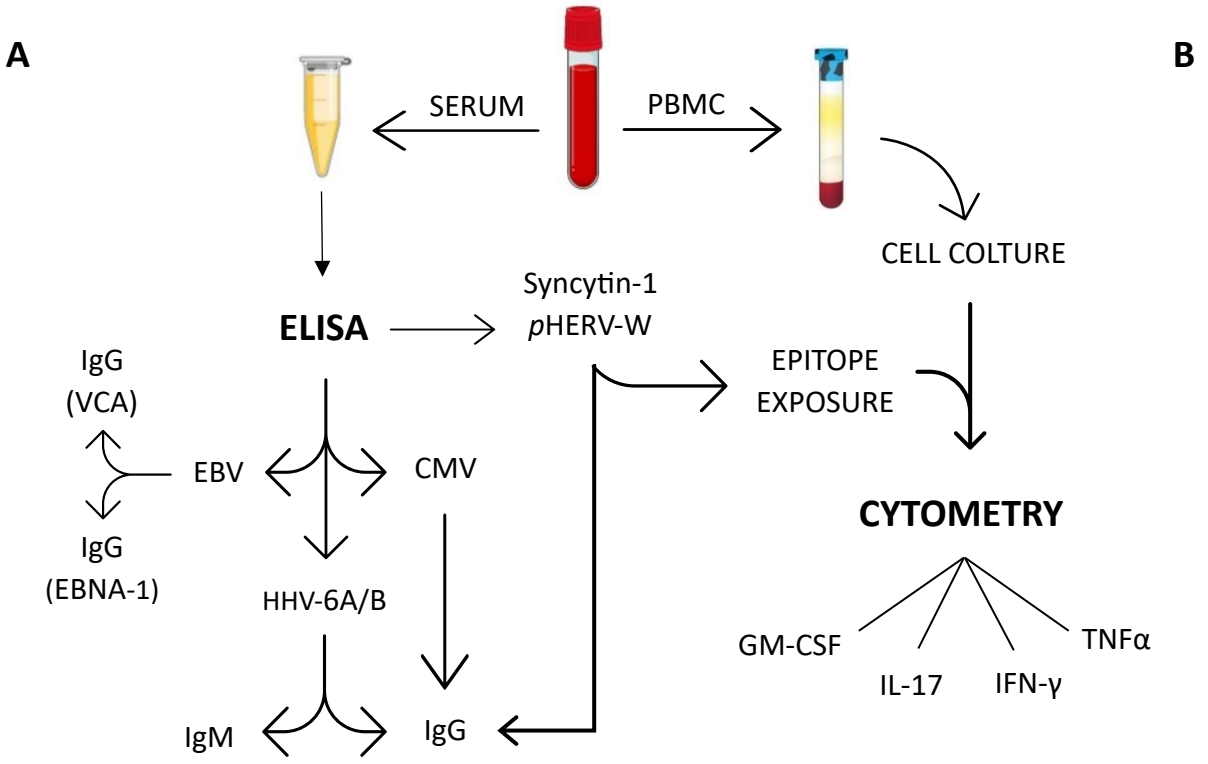


Figure 7.
Experimental design. (A) Separation of serum from peripheral blood for detection of antiviral antibody titers by ELISA assay. (B) PBMCs isolation with subsequent exposure to viral epitopes (*p*HERV-W/Syncytin-1) for detection of proinflammatory cytokines.

The pellet was then resuspended in a mixture of 10% DMSO and 1 ml of PBS. The cells were temporarily stored at -80° in a MrFrostie container before immersion in liquid nitrogen, to avoid drastic temperature change. The cryovials were then placed in a tank containing liquid nitrogen (-196°C) until they were analyzed.

Peptide design

The peptides were designed using the Immune Epitope Database (IEDB) and synthesized with high purity at >95% by LifeTein (South Plainfield, NJ, USA). The peptides were dissolved in DMSO, at the contraction of 10 mM, and stored at -80°C.

In Table 2, the two peptide of envelope protein portions of the Syncytin-1 (UniProt accession no. Q9UQF0) and recombinant protein *p*HERV-W (UniProt accession no. Q9UQF0) are reported.

Table 2.
Amino acid sequence data of peptide epitopes

Protein	**Syncytin-1**	***p*HERV-W**
Epitopes ID	Syncytin-$1_{env\ 486–500}$	*p*HERV-$W_{env\ 486–504}$
Epitope location in env Protein		
Epitope aa portion	486-500	586-504
Epitope sequence	QMEPKMQSKTKIYRR	QIVLQMEPQMQSMTKIYRG

In particular, the regions that fall under this section are located on the portion of the pathological form (*p*HERV-W) that contains four additional amino acids (aa), as evidenced by the section "*Epitope location in env protein*" in table 2.

Indirect Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA technique to detect antibodies against the epitopes of the Syncytin-$1_{env\ 486–500}$ and *p*HERV-$W_{env\ 486–504}$ was performed. The 96-well Nunc immune plates containing a solution of 0.05 M carbonate and bicarbonate in H_2O with pH 9.5, and peptides at 10 ug/mL were incubated at 4 degrees overnight. A blocking solution with 5% nonfat dry milk (NFDM) (Sigma-Aldrich, St. Louis, MO, USA) at RT in Tris-Buffered Saline (TBS) was then added to each well. The plates were washed twice with a solution of TBS and 0.05% Tween-20 (TBS-T), and the serum samples were added in 1:10 concentration and incubated for two hours at RT. Afterwards, the plates were washed in TBS-T and incubated one hour at RT with a solution containing Anti-Human IgG (Fc specific) Alkaline Phosphatase antibody (1:1000, Sigma-Aldrich, St. Louis, MI, USA) dissolved in TBS with 5% NFDM. Next one-hour incubation, the plates were washed in TBS-T, then added p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MI, USA) in milli-Q water and placed at RT in the dark for 30 min. The samples were analyzed in triplicate, and their reactivity was assessed by optical density at 405 nm using SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A negative and positive control sera was then added to confirm the validity of the procedure.

Competitive Assay

Competitive assay was carried out by placing the sera at 4°C with saturating equimolar concentrations (10 µM) of Syncytin-$1_{env\ 486–500}$ or *p*HERV-$W_{env\ 486–504}$ and a scramble peptide. In particular, four sera of RR-MS patients (two SMS and two AMS) and one HC serum, all *p*HERV-$W_{env\ 486–504}$ positive, were incubated. A sero-negative peptides was used as negative controls. Sera were then subjected to ELISA assay on plates coated with *p*HERV-$W_{env\ 486–504}$.

Herpes Virus, Epstein-Barr and Cytomegalovirus by automated ELISA

Serum samples were analyzed using the following commercial tests: anti-EBNA-1 and anti-VCA IgG (Trinity Biotech, USA), anti-CMV IgG (Vircell, USA) and anti-HHV-6A/B IgG and IgM (Vidia, Ltd., Czech Republic) using an automated ELISA processing system (DS2, Dynex Technologies, USA). The results were then presented in artificial units (AU). These were calculated by multiplying the index value by 10 (the index value for each of the samples = sample absorbance/cut-off value) [78,79]. Samples were analyzed in duplicate for each test. All samples analyzed with values less than 11 AU were considered negative. Doubtful samples between 9 and 11 AU were analyzed again.

Cell Culture

The thawing of PBMCs from liquid nitrogen was carried out by performing three washes (400 g, 10 min, RT) in complete medium: RPMI-1640 (Roswell Park Memorial Institute medium; Merck, Darmstadt, Germany), 10% FBS (fetal bovine serum), 0.5% streptomycin-penicillin and 1% L-glutamine. Then, cell viability was assessed by trypan blue exclusion staining and cell counting was performed using a Neubauer chamber. The cells were placed in 12-Well Cell Culture plate at a density of $2x10^6$ PBMCs per ml and cultured overnight in an incubator at 5% CO_2 and 37°C. The next morning, PBMC cultures were washed in complete medium (400 g, 10 min, RT), counted to determine viability and recovery overnight and resuspended at a density of $2x10^6$ per ml.

Antigen Stimulation

Cells of 14 MS patients (7 RR-SMS and 7 PP-MS) and 9 HCs were quickly divided into 5 tubes, each containing 500.000 cells.

The 5 conditions analyzed were:

- Negative control, RPMI to 10% FBS
- Positive control, phorbol 12-myristate 13-acetate (PMA) (50ng/ml) and calcium ionophore (IO) (0.75µg/ml) (Sigma Aldrich)

- DMSO, used as “vehicle control”, at 0.75% (Sigma Aldrich)
- 2 antigen stimulations were performed by exposure of Syncytin-$1_{env\ 486–500}$ and *p*HERV-$W_{env\ 486–504}$ at same concentration (75µM) and costimulatory antibodies anti-CD28/CD49d (5µg/ml) (BD Biosciences, San Diego, CA, USA).

All the cell cultures were incubated at 5% CO_2 and 37°C for 2 h. Subsequently brefeldin A (BFA) (10 µg/ml) and monensin (0.7 µg/ml) (BD Biosciences, San Diego, CA, USA) were added. BFA and monensin are cytokine secretion inhibitors commonly used in intracellular cytokine staining (ICS).

Next 6 h. the cell cultures were washed with saline buffer (PBS) and then centrifugated (300 g, 5 min, RT), discarding the supernatant.

Cell Surface Staining

Pellet samples resuspended in 1 mL of culture medium with mix of cell surface staining (tab.3) were incubated for 20 min at 4°C in the dark. This technique is ideal for staining cell surfaces in flow cytometry. The surface markers can be utilized to define certain sub-population cell types or function when they're labeled with fluorescent antibodies. Subsequently, the cells were washed with saline buffer (PBS) and then centrifugated (500 g, 5 min, RT), discarding the supernatant.

Tab. 3
Mix of cell surface markers labeled with fluorochrome-conjugated antibodies, and their relative provider.

Fluorochrome	Specificity	Sub-Population type	Provider
V500	CD45	Leukocytes	BD
BV421	CD3	T-lymphocytes	BD
APC-H7	CD8	T-lymphocytes	BD
BV421/APC-H7	CD4	T-lymphocytes	BD
PE-Cy7	CD19	B-lymphocytes	BD

PMBCs were analyzed by flow cytometry on a FACS-Canto II 8-color Flow cytometer (BD). In all analysis strategies, leukocytes were identified by facing the lateral Side Scatter Area (SCC-A) *vs.* forward scatter area (FSC-A, Forward Scatter Area) (fig. 8A). Doublets were then excluded using a two-parametric plot of SSC-A and Side Scatter height (SSC-H) (fig. 8B). Total leukocytes were recognized with CD45+ and low SSC-A (fig. 8C), while total T-lymphocytes as CD3+ and SSC-A low (fig. 8D). CD4+ cells were differentiated as CD3+ and CD8- T cells while CD8+ cells were differentiated as CD3+ and CD8+ T cells (fig. 8E). Total B-lymphocytes were recognized with CD19+ and SSC-A low (fig. 8F).

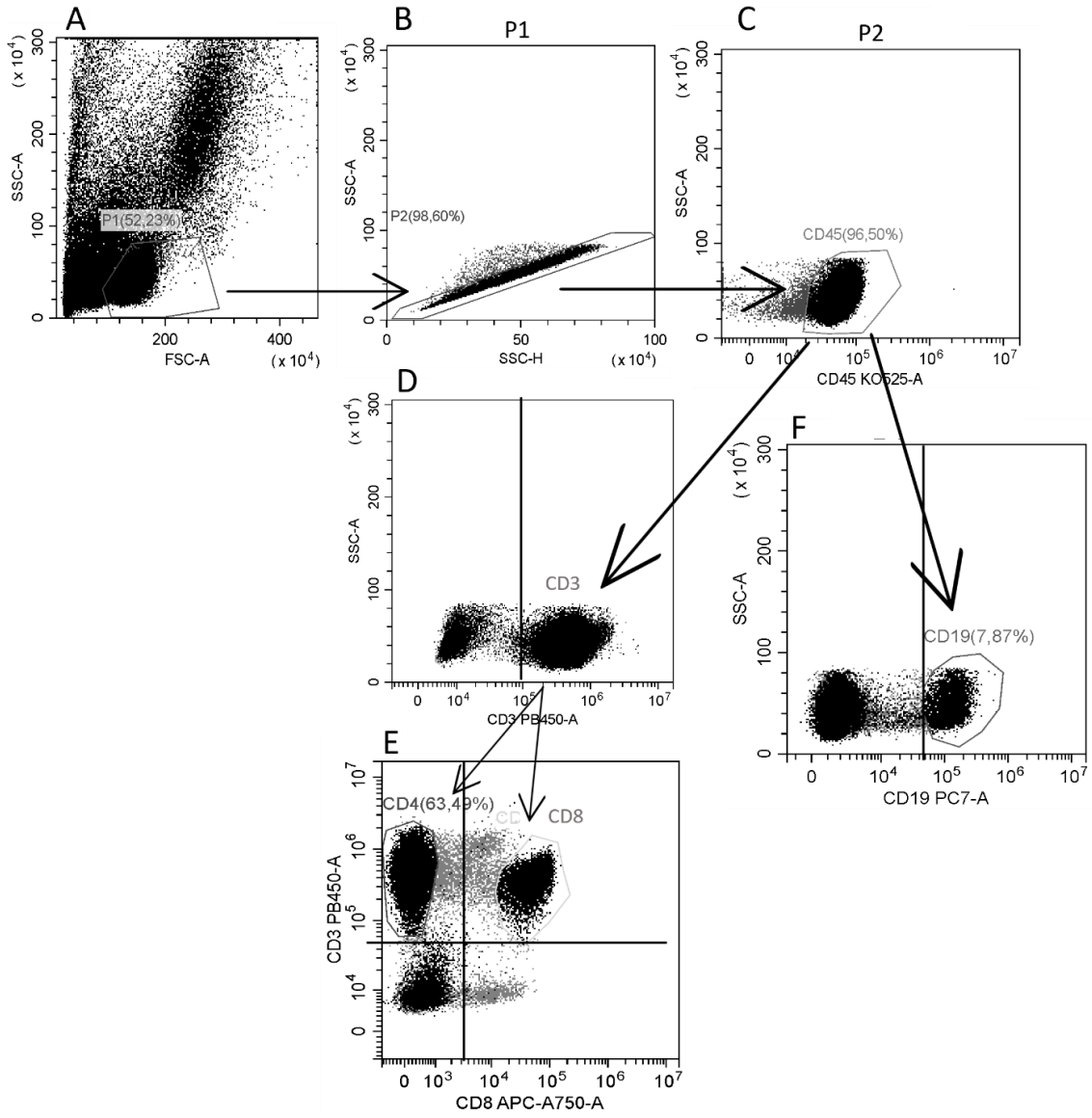


Figure 8.
Strategy for the analysis of lymphocyte subpopulations.

Intracellular Cytokine Staining (ICS)

On the pellets obtained subsequently applied the ICS protocol. It is commonly used to analyze the effect of specific peptides on the immune response in PBMCs. Cytofix/Cytoperm (BD Biosciences, San Diego, CA, USA) was applied to the cells pellet for 20 minutes at 4°C in the dark, to guarantee the cellular fixation and permeabilizing so that cytokines could be labeled within the cells. Next, the cells were washed with PERM-WASH buffer, used to permeabilize cells and as diluent for antibodies, and then centrifugated (500 g, 5 min, RT) discarding the supernatant. The final step involved intracytoplasmic labeling of the cells using the monoclonal antibodies anti-IFN-γ, anti-IL-17, anti-TNF-α, and anti-GM-CSF. These were placed at 4°C for 20 minutes in the dark. Then, the cells were washed with PERM-WASH buffer and then centrifugated (500 g, 5 min, RT) discarding the supernatant. The cell pellets were resuspended in 300 µL of 1% paraformaldehyde in PBS for analysis in the flow cytometer.

Statistical Analysis

The data analysis process was carried out using the GraphPad Prism software, versions 8.0/ 9.0 (San Diego, CA, USA) SPSS software package (version 28.0 SPSS, Inc) and R-studio. The receiver operator characteristic (ROC) curves were then utilized to evaluate the experiments' accuracy. The variation coefficient (CV) of intraassay was set at maximum of 10%. Outliers were identified by the Interquartile Range (IQR) method by imposing a no-negative constant (*K*) of three. The cut-off value for positivity in each test was set at 95% specificity and the sensitivity was calculated accordingly. Shapiro-wilk test was performed to evaluate the normality of data distribution. Non-parametric test, Mann-Whitney and Kruskal-Wallis were utilized for the multiple comparisons, according to data distribution. The Spearman test was then utilized to analyze the correlation between two variables. Logistic regression analysis was applied to analyze the influencing clinical and demographic data on patients. The principal component analysis (PCA) was also utilized to investigate the unsupervised patterns in the data, allowed the identification of similarities and trends between the samples. Statistical significance was set at *p*-value of 5%.

RESULTS

*p*HERV-W envelope protein and the inflammatory phase of multiple sclerosis in Sardinia population (Italy)

The results showed in this chapter have been published in:

"Ruberto S, Cossu D, Sechi LA. Correlation between antibodies against the pathogenic *p*HERV-W envelope protein and the inflammatory phase of multiple sclerosis. *Immunology*. 2024, 171(2), pp. 270–276. https://doi.org/10.1111/imm.13712".

The peptides used in the indirect ELISA experiments derive from the portions of the two forms of HERV-W envelope, syncytin-1 and *p*HERV-W. The detection of the specific autoantibody response in serum was applied to a Sardinia population composed of 46 HCs, 36 RR-MS in stable form (SMS) and 46 RR-MS in acute form (AMS). As shown in figure 9A the antibody response to *p*HERV-$W_{env\ 486–504}$ was higher in AMS patients (median: 0.06, interquartile range [IQR]: 0.17–0.03) than controls (median: 0.04, IQR: 0.07–0.02, $p \leq 0.01$) and SMS patients (median: 0.05, IQR: 0.08–0.03, $p \leq 0.001$). The humoral response of patients against the Syncytin-$1_{env\ 486–500}$ peptide was not significantly different between the groups (fig. 9B).

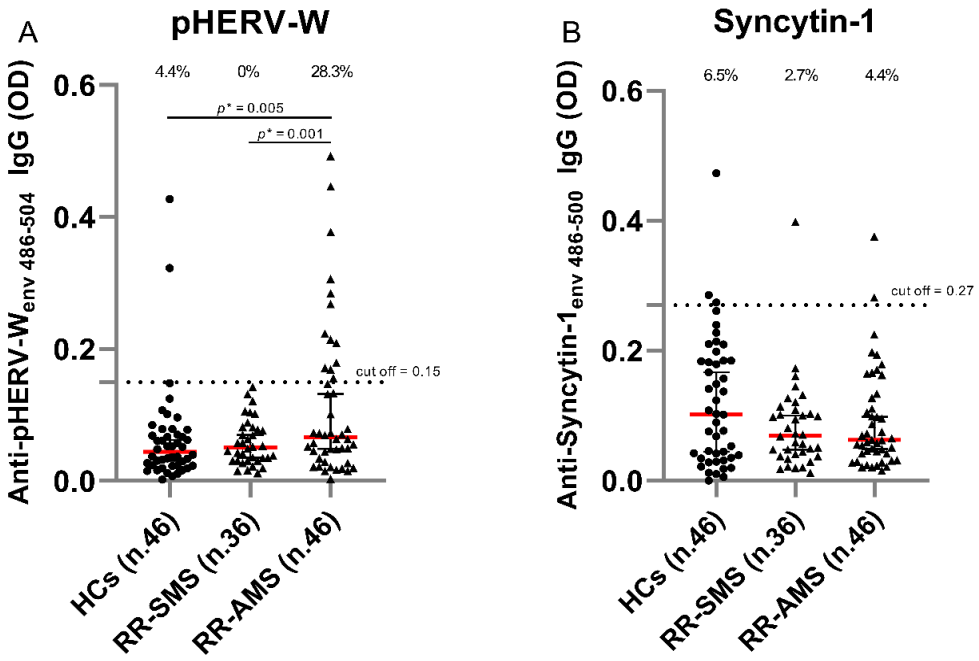


Figure 9.
IgG-antibody titers against *p*HERV-$W_{env\ 486–504}$ and Syncytin-$1_{env\ 486–500}$ were detected in sera of patients with stable MS (SMS), acute MS (AMS), and healthy controls (HC). The dots represent the titer of each patient. The figure displays the interquartile range with the median (red line) of anti-*p*HERV-$W_{env\ 486–504}$ antibodies (A), anti-Syncytin-$1_{env\ 486–500}$ antibodies (B). The percentage of positive results for each group was displayed in the upper part of the graph. These results were derived from the exact tests performed by Shapiro-Wilk, Fisher and Chi-square.

According to the competition assay, five anti-*p*HERV-$W_{env\ 488-504}$ positive sera were pre-incubated, with reactive peptides prior to being subjected to ELISA the day after on *p*HERV-$W_{env\ 486-504}$-coated plates. As shown in figure 10, the competitivity effects of the Syncytin-$1_{env\ 486–500}$ peptide on the *p*HERV-$W_{env\ 486-504}$ antibody were equal to 20-23% of inhibition. The scrambled peptide (case-control) did not trigger a significant inhibition of the signal.

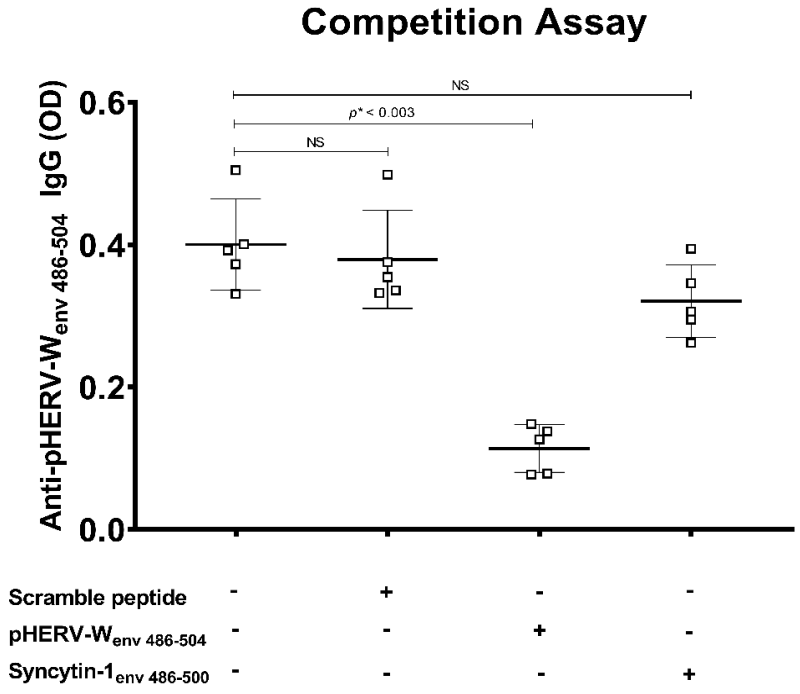


Figure 10.
The antibody titers against the *p*HERV-$W_{env\ 486-504}$ detected in the preincubated positive sera of MS patients (2 RR-AMS, 2 RR-SMS) and one HC. Preincubation with Syncytin-$1_{env\ 486–500}$ (negative control), *p*HERV-$W_{env\ 486-504}$ (positive control), and scramble peptide (case-control). Mann–Whitney test and Kruskal–Wallis with Dunn's test. NS, non-statistically significant. $p \leq 0.05$ was considered statistically significant.

A positive correlation was detected between the anti-*p*HERV-$W_{env\ 486–504}$ and the anti-Syncytin-$1_{env\ 486–500}$ antibodies in AMS patients ($r = 0.65$, $p \leq 0.001$) (fig. 11A). A positive correlation was also detected between the anti-*p*HERV-$W_{env\ 486–504}$ and the Syncytin-$1_{env\ 486–500}$ antibodies in HCs ($r = 0.68$, $p < 0.0001$) (fig. 11B). There was no apparent relationship between the response of the autoantibodies to EDSS, age, gender.

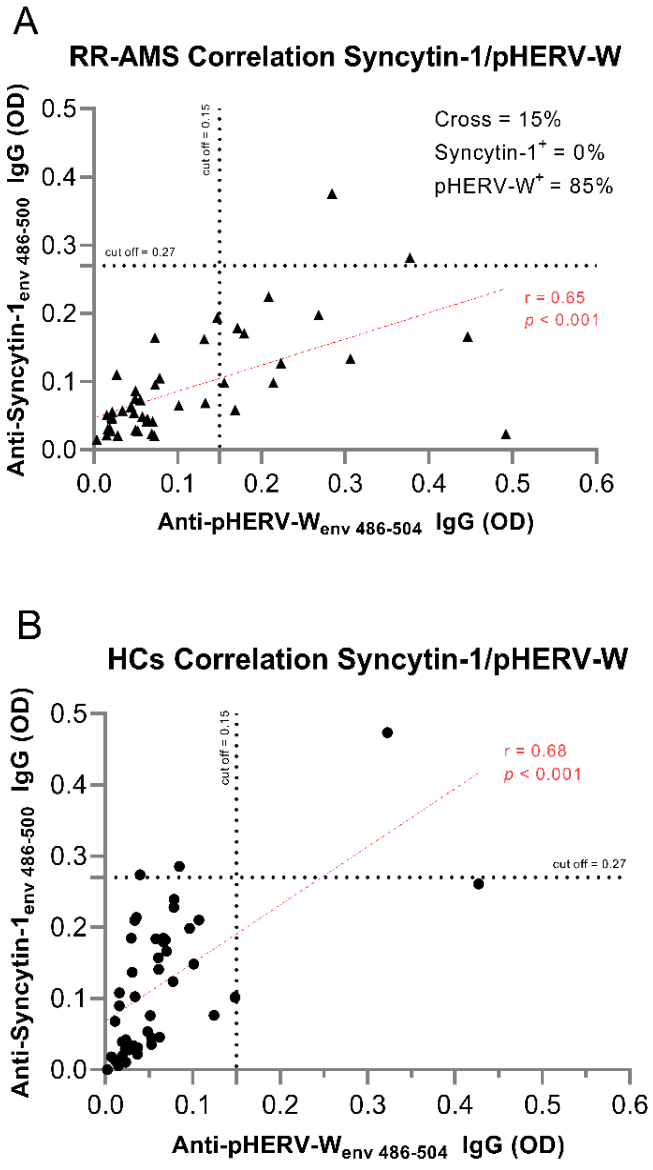


Figure 11.
The scatter plot shows the Spearman's correlation (red line) between the anti-Syncytin-$1_{env\ 486-500}$ and *p*HERV-$W_{env\ 486-504}$ antibody levels in patients with acute MS (AMS) (A) and control subjects (HC) (B).

*p*HERV-W and syncytin-1 envelope protein in Spanish population

This chapter summarizes the results of the study, collected, analyzed, and processed at San Carlos Hospital in Madrid, Spain.

Indirect-ELISA in Spanish and Italian (Sardinia) population

The peptides used in the previously indirect ELISA experiments were used to analyze the humoral response in MS patients and HCs of Hispanic ethnicities, from Spain. Initial results of the study revealed lower levels of antibody titers against *p*HERV-$W_{env\ 486\text{-}504}$ and anti-Syncytin-$1_{env\ 486\text{-}500}$ in Spanish subjects compared with values found in the Sardinian population (fig.12).

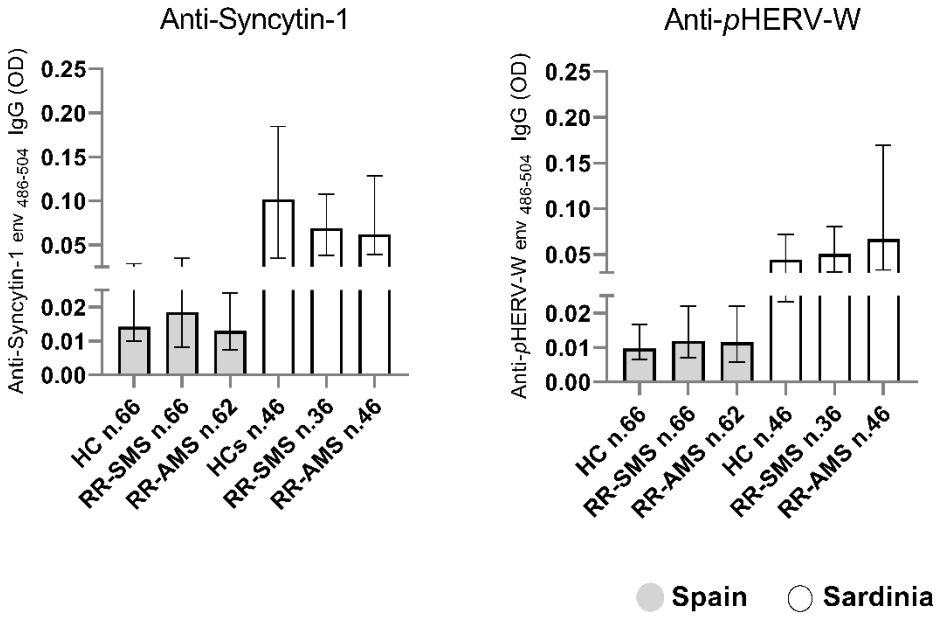


Figure 12.
The antibody titers against the Syncytin-$1_{env\ 486–500}$ and *p*HERV-$W_{env\ 486\text{-}504}$ detected in sera of MS patients (RR-AMS, RR-SMS) and HCs, in Spanish (grey) and Sardinian (white) population. Median with the interquartile range (IQR).

Since of the great difference between the levels of autoantibodies found between the two populations, to assess the differences with respect to the ethnicity of the subjects, the results of the humoral response were reported as the *ratio* of anti-*p*HERV-$W_{env\ 486\text{-}504}$ to anty-Syncytin-$1_{env\ 486\text{-}500}$ response. The *ratio* (anti-pHERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$), for each category, HC, RR-SMS and RR-AMS, were compared between the Spanish and Sardinian populations and no difference was found in any of the cases investigated (fig.13).

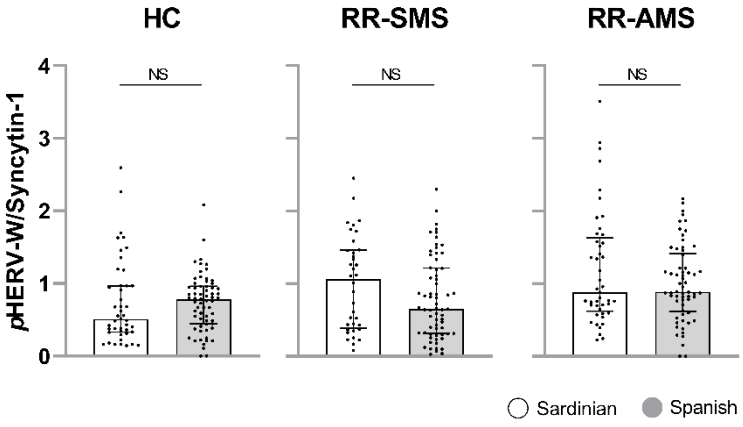
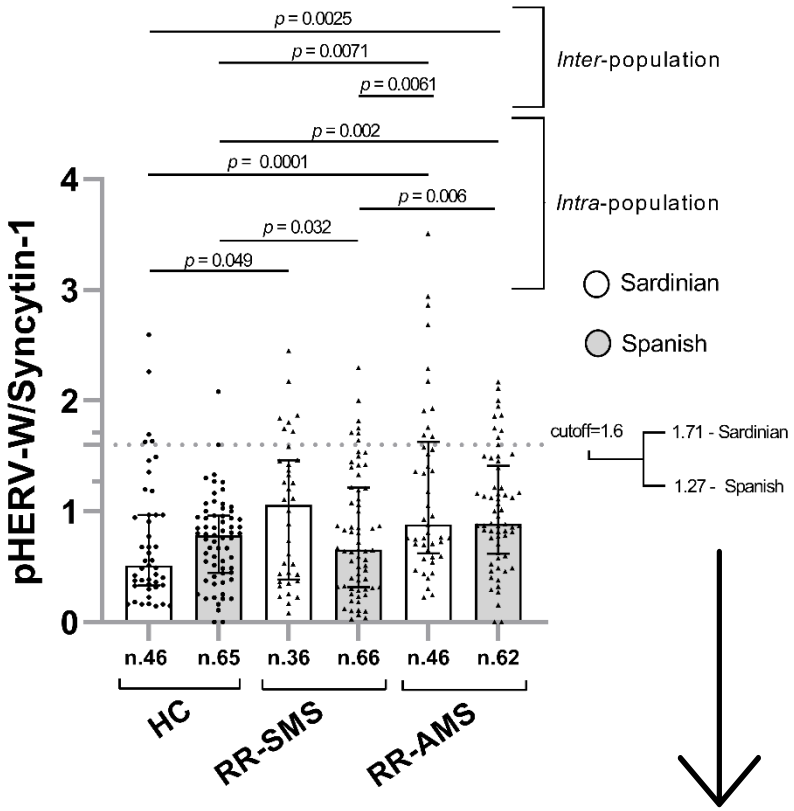


Figure 13.
The *ratio* of IgG-antibody titers detected (anti-pHERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) in sera of MS patients (RR-AMS, RR-SMS) and HCs, in Sardinian (white) and Spanish (grey) population. Median (column height) with the interquartile range (IQR). NS, non-statistically significant.

According to the *intra*-population analysis, both in Sardinia and Spanish populations the ratio of IgG-antibody titers detected (anti-*p*HERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) was higher in RR-SMS and -AMS patients than HC. Otherwise, only Spanish patients had differences in humoral responses, between RR-SMS and -AMS. Based on *inter*-population analysis, Spanish RR-AMS patients showed higher levels than HCs from Sardinia. The same difference was found between Sardinian RR-AMS patients and Spanish HCs. Finally, significantly higher levels in Sardinian RR-AMS patients than Spanish RR-SMS patients were found (fig. 14A/B).

A



B

		Inter		*Intra*	
Population	Ethnicity	+/-	+%	+/-	+%
HC	Sardinian	2/44	4.3	7/104	6.3
	Spanish	3/62	4.6		
RR-SMS	Sardinian	6/30	16.6	14/88	13.7
	Spanish	15/51	22.7		
RR-AMS	Sardinian	9/37	19.6	20/88	18.5
	Spanish	16/46	25.8		

Figure 14.

Ratio of IgG-antibody titers (anti-*p*HERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) detected in sera of healthy controls (HC) and RR-SMS and RR-AMS patients in the Sardinian and Spanish population (A). The figure displaying the median (column height) with IQR. The dots represent the titer of each patient. (B) Positivity values compared with cutoffs calculated on individuals (*Inter*) and whole (*Intra*) populations. Mann–Whitney test, Chi-Square and Kruskal–Wallis with Dunn's test. $p \leq 0.05$ was considered statistically significant.

***p*HERV-W envelope protein and the clinical forms of MS in Hispanic population (Spain)**

The set of the total Spanish MS patients detected a *ratio* higher (median: 1.01, IQR: 0.72-1.32) than controls (median:0.78, IQR: 0.95-0.47) (fig. 15A). Firstly, the MS population has been divided into two macro-clinical forms, the relapsing-remitting (RR-SMS and RR-AMS, REM.) and progressive (PP-MS and SP-MS, PROG.). The *ratio* of progressive forms (median: 1.15, IQR: 1.37-0.95) was higher than remission form (median: 0.85, IQR: 1.22-0.47) and HC (median: 0.79, IQR: 0.96-0.45) (fig. 15B).

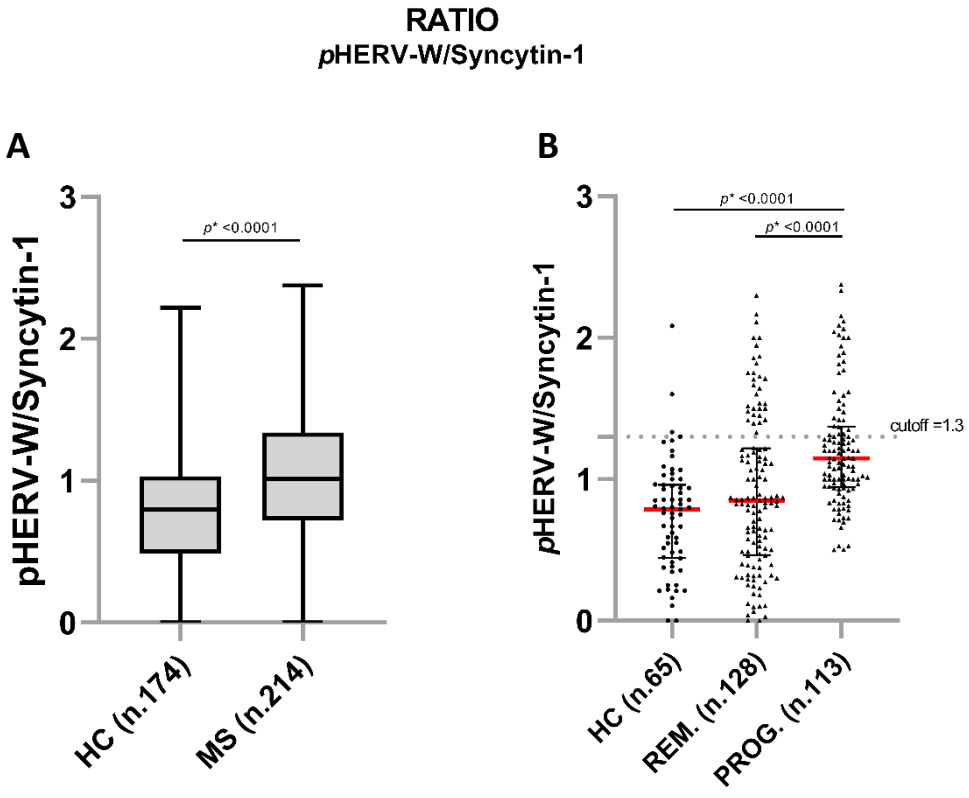


Figure 15.

The ratio of IgG-antibody titers (anti-*p*HERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) detected in sera of healthy controls (HC) and MS patients (A). *Ratio* of IgG-antibody titers in HC and MS patients in remission (REM.) and progressive (PORG.), displaying the median (red line) with IQR (B). The dots represent the titer of each patient. Mann–Whitney test and Kruskal–Wallis with Dunn's test. $p \leq 0.05$ was considered statistically significant.

Finally, the *ratio* in each clinical form of MS form and HCs was studied. The ratio was significantly higher in RR-AMS, PP-MS and SP-MS than in RR-SMS and HCs (fig. 16A). In particular, the ELISA performance was also assessed by the area under the receiver operating characteristic curve (AUC-ROC = 0.76, p ≤ 0.0001). The cut-off value for positivity was set at a ratio 1.3, with 95% specificity and accordingly sensitivity (fig. 16B).

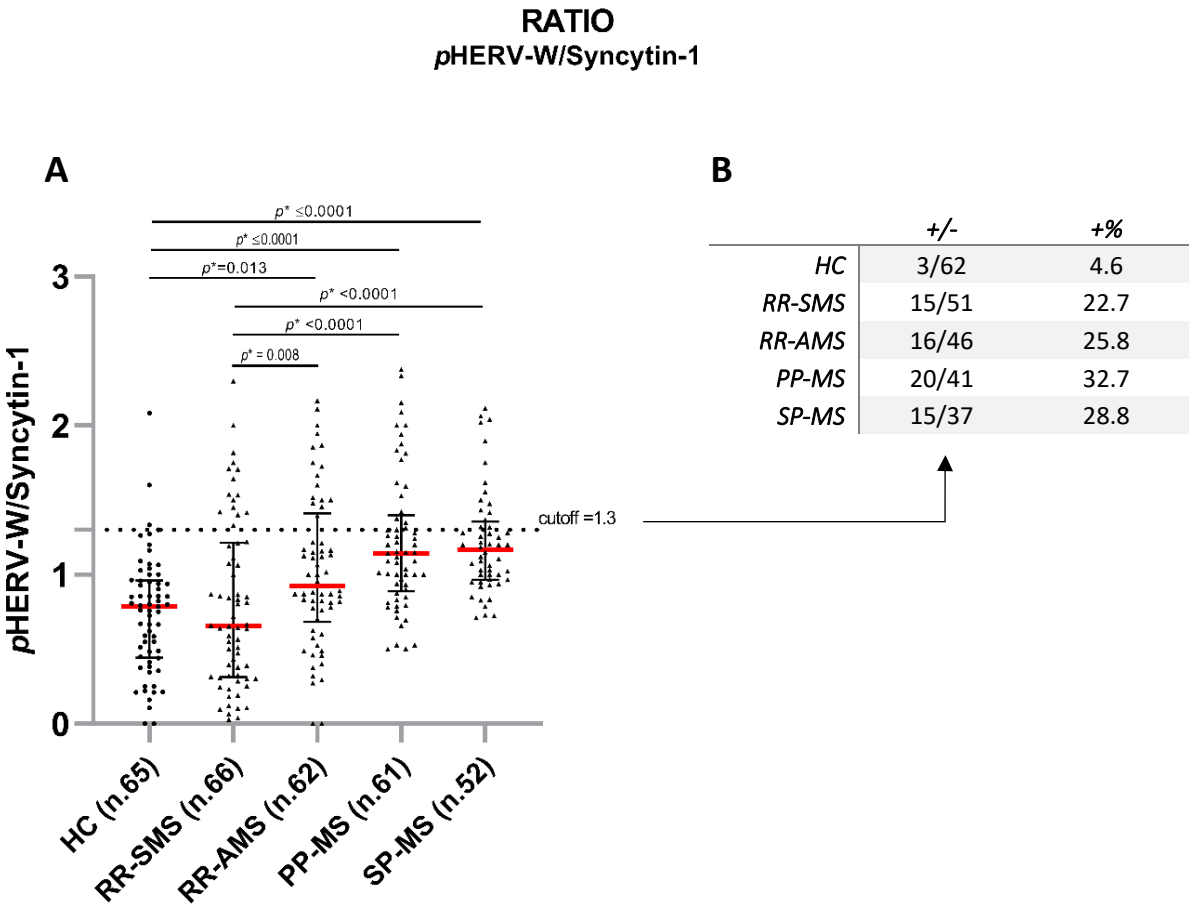


Figure 16.
Ratio of IgG-antibody titers (anti-pHERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) detected in sera of healthy controls (HC) and MS patients (A). Ratio [median, P75-P25]: HC= 0.79, 0.96-0.45. RR-SMS=0.65, 1.02-0.31. RR-AMS=0.92,1.35-0.72. PP-MS=1.14, 1.37-0.9. SP-MS=1.17,1.33-0.97. The positivity values than cutoff (B). The figures display the median (red line) with IQR. The dots represent the titer of each patient. Mann–Whitney test, Kruskal–Wallis with Dunn's test an. $p \leq 0.05$ was considered statistically significant.

Clinical Variability on Humoral Response

Based on the EDSS score, RR-SMS patients, were classified into two groups:

- Patients with EDSS less than 2 (RR-SMS < 2)
- Patients with EDSS greater than 4 (RR-SMS > 4)

RR-SMS patients with an EDSS major than 4 showed higher levels of the ratio of IgG-antibody titers (anti-*p*HERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) than HCs and RR-SMS patients with EDSS score lower than 2 (fig. 17).

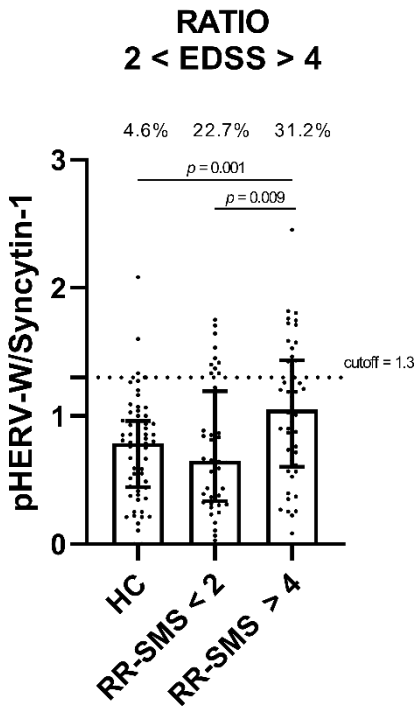


Figure 17.
Ratio of IgG-antibody titers (anti-*p*HERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) detected in sera of healthy controls (HC) and RR-SMS with EDSS score < 2 and > 4. The figure displays the median (column height) and IQR. The dots represent the titer of each patient. The percentage of positive results, relative to the cutoff, for each group was displayed in the upper part of the graph. Mann–Whitney test and Chi-Square. $p \leq 0.05$ was considered statistically significant.

Epstein-Barr and *Cytomegalovirus* IgG and *Herpes Virus* IgM/G ELISA in MS

The analysis of ELISA results was first conducted using qualitative serological results, *i.e*. positive and negative, among the different MS clinical forms and HC. The prevalence of IgG antibodies against EBNA-1 was higher in RR-AMS and PP-MS than in controls. Although not statistically significant, almost all MS patients tested positive for the two Epstein-Bar antigens. Table n. 4 summarizes the prevalence found in the patient and control populations as well as the differences between the two populations.

Table 4.

Prevalence of seropositivity of IgM and IgG anti-HHV-6A/B, anti-EBNA-1 and anti-VCA IgG anti-CMV IgG in MS patients and HC. The reported values reflect the percentage of seropositivity. *Significantly differ with respect to HCs (Chi-square test).

	IgM$^+$	*IgG*$^+$			
%	HVV-6A/B	HVV-6A/B	EBV/EBNA-1	EBV/VCA	CMV
HC	28	86	86	95	69
RR-SMS	22	84	95	97	65
RR-AMS	33	93	100*	100	69
PP-MS	35	76	97*	100	69
SP-MS	35	88	93	100	67

Subsequently, the analysis was performed on a quantitative basis to quantify the antibody titers. Significant differences in EBNA-1, HVV-6A/B IgG and IgG$^+$ and HVV-6A/B IgM$^+$ antibody titers were found between patients and HCs (tab. 5). Finally, correlation analysis detected a moderate degree of correlation between the *ratio* (anti-*p*HERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) and HVV-6A/B IgM$^+$ (N.=18, r=0.6, p=0.009) and IgG$^+$ (N.=44, r=0.3, p=0.05) levels in PP-MS patients.

Table 5.

Quantification of antibody titers of HVV-6A/B IgM and IgG, and EBV/EBNA-1, EBV/VCA and CMV IgG in patients and controls [Mean +/- SD and median (IQR)]. Kruskal–Wallis and Mann–Whitney test. *Significantly differ with respect to HCs.

Mean ± SD Median (IQR)		***HC***	***RR-SMS***	***RR-AMS***	***PP-MS***	***SP-MS***
HVV-6A/B	IgM	7.8 ± 6.9 5.9 (3.2-9.8)	7.4 ± 8.2 5.2 (3-8.5)	9.7 ± 12.4 5.5 (2.6-10.9)	7.3 ± 6.6 4.5 (1.9-11.3)	9.3 ± 10.1 6.1 (2.7-12.0)
	IgM^+	15.8 ± 5.5 13.8 (10.4-17)	18.3 ± 11.6 15.1 (12-18)	**23*± 14.9 19.4(17.1-25.5)**	15.2 ± 4.4 14.7 (10.8-18.3)	19.1 ± 10.1 16.4 (12.0-23.1)
HVV-6A/B	IgG	16.7 ± 8.6 15.1 (12.5-19.1)	20.6 ± 12.9 18 (11.8-26.3)	**23.6**± 10.8 21.4(17.4-31.7)**	21.3 ± 15.6 16.9 (9.3-29.6)	16.6 ± 7.7 15.8 (11.6-19.6)
	IgG^+	18.5 ± 8.2 16.5 (13.5-20.1)	**23.5*± 11.9 19 (16.0-28.4)**	**24.2**± 10.1 21.7(17.9-32.4)**	**26.5**± 14.1 20 (16.2-38.7)**	18 ± 7.1 17.2 (14.5-20.8)
EBV/EBNA-1	IgG	17.6 ± 8.36 17.9 (12.6-22.4)	**21.6**± 6.6 22 (18.8-24.6)**	**22.2**± 5.1 21.8(18.2-25.9)**	19.6 ± 6.6 19 (15.9-22.9)	20.7 ± 7.17 20.4(18.0-24.9)
	IgG^+	19.5 ± 6.6 19.5 (14.7-23.4)	**22.5*± 5.4 22.3 (19-24.7)**	22.2 ± 5.1 21.8 (18.2-26)	20.1 ± 6.1 19.2 (16.4-23.4)	22.1 ± 5.4 21 (18.3-25.2)
EBV/VCA	IgG	48.3 ± 17.06 50.8 (41.1-62.1)	47.9 ± 14.3 49.9 (39.7-56)	52.9 ± 12.2 55.5 (45.5-61.5)	55.1 ± 12.2 56.2(47-63.3)	49.3 ±11.7 49.8 (44.4-58.5)
	IgG^+	50.6 ± 14.1 52.7 (42.3-62.2)	49.4 ± 12.1 50 (41,6-56,0)	52.9 ± 12.2 55.5 (45.5-61.5)	55.1 ± 12.2 56.2(47-63.3)	49.3 ±11.7 49.8 (44.4-58.5)
CMV	IgG	20.15 ± 13.2 21.8 (5.6 -29)	17.2 ± 13.3 19.7 (1.9-27)	18.2 ± 13.1 20.3 (2.3-28.5)	22.1 ± 15 26.5(4.6-32.1)	19.6 ±14.6 25.3(2.3-30.9)
	IgG^+	27.1 ± 7.8 26.7 (21.1-31.9)	25.7 ± 8.1 25.3 (20.9-31.0)	25.9 ± 8.1 26.3 (19.6-31.3)	30.9 ± 8.5 30.2 (25.9-36.6)	28.5 ±7.7 29.2 (25.4-31.8)

*p=0.02 significantly differ with respect to HCs

**$p\leq$ 0.009 significantly differ with respect to HCs

Multivariable Logistic Regression

The multivariable regression analysis was applied considering one dependent variable (or outcome), and multiple independent variables. The analysis was performed on two groups of patients, RR-SMS and PP-MS (outcome), taking into account the significant variability in clinical and demographic data found between the two groups (Independent variables).

Linear regression equation:

$$RR - SMS \ \& \ PP - MS \sim$$

$$Intercept + RATIO(\textit{anti-pHERV-W}_{\textit{env 486–504}}\textit{/anti-Syncytin-1}_{\textit{env 486–500}}\textit{) + Age + EDSS + Age of onset of illness + Duration of illness (month)}$$

The table below (tab. 6) shows the significant values generated by the model for the different independent variables. Positive estimates for the EDSS, ratio, disease duration, and age of onset were reported with *Odd Ratio* (*OR*) greater than 1. On the other hand, negative estimate for the Age parameter was only reported with an *OR* less than 1.

Table 6.
Parameters generated by the Multivariable Logistic Regression model. Dependent variables: MS clinical forms (RR-SMS and PP-MS). Independent variables: Ratio (anti-pHERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$), Age, EDSS, Age of onset and disease duration. Parameters generated: Estimate, Standard Error (S.E), Odd Ratio (OR), Z Score (Estimate/S.E.). $p \leq 0.05$ was considered statistically significant.

Dependent Variable **RR-SMS/PP-MS**					
Independent Variable	**Estimate**	**S.E.**	***OR***	**[Z]**	***p*-value**
RATIO	1.432	0.571	4.186	2.507	0.0122*
Age	-0.1920	0.083	0.8253	2.268	0.0233*
EDSS	0.9941	0.204	2.702	4.819	<0.0001***
Age of onset of illness	0.2975	0.087	1.347	3.392	0.0007**
Duration of illness (month)	0.01885	0.007	1.019	2.498	0.0125*

Subsequently, the application of the model allowed us to estimate the distribution of each element based on their predicted values and the observed ones (AUC-ROC 0.94. $p < 0.0001$). The results of the model application revealed that the distribution of the correct classification elements was 86.67% accuracy for the RR-SMS and 89.66% for the PP-MS groups (fig. 18), with 97.7% and 88.9% positive and negative predictive power, respectively.

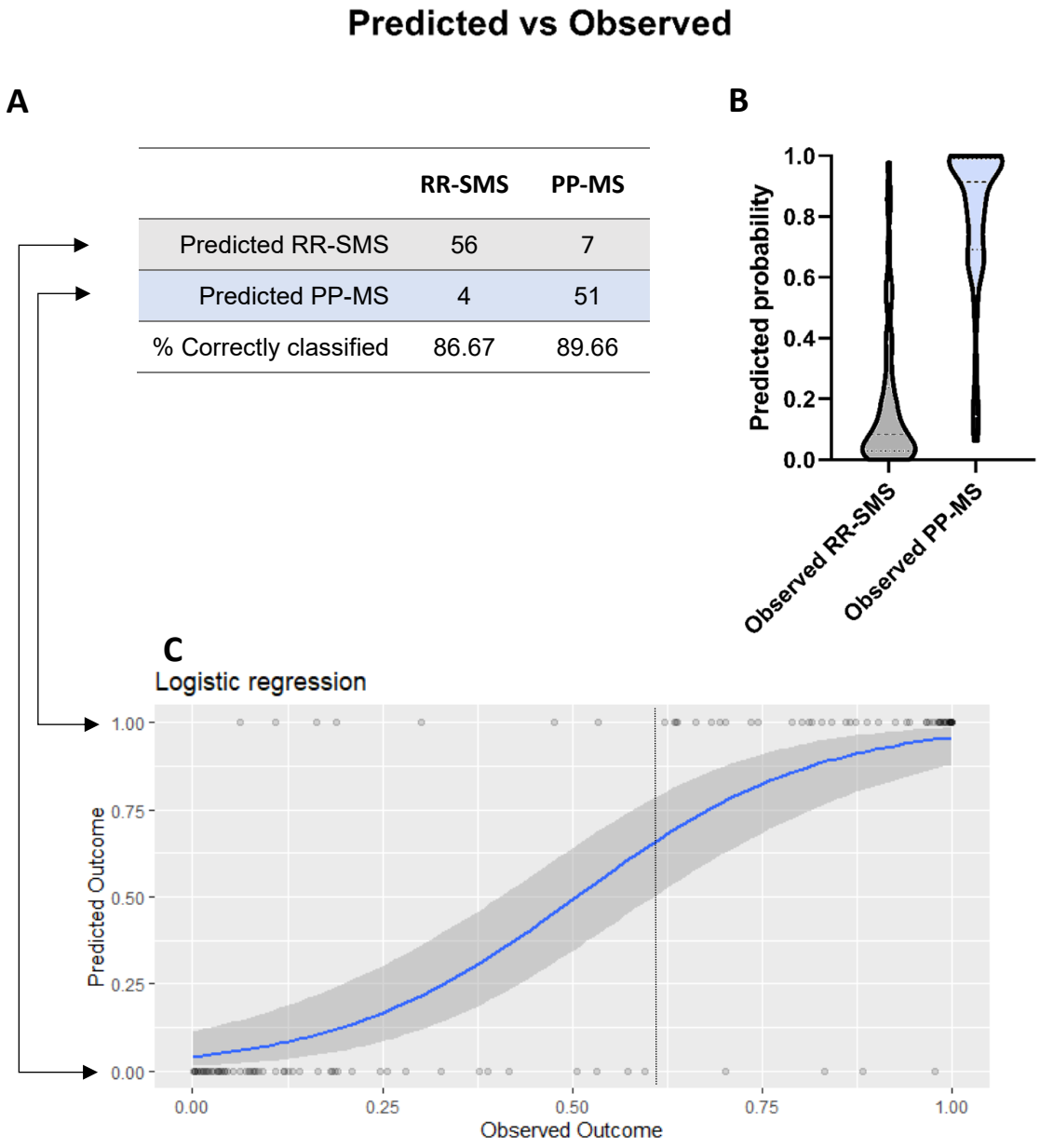


Figure 18.
Application of the multivariable logistic regression model. (A) Classification table of predicted *vs.* observed values with percentage of correct ranking. (B) Probability prediction plot, based on the applied model. (C) Fitted logit function and observed values (0=RR-SMS, 1=PP-MS), with classification cutoff (0.6).

The same analysis was applied considering other categories of dependent variables, and multiple independent variables. The analysis was performed on two groups of patients, RR-SMS and SP-MS (dependent variable), taking into account the significant variability in clinical and demographic data found between the two groups (Independent variable).

Linear regression equation:

$$RR - SMS \,\&\, SP - MS \sim$$
$$Intercept + RATIO(\textit{anti-pHERV-W}_{\textit{env 486–504}}\textit{/anti-Syncytin-1}_{\textit{env 486–500}}) + Age + EDSS$$

The table below (tab. 7) shows the significant values generated by the model for the different parameters. Positive estimates for the EDSS, Ratio, and Age were reported with *Odd Ratio* (*OR*) greater than 1.

Table 7.
Parameters generated by the Multivariable Logistic Regression model. Dependent variables: MS clinical forms (RR-SMS and SP-MS). Independent variables: Ratio (anti-*p*HERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$). Age, EDSS. Parameters generated: Estimate, Standard Error (S.E), Odd Ratio (OR) and Z Score (Estimate/S.E.). $p \leq 0.05$ was considered statistically significant.

Dependent Variable RR-SMS/SP-MS					
Independent Variable	**Estimate**	**S.E.**	**OR**	**[Z]**	**p-value**
RATIO	1.768	0.7452	5.860	2.373	0.0177*
Age	0.1166	0.04673	1.124	2.496	0.0126*
EDSS	1.239	0.2688	3.452	4.609	<0.0001***

The application of the model allowed us to estimate the distribution of each element based on their predicted values and the observed ones (AUC-ROC 0.96. p<0.0001). The results of the model application revealed that the distribution of the correct classification elements was 90%

accuracy for the RR-SMS and 94% for the SP-MS groups (fig. 19), with 86.7% and 94.7% positive and negative predictive power, respectively.

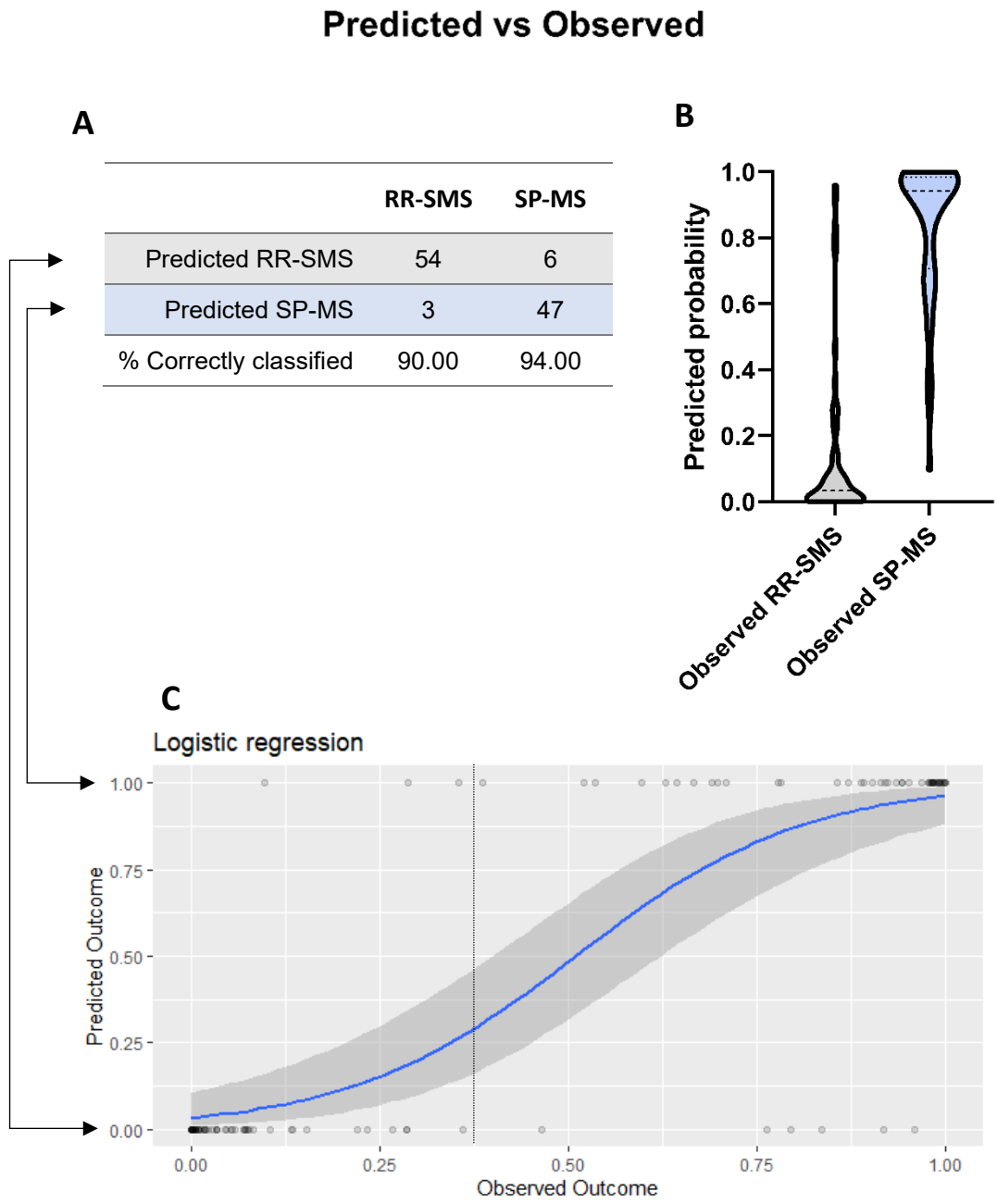


Figure 19.
Application of the multivariable logistic regression model. (A) Probability prediction plot, based on the applied model. (B) Classification table of predicted *vs*. observed values with percentage of correct ranking. (C) Fitted logit function and observed values (0=RR-SMS, 1=SP-MS), with classification cutoff (0.37).

Finally, multivariable regression analysis was applied considering remission form (RR-SMS) and disease progressiveness (Progressive, PP- and SP-MS) as dependent variables. The analysis was performed, considering the significant variability in clinical and demographic data found between the two groups (Independent variable).

Linear regression equation:

$$RR - SMS \ \& \ Progressive \sim$$

$$Intercept + RATIO(\textit{anti-pHERV-W}_{\textit{env 486–504}}\textit{/anti-Syncytin-1}_{\textit{env 486–500}}\textit{) + Age of onset + EDSS+ Duration of illness (month)}$$

The table below (tab. 8) shows the significant values generated by the model for the different parameters. Positive estimates for the EDSS, Ratio, and Age of onset and Duration of illness were reported with *OR* greater than 1.

Table 8.
Parameters generated by the Multivariable Logistic Regression model. Dependent variables: MS clinical forms (RR-SMS and Progressive forms). Independent variables: Ratio (anti-pHERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$)., EDSS, Age of onset and Duration of illness. Parameters generated: Estimate, Standard Error (S.E), Odd Ratio (OR), Z Score (Estimate/S.E.). $p \leq 0.05$ was considered statistically significant.

Dependent Variable **RR-SMS/Progressive**					
Independent Variable	**Estimate**	**S.E.**	***OR***	**[Z]**	***p*-value**
RATIO	1.723	0.5731	5.601	3.006	0.0026**
EDSS	1.127	0.1978	3.088	5.699	<0.0001***
Age of onset of illness	0.1261	0.03485	1.134	3.619	0.0003***
Duration of illness (month)	0.007412	0.003365	1.007	2.203	0.0276*

The application of the model allowed us to estimate the distribution of each element based on their predicted values and the observed ones (AUC-ROC 0.94, p<0.0001). The results of the

model application revealed that the distribution of the correct classification elements was 86.67% accuracy for the RR-SMS and 92.45% for the progressive groups (PP-, SP-MS) (fig. 20), with 89.9% and 85.9% positive and negative predictive power, respectively.

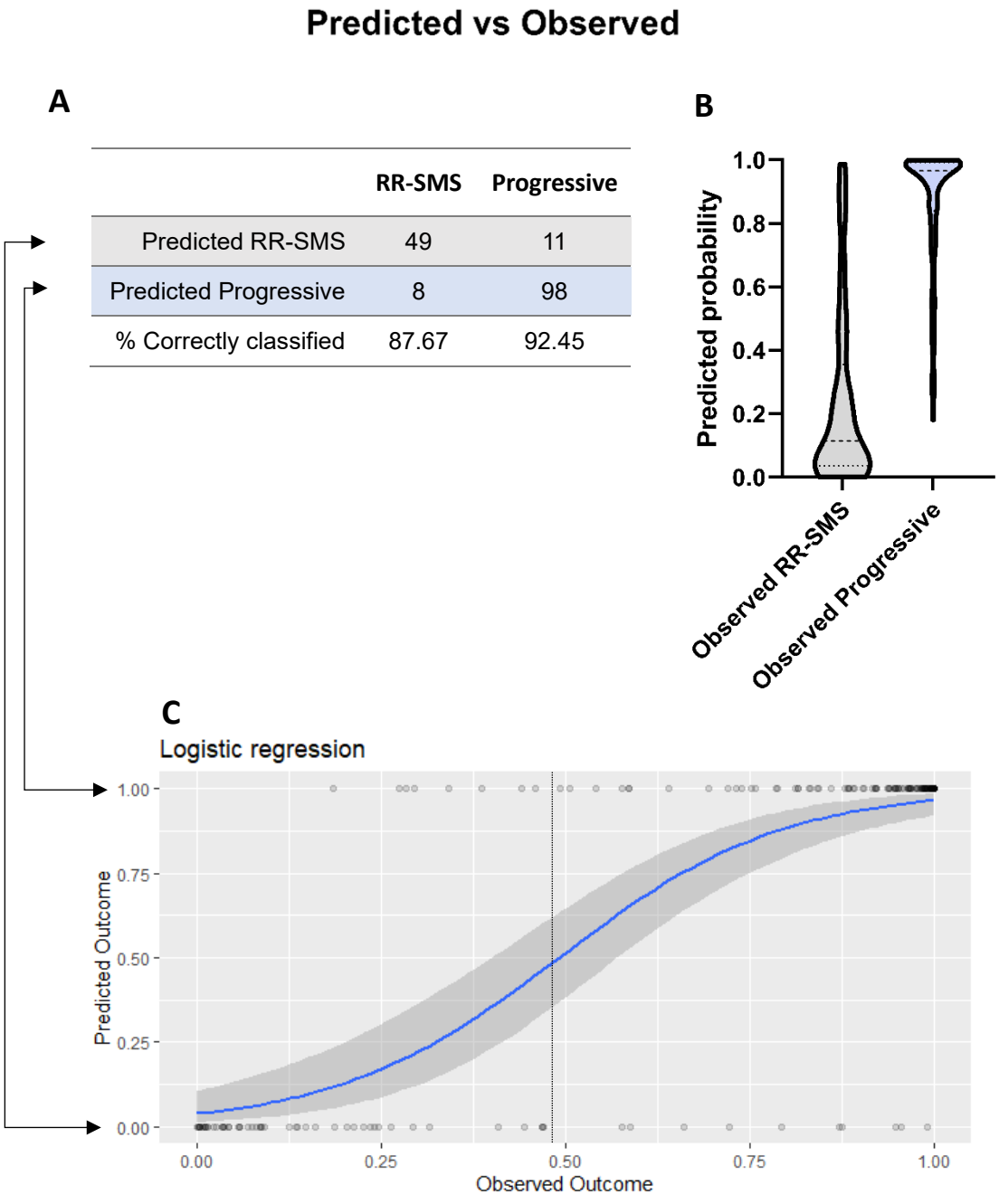


Figure 20.
Application of the multivariable logistic regression model. (A) Probability prediction plot, based on the applied model. (B) Classification table of predicted *vs*. observed values with percentage of correct ranking. (C) Fitted logit function and observed values (0=RR-SMS, 1=Progressive), with classification cutoff (0.47).

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was carried out taking into account the several clinical variables. PCA of the data revealed three components (PC1, PC2, PC3), explaining 77.89% of the total variation in the data (fig. 21). There was a big drop in eigenvalue between PC1 (2.03) and PC2 (1.03), and a smaller drop between PC2 and PC3 (>0.8). Together, PC1 and PC2 accounted for 61.19% of the total variance (40.63% and 20.56%, respectively). Therefore. the first two components were the best to investigate the data distribution, with an eigenvalue >1. On the other hand, according to Bjrklund (2019)[80], the analysis of PC3 was carried out as it allowed for different interpretations also because to the small difference of the percentage of variance with PC2 (3.8%).

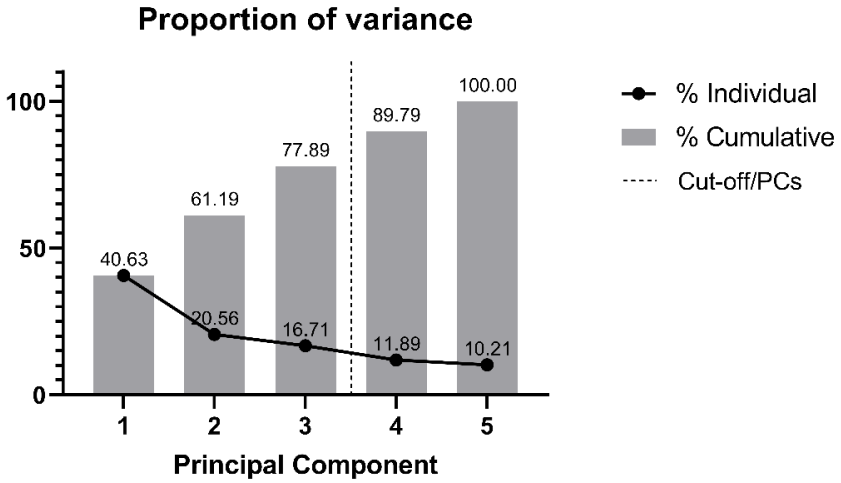


Figure 21.
Scree plot of proportion of variance. Components with cumulative and individual variance explained. Cutt-Off variance of 77.89%.

All the loadings for PC1 were positive, otherwise two loadings of the region of interest (ROI) PC2 (Age, CMV-IgG) and PC3 (CMV-IgG, EBV/VCA-IgG) were negative. In the biplot by PC1 *vs*. PC2, the RR-SMS PC's scores are strongly differentiated from those of progressive (PP-, SP-MS) especially according to the PC1' distribution (fig. 22A). Similar conclusions can be drawn by analysing the results of PC1 *vs.* PC3 biplot, where the PC3 axes higlights a slight partition between the primary (PP-MS) and secondary prograssive (SP-MS) PC's scores (fig. 22B).

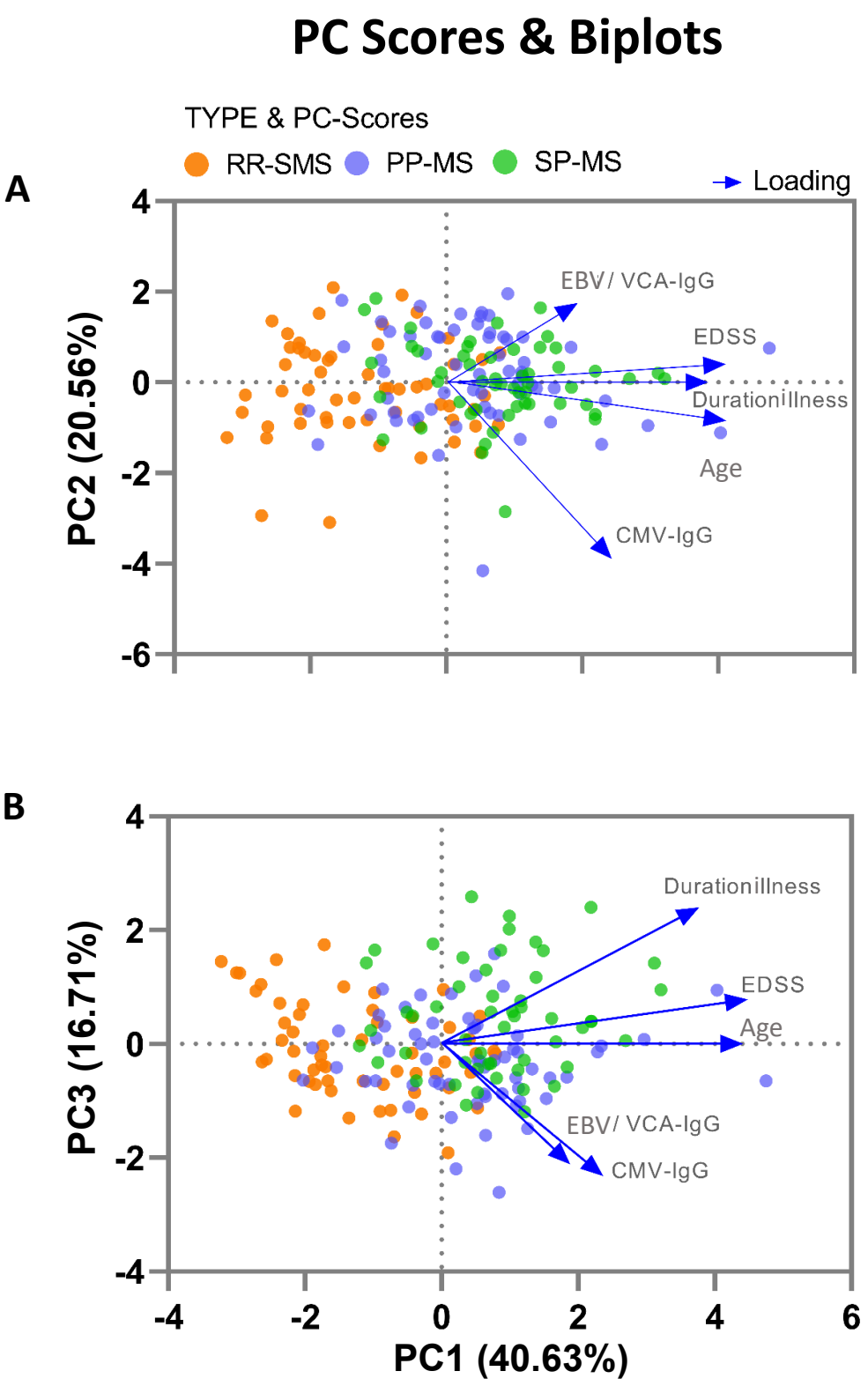
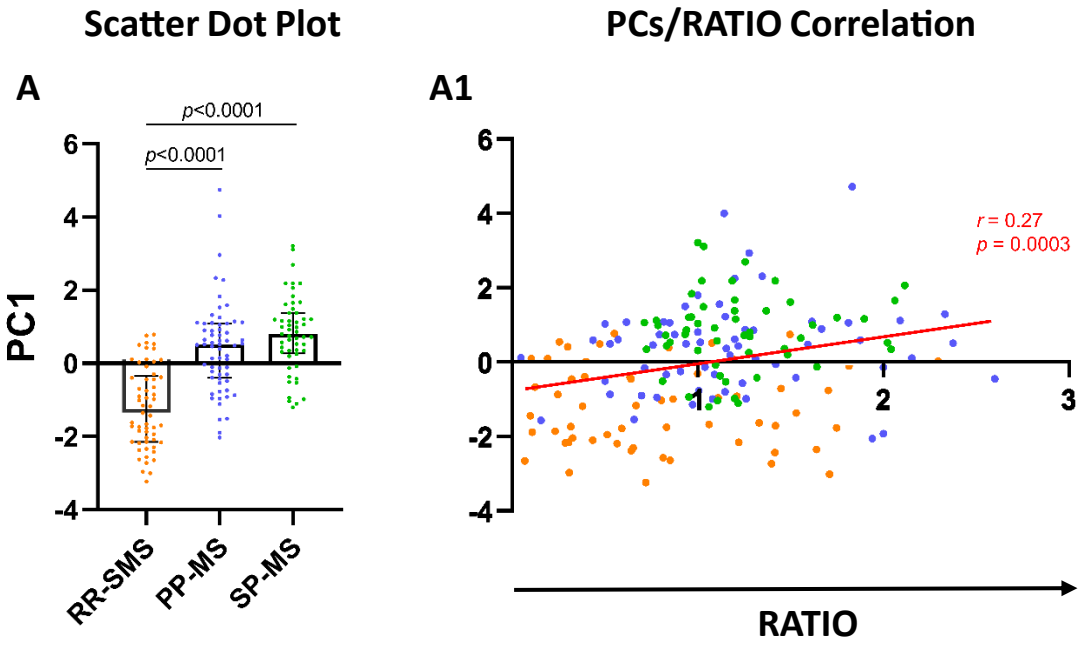


Figure 22.
Biplots of principal components. Biplots were constructed for (A) PC1 *vs.* PC2, (B) PC1 *vs.* PC3. The percentage variance indicated by each PC is highlighted in the axes' titles. Loadings are marked with blue lines and subject PC scores with filled circles, color coded by diagnosis (see the legend).

PC's score and Ratio correlation in MS patients

The study of PC1 subjects' scores revealed significant group differences between RR-SMS (median=-1.35, IQR: -2.14 - -0.29) compared with PP-MS (median=0.51, IQR: -0.42-1.1, *p* <0.0001) and SP-MS (median=0.69, IQR: 0.80-1.38, *p* <0.0001) (fig. 23A). According to PC2 subjects' scores no difference between groups was found (fig. 23B). Finally, in PC3 it was detected significant group differences in subject scores between SP-MS (median=0.39, IQR: -0.33 – 1.3) compared with PP-MS (median=-0.23, IQR: -0.29-0.22, *p* <0.0001) and RR-SMS (median=-0.17, IQR: -0.71-0.46, *p* <0.0001) (fig. 23C).

Finally, correlation analysis showed a moderate and significant degree of correlation between the PC1 subjects' scores and ratio (anti-pHERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) (r = 0.27, $p \leq 0.001$) (fig. 23A.1). On the other hand, negative but non-significant correlations were also detected in the PC2 (r = -0.08) (figure 23B.1) and PC3 (r = -0.11) (figure 23C.1) with respect to ratio.



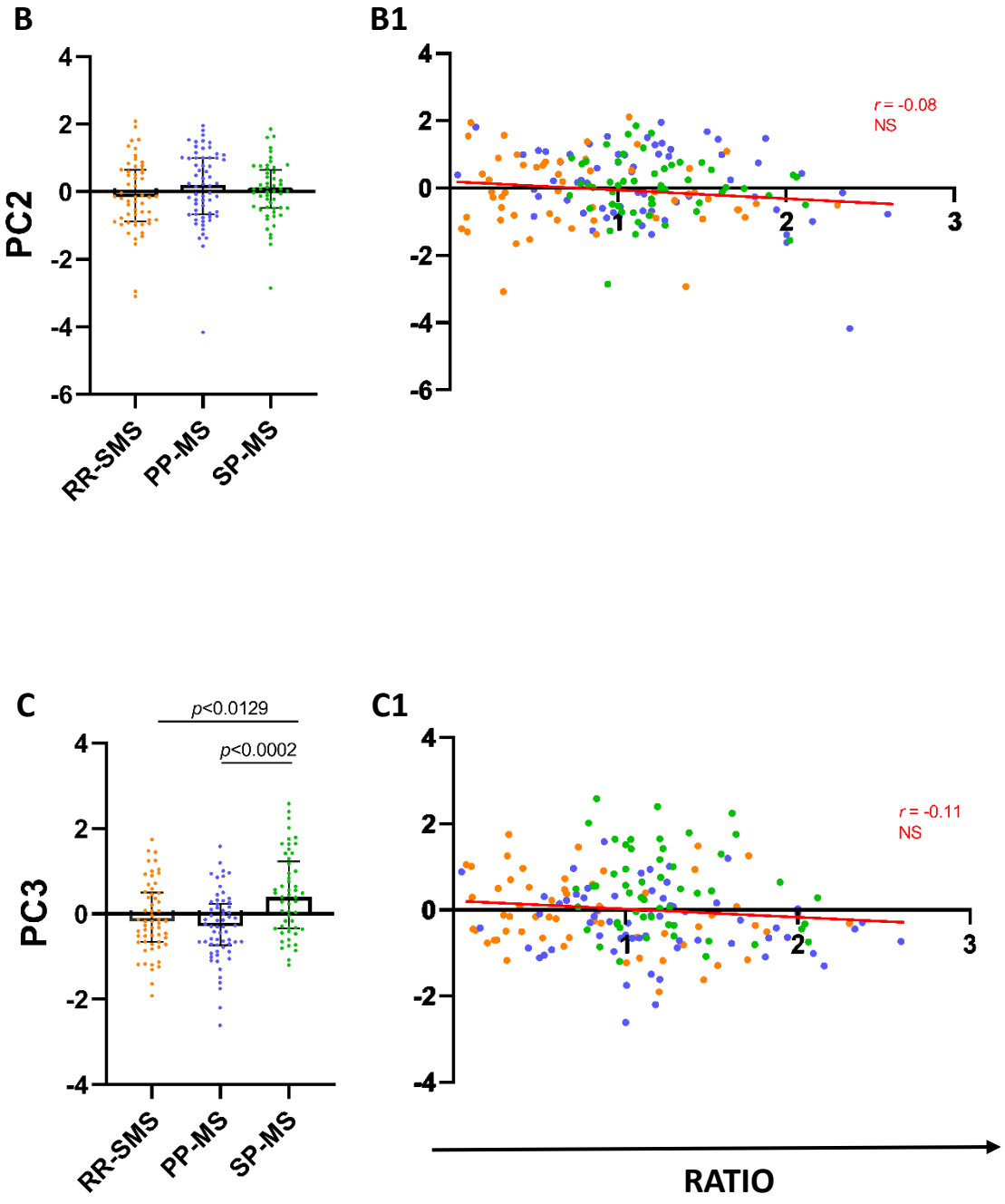


Figure 23.

Comparison of principal component subject scores in MS groups, median with interquartile range. PC1 (A), PC2 (B), and PC3 (C) subject scores were compared between RR-SMS, PP-MS and SP-MS groups. The scatter plot shows the correlations between the anti-Syncytin-$1_{env\ 486\text{-}500}$ and anti-*p*HERV-$W_{env\ 486\text{-}504}$ antibody levels (RATIO) in RR-SMS, PP-MS and SP-MS with respect to PC1 (A1), PC2 (B1) and PC3 (C1). Dots represent the PC component score for each participant, coded according to diagnosis (see PCA legend). Kruskal–Wallis with Dunn's test, Mann–Whitney test and Spearman correlation. $p \leq 0.05$ was considered statistically significant.

Flow cytometric quantification of positive T and B lymphocytes

Flow cytometric evaluation of both peptide-specific $CD8^-$ and $CD8^+$ (i.e. CD4) T lymphocytes (fig. 24) and CD19 B lymphocytes (fig. 27) after eight-hour stimulation of PBMCs from seven RR-SMS, seven PP-MS patients and nine HCs was performed. It was analyzed the expression of IFN-γ, GTM-CSF, TNF-α and IL-17 upon stimulation with the following peptides: Syncytin-$1_{env\ 486\text{-}500}$ and *p*HERV-$W_{env\ 486\text{-}504}$.

$CD4^+$ T-Cells Stimulation

It was detected in MS patients and HCs the percentage of $CD4^+$ cells secreting IFN-γ, GTM-CSF, TNF-α and IL-17 (fig. 25). RR-SMS patients exhibit a significant difference in the expression of TNF-α in $CD4^+$ exposed to Syncytin-$1_{env\ 486\text{-}500}$ (median: 0.21; P75-P25: 0.24-0.16; p=0.05) and *p*HERV-$W_{env\ 486\text{-}504}$ (median: 0.18; P75-P25: 0.11-0.25; p=0.02) than DMSO (median: 0.07; P75-P25: 0.11-0.06). In PP-MS patients a significantly increasing TNF-α in $CD4^+$ exposed to *p*HERV-$W_{env\ 486\text{-}504}$ (median: 0.24; P75-P25: 0.20-0.37; p=0.04) than DMSO (median: 0.15; P75-P25: 0.15-0.1) was detected. Regarding the HCs, none of the tested conditions was capable of inducing cytokines production by $CD4^+$ (fig. 25). Similarly, no significant difference was observed in cytokines production by $CD4^+$ exposed to *p*HERV-$W_{env\ 486\text{-}504}$ among HC, RR-SMS and PP-MS (fig. 26).

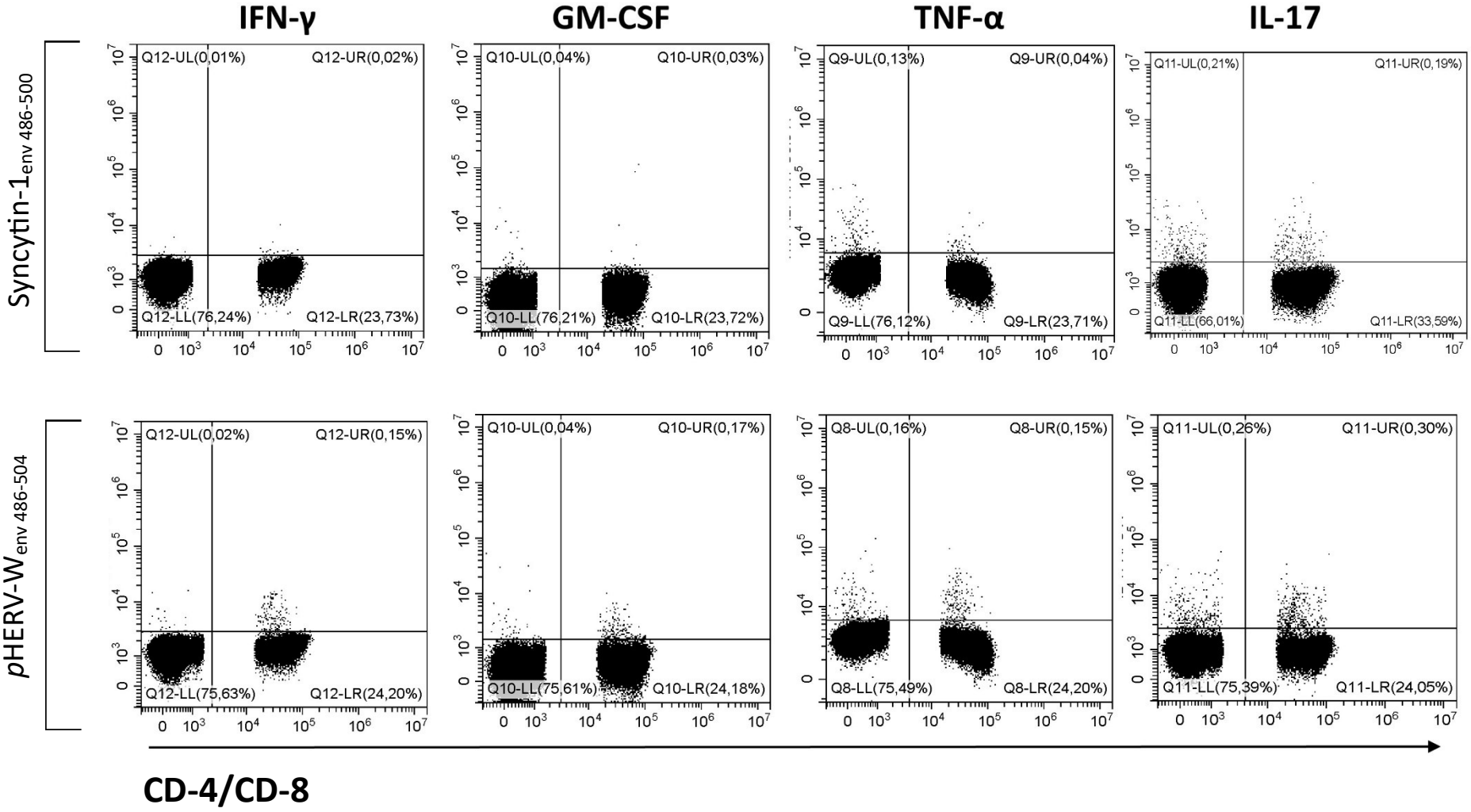


Figure 24.

Detection of Syncytin-$1_{env\ 486\text{-}500}$ and pHERV-$W_{env\ 486\text{-}504}$-specific T-cells by intracytoplasmic cytokine expression assay. PBMCs were stimulated for eight hours with the identified peptides in the presence of anti-CD28/CD49d antibody and BFA. Gated to $CD3^+CD8^+$ cells, the expression of IFN-γ, GM-CSF, TNF−α and IL-17 in $CD4^+$ T cells (left) and $CD8^+$ T cells (right) was measured in RR-SMS and PP-MS. UL and UR quadrants report the % positivity. PBMCs: peripheral blood mononuclear cells; BFA: Brefeldin A; IFN-γ: interferon gamma; GM-CSF: Granulocyte-macrophage colony-stimulating factor; TNF: tumor necrosis factor alpha; IL: interleukin.

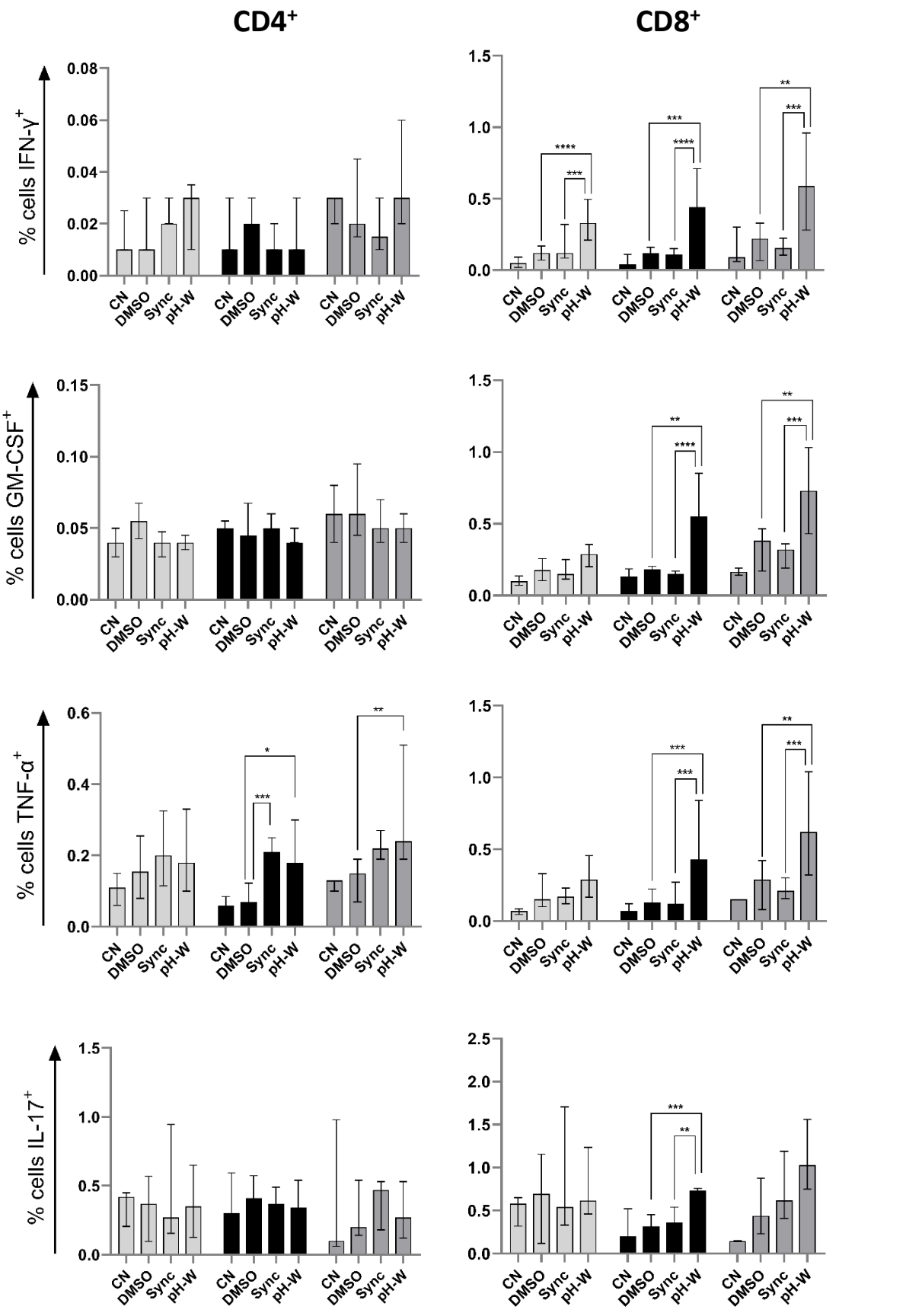

CD4+
CD8+
% cells IFN-γ+
% cells GM-CSF+
% cells TNF-α+
% cells IL-17+
CN
DMSO
Sync
pH-W
HC
RR-SMS
PP-MS
*p = 0.05; **p = 0.04-0.03; ***p = 0.02-0.01; ****p < 0.01

Figure 25.

Detection of CN, DMSO (vehicle-control), Sync, and *p*H-W-specific cytokine-positive T-cells by intracytoplasmic cytokine expression assay. Percentage of cytokine-positive CD4 (left) and CD8 (right) in HC, RR-SMS and PP-MS. The figures display the median with interquartile range. Mann–Whitney test and Kruskal–Wallis with Dunn's test. $p \leq 0.05$ was considered statistically significant. CN: negative control; DMSO: Dimethyl sulfoxide; Sync: syncytin-$1_{env\ 486\text{-}500}$; *p*H-W: *p*HERV-$W_{env\ 486\text{-}504}$; PBMCs: peripheral blood mononuclear cells: IFN-γ: interferon-gamma; GM-CSF: Granulocyte-macrophage colony-stimulating factor; TNF: tumor necrosis factor-alpha; IL: interleukin.

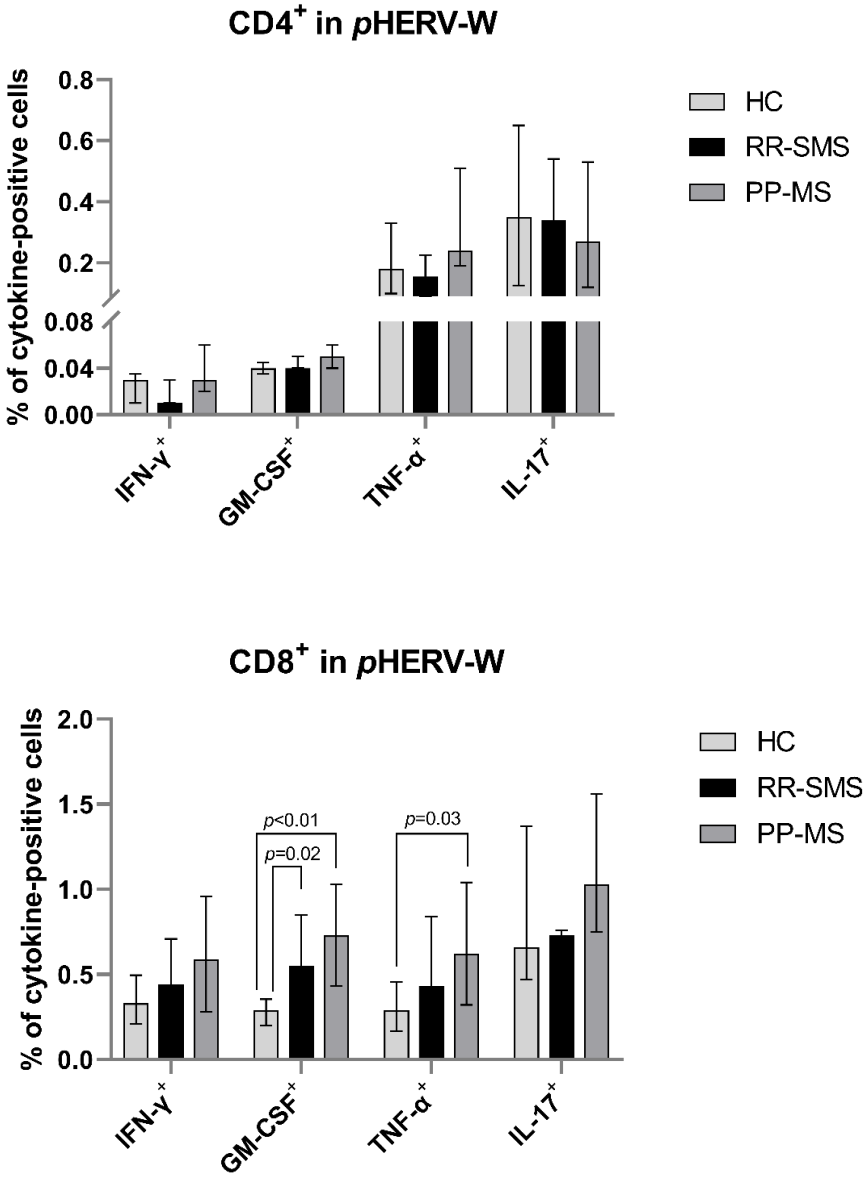


Figure 26.

Cytokine-positive CD4 (above) and CD8 (below) detection in PBMCs of HC, RR-SMS and PP-MS. Percentage of $CD4^+$-$CD8^+$/T cells secreting IFN-γ, GM-CSF, TNF-α and IL-17 after 8 h incubation with *p*HERV-$W_{env\ 486\text{-}504}$. Mann–Whitney test and Kruskal–Wallis with Dunn's test. $p \leq 0.05$ was considered statistically significant.

CD8⁺ T-Cells Stimulation

The same conditions studied in CD4 T-cells were evaluated for CD8. PBMCs from MS patients, exposed to *p*HERV-W, showed $CD8^-$ and $CD8^+$ T–cells expressing all cytokines at a frequency higher of 0.5% and 0.4% than DMSO and Syncytin-1, respectively (fig. 24). In RR-SMS patients was detected significant increase in the expression of all cytokines in CD8 exposed to *p*HERV-$W_{env\ 486\text{-}504}$ than DMSO and Syncytin-$1_{env\ 486\text{-}500}$ (IFN-γ= median: 0.44; P75-P25: 0.65-0.3; *p*=0.014 *vs* DMSO = median: 0.12; P75-P25:0.15-0.1, *p*=0.002 *vs.* Sync. = median: 0.11; P75-P25:0.13-0.07; GTM-CSF= median: 0.55; P75-P25: 0.80-0.40; *p*=0.04 *vs*. DMSO = median: 0.18; P75-P25: 0.19-0.13, *p*<0.001 *vs* Sync. = median: 0.15; P75-P25: 0.16-0.11; TNF-α= median: 0.43; P75-P25: 0.80-0.32; *p*=0.01 *vs.* DMSO = median: 0.13; P75-P25: 0.18-0.07, and Sync. = median: 0.12; P75-P25: 0.22-0.1; IL-17= median: 0.73; P75-P25: 0.76-0.68; *p*=0.01 *vs.* DMSO = median: 0.32; P75-P25: 0.43-0.20, *p*=0.03 *vs.* Sync. = median: 0.36; P75-P25: 0.52-0.22) (fig. 25). Significant increments were detected in PP-MS patients in the expression of IFN-γ, GTM-CSF and TNF-α cytokines in CD8 exposed to *p*HERV-$W_{env\ 486\text{-}504}$ than DMSO and Syncytin-$1_{env\ 486\text{-}500}$ (IFN-γ= median: 0.59; P75-P25: 0.8-0.35; *p*=0.03 *vs.* DMSO = median: 0.22; P75-P25: 0.31-0.07, *p*=0.01 *vs.* Sync. = median: 0.15; P75-P25: 0.21-0.12; GTM-CSF= median: 0.73; P75-P25: 0.88-0.51; *p*=0.04 *vs.* DMSO = median: 0.38; P75-P25: 0.45-0.23, *p*=0.02 *vs.* Sync. = median: 0.32; P75-P25: 0.35-0.02; TNF-α = median: 0.62; P75-P25: 0.85-0.44; *p*=0.04 *vs.* DMSO = median: 0.29; P75-P25: 0.37-0.08, *p*=0.02 *vs.* Sync. = median: 0.21; P75-P25: 0.29-0.16). In spite of the non-significant results, a positive trend emerged in the IL-17 expression by $CD8^+$ T-cells exposed to *p*HERV-$W_{env\ 486\text{-}504}$ than DMSO and Syncytin-$1_{env\ 486\text{-}500}$. In HCs significant difference was observed in IFN-γ production by CD8 exposed to *p*HERV-$W_{env\ 486\text{-}504}$ than DMSO and Syncytin-$1_{env\ 486\text{-}500}$ (IFN-γ = median: 0.34; P75-P25: 0.41-0.26; *p*≤0.01 *vs*. DMSO = median: 0.12; P75-P25: 0.16-0.07; *p*≤0.01 *vs.* Sync. = median: 0.12; P75-P25: 0.22-0.09). In addition, the levels of cytokines produced by $CD8^+$, exposed to *p*HERV-$W_{env\ 486\text{-}504}$, were significantly different between HC, RR-SMS and PP-MS (fig. 26). $CD8^+$ T-cells in PP-MS patients exhibited a notable increase in GM-CSF (+0.42%; *p*<0.01) and TNF-α (+0.34%; *p*=0.03) levels than HC. Similarly, in RR-SMS patents a significant increase of GM-CSF levels (+0.30%; *p*=0.02) than HC was detected.

CD19⁺ B-Cells Stimulation

Intracytoplasmic cytokines levels were detected in CD19 B-cells exposed to peptides syncytin-$1_{env\ 486\text{-}500}$ and *p*HERV-$W_{env\ 486\text{-}504}$ (fig. 27). In RR-SMS patients were detected significant increase in CD19 expression of TNF-α and IL-17 exposed to *p*HERV-$W_{env\ 486\text{-}504}$ than DMSO and Syncytin-$1_{env\ 486\text{-}500}$ (TNF-α = median: 4.21; P75-P25: 4.67-2.67; p=0.05 *vs.* DMSO = median: 1.4; P75-P25: 2.19-1.07, p<0.01 *vs.* Sync. = median: 1.24; P75-P25: 1.61-1.2; IL-17= median: 1.28; P75-P25: 1.56-1.00; p=0.05 *vs.* DMSO = median: 0.59; P75-P25: 0.68-0.46, p<0.01 *vs.* Sync. = median: 0.62; P75-P25: 0.95-0.6), and an increase in the expression of GM-CSF than DMSO (GM-CSF= median: 0.96; P75-P25: 1.01-0.86; p=0.05 *vs.* DMSO = median: 0.34; P75-P25: 0.54-0.24) (fig. 28). Significant increment was detected in PP-MS patients in the CD19 expression of TNF-α cytokine, exposed to *p*HERV-$W_{env\ 486\text{-}504}$ than DMSO and Syncytin-$1_{env\ 486\text{-}500}$ exposure (TNF-α = median: 2.96; P75-P25: 4.08-2.36; p=0.01 *vs.* DMSO = median: 1.19; P75-P25: 1.23-1.03, p=0.05 *vs.* Sync. = median: 1.76; P75-P25: 2.09-1.75). Regarding the HCs, under none of the conditions tested was detected a significant increase in cytokine production by $CD19^{+}$. Furthermore, the levels of cytokines produced by $CD19^{+}$ exposed to pHERV-$W_{env\ 486\text{-}504}$ were significantly different among HC, RR-SMS and PP-MS (fig. 29). CD19+ T-cells in PP-MS patients exhibited a notable increase in GM-CSF (+1.03%; p<0.01) levels than HC. Similarly, in RR-SMS patents a significant increase of TNF-α levels (+1.87%; p=0.05) than HC was detected.

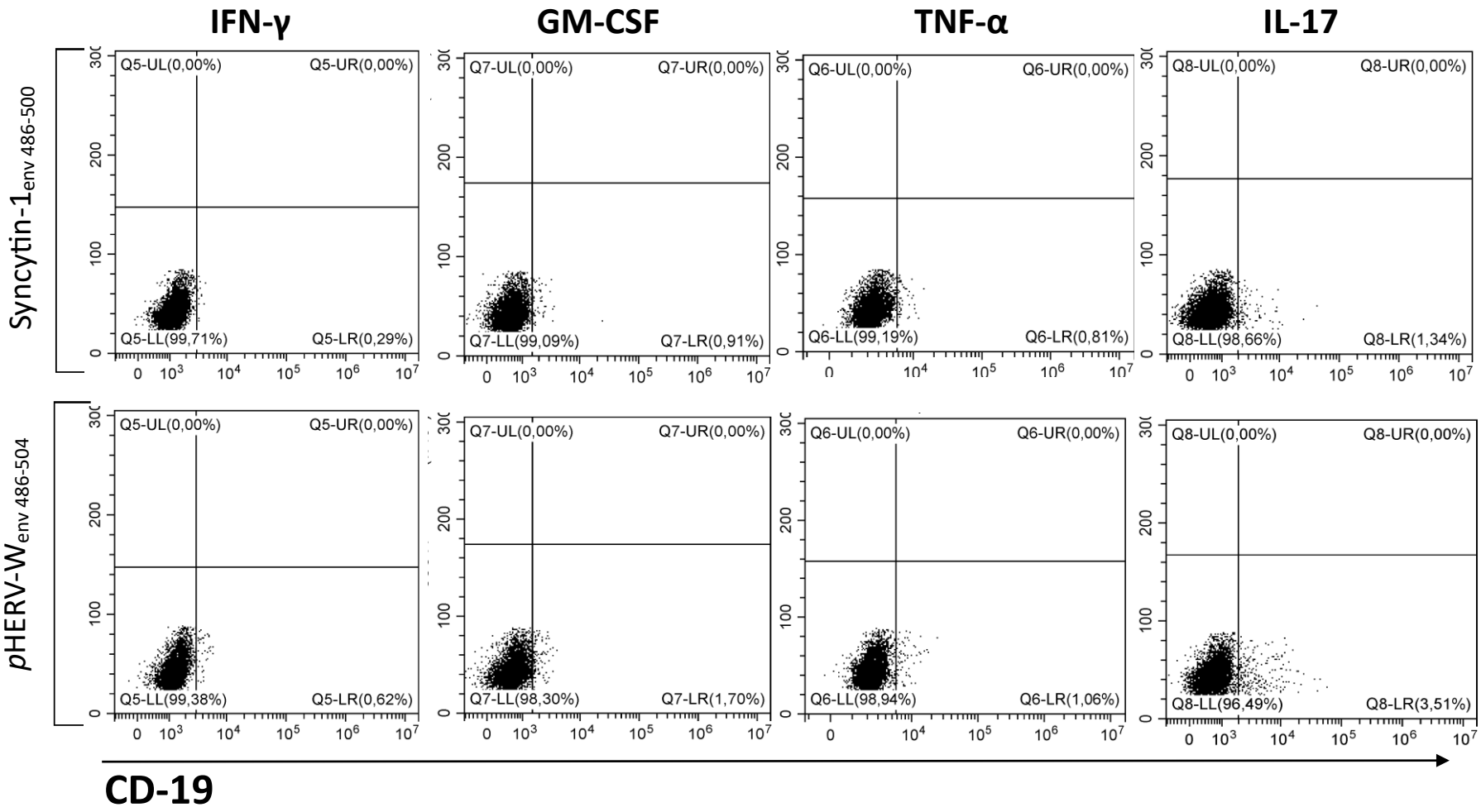


Figure 27.

Detection of Syncytin-$1_{env\ 486\text{-}500}$ and *p*HERV-$W_{env\ 486\text{-}504}$-specific B-cells by intracytoplasmic cytokine expression assay. PBMCs were stimulated for eight hours with the identified peptides in the presence of anti-CD28/CD49d antibody and BFA. Gated to $CD19^{+}$ cells, the expression of IFN-γ, GM-CSF, TNF−α and IL-17 was measured in RR-SMS and PP-MS. LR quadrant report the % positivity. PBMCs: peripheral blood mononuclear cells; BFA: Brefeldin A: IFN-γ: interferon gamma; GM-CSF: Granulocyte-macrophage colony-stimulating factor: TNF: tumor necrosis factor alpha: IL: inter leukin.

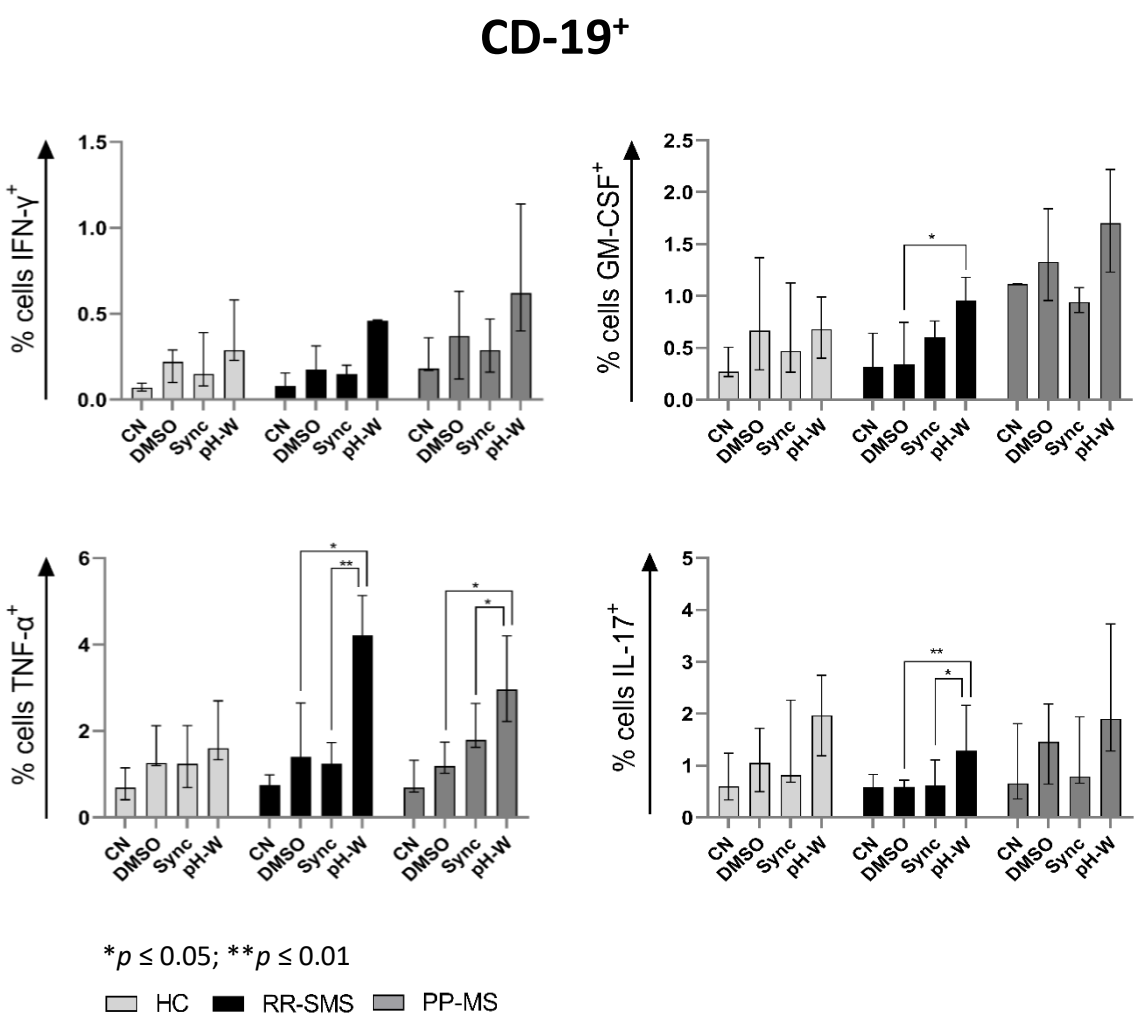


Figure 28.

Detection of CN, DMSO (vehicle-control), Sync and *p*H-W-specific cytokine-positive B-cells by intracytoplasmic cytokine expression assay. Percentage of cytokine-positive CD19 in HC, RR-SMS and PP-MS patients. The figures displaying the median with interquartile range. Mann–Whitney test and Kruskal–Wallis with Dunn's test. $p \leq 0.05$ was considered statistically significant. CN: negative control; DMSO: Dimethyl sulfoxide; Sync: syncytin-$1_{env\ 486\text{-}500}$; pH-W: pHERV-$W_{env\ 486\text{-}504}$; PBMCs: peripheral blood mononuclear cells; IFN-γ: interferon gamma; GM-CSF: Granulocyte-macrophage colony-stimulating factor; TNF: tumor necrosis factor alpha; IL: interleukin.

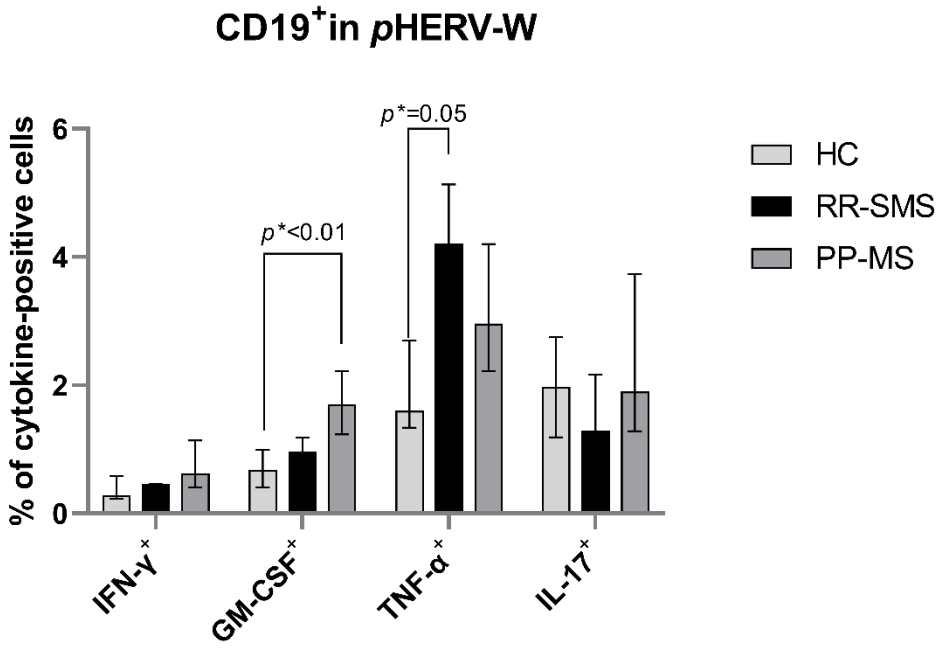
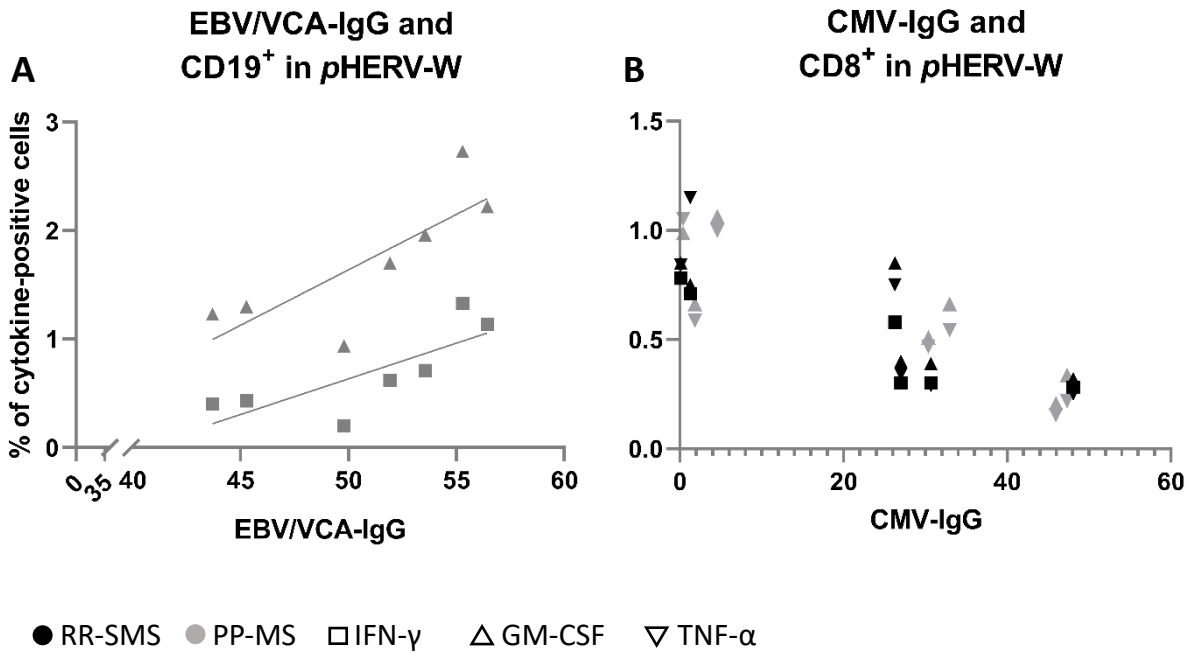


Figure 29.

Cytokine-positive CD19 detection in PBMCs of HC, RR-SMS and PP-MS. Percentage of $CD19^+$/B cells secreting IFN-γ, GM-CSF, TNF-α and IL-17 after 8 h incubation with *p*HERV-$W_{env\ 486\text{-}504}$. Mann–Whitney test and Kruskal–Wallis with Dunn's test. $p \leq 0.05$ was considered statistically significant.

Epstein-Barr, Cytomegalovirus IgG and *p*HERV-W B/T-cells cytokine expression

Correlation analysis showed a high degree of correlation between EBV/VCA, CMV-IgG antibody titers and the *p*HERV-$W_{env\ 486-504}$ intracytoplasmic cytokine-positive CD19 and CD8, evaluated in B/T cells of RR-SMS and PP-MS patients (fig 30A/B).



A1

EBV/VCA-IgG	**PP-MS**	
vs	*IFN-γ⁺*	*GM-CSF⁺*
r	0.857	0.857
R^2	0.609	0.632
***p*-value**	0.024*	0.024*

B1

CMV-IgG	**MS**		**RR-SMS**			**PP-MS**	
vs	*GM-CSF⁺*	*TNF-α⁺*	*IFN-γ⁺*	*GM-CSF⁺*	*TNF-α⁺*	*GM-CSF⁺*	*TNF-α⁺*
r	-0.809	-0.901	-0.985	-0.898	-0.943	-0.775	-0.893
R^2	0.663	0.760	0.789	0.588	0.744	0.761	0.788
***p*-value**	0.001*	<0.001*	0.006*	0.028*	0.017*	0.049*	0.012*

Figure 30.

Scatter plot showing the correlations between the EBV/VCA-IgG (A) and CMV-IgG (B) antibody titers and the *p*HERV-W cytokine-positive CD-19 and CD-8, respectively, in RR-SMS and PP-MS patients. Below, nonparametric Spearmen correlation coefficient (*r*) and R^2 from simple linear regression analysis, both for the entire population (MS) and the two MS clinical forms (RR-SMS and PP-MS), with regard to EBV/VCA-IgG (A1) and CMV-IgG (B1). $p \leq 0.05$ was considered statistically significant.

Significant correlations were detected between EBV/VCA-IgG antibody titers and the *p*HERV-$W_{env\ 486\text{-}504}$ cytokine-positive CD19 evaluated in B cells of PP-MS patients (fig 28A). High degrees of correlations were detected for IFN-γ and GM-CSF-positive cells in PP-MS (fig. 30A.1). Otherwise, correlation analysis showed an opposite and high degree of correlation between IFN-γ, GM-CSF and TNF-α levels in RR-SMS and for GM-CSF, TNF-α in PP-MS, *vs.* CMV-IgG antibody titers (fig. 30B.1). No correlation was found in HC, nor with syncytin-$1_{env\ 486\text{-}500}$ cytokine-positive CD8 and CD19 cells for control and patient populations.

DISCUSSION

- *IgG Humoral response levels of anti-pHERV-W$_{env\ 486-504}$/anti-Syncytin-1$_{env\ 486-500}$ are significantly higher in MS patients, both acute relapsing and progressive forms, with respect to HC and RR-MS in stable inflammatory phase. This fact would support the involvement of pHERV-W env/syncytin-1 in MS pathogenesis and exacerbation.*

One of the aims of this study was to analyze the serology of *p*HERV-W envelope protein(s) in patients with MS categorized according to their disease activity. The results suggest that the levels of *p*HERV-W antibodies titers were higher in the serum of MS than HC both in Italian and Spanish population. In addition, the increased IgG humoral response against *p*HERV-W$_{env\ 486-504}$/Syncytin-1$_{env\ 486-500}$ observed in the group of patients with acute inflammatory phase (RR-AMS) and progressive clinical forms compared with the RR-SMS group could confirm the involvement of *p*HERV-W env/syncytin-1 in MS exacerbation.

Several studies investigating the immunopathogenic properties of *p*HERV-W env proteins have revealed their ability to trigger an inflammatory response and a neurodegenerative phenotype in MS[81]. These studies suggest that *p*HERV-W env can promote the diapedesis, activation and infiltration of immune cells, thereby preventing the remyelination and stimulating the release of pro-inflammatory cytokines by lymphocytes, monocytes, macrophages, and microglia[82,83]. *In vitro* studies have shown a strong molecular recognition between the anti-*p*HERV-W env antibodies and epitopes of oligodendrocyte glycoproteins (MOG) epitopes[84]. Animal studies have further supported these findings, demonstrating that *p*HERV-W env can activate the EAE model when combined with the MOG$_{35–55}$ peptide[72]. Moreover, in MS patients, the antibody response to HERV-W env peptides was found to be superior to that of patients with neuromyelitis spectrum disorder (NMOSD) positive for antibodies to aquaporin-4[85]. Similarly to another retrovirus, the HERV-Fc1 *gag* gene expression and its extracellular RNA viral loads were significantly higher in patients with RR in acute phase (AMS) compared to those stable phase (RR-SMS) and HCs. *De facto*, the observed patterns of episodic attacks and periods of recovery in patients with AMS and progressive forms are similar to those seen in retroviral infections[86].

The findings of this study suggest that the link between the *p*HERV-W and disease severity may be explained by the variations in amino acid sequences compared to its homologous

protein, syncytin-1. Consistent with these findings, previous studies have shown that *p*HERV-W env gene expression levels were higher than syncytin-1 in RR-MS and PP-MS patients[76].

Although the structural similarities between the two env proteins are significant, with 94% homology, they exhibit distinct functional characteristics. For instance, while syncytin-1 has fusogenic properties, the domain of *p*HERV-W env lacks these characteristics. It is important to note that only *p*HERV-env can be observed both membrane-associated or soluble forms, forming monomers trimers or hexamers[87,88].

Several studies have reported that genetic elements could contribute to the persistence and development of MS[27,29]. In Europe, the geographical distribution of MSRV copies has been associated with the prevalence of MS. In accordance with the results of this study, the small geographic community of Sardinia showed a higher number of MSRV copies compared to other areas studied in Italy, Spain and Europe[89.] Arru *et al.* (2007) have detected over 2.000 HERV-W/MSRV copies *per* ml in MS patients from Sardinia and about 70 in the Spanish patients. The same trend was observed in healthy controls, detecting 312 HERV-W/MSRV copies *per* ml in the Sardinian population, while 15 in the Spanish participants[89]. This high difference might explain the large difference observed in the humoral response between the two populations analyzed in this study (fig.12). The presence of several thousand copies of the HERV-W and MSRV in Sardinia may have been caused by the region's geographic isolation. Therefore, to minimize the evolutionary pressure factor, the present study focused on the relationship between *p*HERV-W and syncytin-1 env proteins.

The ratio (anti-pHERV-$W_{env\ 486–504}$/anti-Syncytin-$1_{env\ 486–500}$) analysis revealed a difference between RR-SMS and -AMS cases in Spanish individuals, but not in the population of Sardinia (fig. 14). In the Spanish MS population, a significant increase of humoral response was detected in both the acute phase and progressive forms of the disease (fig. 16).

The observed difference in the ratio analysis between RR-SMS and -AMS cases in Spanish individuals, but not in the population of Sardinia, further suggests that there may be distinct genetic or environmental factors influencing the expression of *p*HERV-W and syncytin-1 env proteins in these populations. For instance, the ERVWE2 *locus* on chromosome X has been proposed for the genomic origin of the MSRV env proteins, *p*HERV-W[73]. Genotyping analysis of ERVWE2 insertion detected several polymorphisms and susceptibility variants in a large group of individuals, reporting a significant association with MS susceptibility and

polymorphisms rs1290413 and rs6622139. These polymorphisms would induce variations in *p*HERV-W amino-acid sequence with a hydroxyl (polar) group by an aliphatic residual, leading to a protein's tridimensional structure changing, that might affect its antigenic properties by making the epitopes more accessible to immune cells. Moreover, the changes in the sequence of the RNA could affect its stability and affect the level of RNA[90]. In addition, the higher number of copies of the HERV-W/MSRV gene in Sardinian population[89] could mask the increase in humoral levels between the stable and acute phases of RR-MS observed in Spanish patients. On the other hand, in the Spanish MS population, there may be a heightened immune response targeting *p*HERV-W proteins, contributing to the increased neuroinflammatory cascade and oligodendrocyte death observed in progressive forms of MS[13,15]. However, the anti-Human (FC-specific) IgG used in the study of Spanish population was slightly different from that employed in the Sardinia investigation (whole molecule). Binding to the FC portions of the antibody may provide a more truthful result, minimizing the possible background noise given by incorrect binding.

Furthermore, longitudinal studies have shown that the presence of *p*HERV-W in the CSF of early MS patients is linked to higher relapse rates, worse EDSS scores, and a higher likelihood of reaching secondary progression[81]. Additionally, an upward trend in *p*HERV-W humoral response was detected in RR-MS patients in the stable phase with EDSS score greater than 4, indicating people with relatively severe disability (fig. 17). This suggests that the presence of *p*HERV-W may serve as a biomarker for disease severity and progression in MS.

- *Patients with acute and progressive phase MS showed a major prevalence of antibodies to EBV infection. In addition, patients with relapsing remitting and primary progressive MS had higher IgG antibody titers against HVV-6 and EBV infections, supporting the idea that viral elements may contribute to the MS evolution.*

MS has been associated with various viruses over time, such as *Herpesvirus 6* (HHV-6) and *Epstein-Barr virus* (EBV). The evidence supporting the link between EBV and neuroinflammation has been consistent. Common patterns in the epidemiology of MS and infectious mononucleosis have been observed[41]. A longitudinal study revealed that young

adults who were EBV-negative at the baseline of the study did not develop MS symptoms until several months after the infection[91]. In addition, a meta-analysis conducted from 1960 to 2012 found that in MS patients, the presence of anti-EBNA (EBV nuclear antigens) and anti-VCA (EBV viral capsid antigen) IgG antibodies was associated with an increased risk of developing the condition[92]. Finally, there is significant evidence that high levels of EBV seroprevalence in pediatric MS play a unique role in the development of this condition[93]. In accordance with that, quantitative results in the Spanish MS population revealed higher frequencies of anti-EBNA-1 IgG positive patients with RR-AMS (100%) and PP-MS (97%) compared to HCs (86%). Similarly, qualitative analysis detected greater levels of anti-EBNA-1 IgG in RR-MS, both in stable (21.6 AU) and acute (22.2 AU) phases, compared to HCs (17.6 AU).

Another virus associated with MS is HHV-6. The neurotropic HHV-6 virus may play more active role in the development of MS lesions[94]. Studies have reported higher serum levels of anti-HHV-6 antibodies in MS patients compared to HCs[95]. Various hypotheses have been proposed to explain how HHV-6 infection can trigger the development of MS, including molecular mimicry, viral inhibition, bystander effects, and failure of myelin repair [96].

In the results of this study, the frequencies of anti-HHV-6 IgM positive and IgG positive individuals detected in the Spanish population with MS showed no difference from HCs subjects, for all categories. However, quantitative analysis revealed higher levels of anti-HHV-6A/B IgM in RR-AMS (23 AU) and anti-HHV-6A/B IgG in RR-MS, both in stable (23.5 AU) and acute (24.2 AU) phases, as well as in PP-MS (26.5 AU) compared to HCs (IgM 15.8 AU, IgG 18.5 AU)

Several studies have reported the activation of HERV-W and MSRV-associated genes by HHV-6 and EBV viral infections, which stimulate the dormant sequences of the HERV genes. Epigenetic dysregulations affecting the expression of the HERV gene are known to occur in various forms of chronic and neurodegenerative disorders. The regulation of HERVs expression may be defective due to accumulated recombination and mutation through evolution[97]. *In vitro* studies conducted on EBV revealed that its major envelope protein, gp350, can activate the transcription of syncytin-1 and MSRV genes, both in astrocytes and PBMCs[98]. Regarding the HHV-6 virus infection, a positive correlation was observed between the levels of the *p*HERV-W protein expression and the levels of anti-HHV-6A/B IgG antibodies levels in

NK cells and T-lymphocytes [99], supporting the positive association between *p*HERV-W and HVV-6A/B IgG and IgM humoral response detected in this study.

Therefore, it has been suggested that interactions between various viral risk factors and the host's environmental and genetic components, such as ethnicity[89] or alteration in HLA class II genes[26,27], may trigger disease progression and/or onset[96].

- *Humoral response against pHERV-W$_{env\ 486\text{-}504}$/Syncytin-1$_{env\ 486\text{-}500}$ combined with clinical and demographic variables allowed the design of mathematical and statistical models aimed at differentiating MS remissive and progressive forms.*

The ability to differentiate between classes of MS individuals could be possible by combining various clinical variables. The development of a classification method for MS using a noninvasive approach was demonstrated by the results of the study. Several clinical factors, including EDSS, age, duration of the disease, and humoral response, allowed for the creation of a high-performing logistic model. In all cases studied, the ratio showed a *odds ratio* greater than *1*, supporting the positive association between *p*HERV-W humoral response and progressive forms of MS compared with relapsing MS. Therefore, the development of a logistic model would provide a rationale for establishing that the distinction between relapsing and progressive MS can also be based on a combination of demographic and clinical factors. The use of such a model can help clinicians better understand the disease trajectory of individual patients, potentially leading to more tailored treatment approaches. In addition, the model would identify MS-individuals who have been misclassified, providing valuable information about the course of the disease.

Principal component analysis allowed us to identify spatial covariance patterns of MS factors in a sample of RR-SMS and progressive MS patients. Three principal components (PCs) explained 77.9% of the total variance. The first PC, accounting for 40.6% of the variance, was characterized by only positive loadings, with a minority contribution from EBV/VCA-IgG variable coefficient (ROI eigenvectors). The second PC, explaining 20.6% of the variance, displayed both positive and negative loadings, while PC3, representing 16.7% of the variance,

also showed positive and negative loadings, with the strongest contributions corresponding to ROIs by EDSS, Age and Duration of illness.

Exploratory analyses first examined group differences in the PC subjects' scores of MS patients, detecting significant differences and the largest effect size in PC1. In addition, a modest positive and significant trend (r = 0.27) was detected between the PC1 subjects' scores and ratio (fig. 23A.1). These findings suggest that the loadings in PC1 are positive indicators of the progression of MS, in accordance with several previous studies[101,102]. Moreover, the positive correlation between PC1 subjects' scores and the anti-*p*HERV-W humoral response supports the idea that the latter could play a role in disease progression[65,102].

A moderate effect size in the progressive group was detected in PC3 spatial variance patterns, with significant differences in PC3 subjects' scores. Exploratory analyses on PC3 detected negative contributions by EBV/VCA-IgG and CMV-IgG loadings, with significant and positive PC3 subjects' scores of SP-MS patents compared to PP-MS and RR-SMS. In accordance with that, Farrell et al. (2009) revealed in PP-MS patients lower levels of anti-EBNA-1 but higher anti-VCA IgG levels compared to those with RR-MS[103]. This study also found significantly positive levels of anti-EBNA-1 IgG in RR-MS, while positive levels of anti-VCA IgG, combined with other MS factors, appear to be linked to the primary progressive variant. Moreover, studies have detected that the presence of grey matter atrophy was associated with the anti-EBV/VCA antibody status of the MS, with higher T2 and T1 lesion volumes[104]. This finding supports the results of a previous study analyzing data collected from high-risk individuals with CIS, which revealed that the highest levels of anti-EBV/VCA antibodies were associated with a reduction in the volume of *thalamus* and cortical volume[105]. Thalamic volume loss is associated, in turn, with disability progression, as measured by increased EDSS score, and cognitive impairment[106].

The role of CMV in MS is more controversial. It may play a harmful or beneficial role in MS pathology and exacerbation. On the one hand, the expansion and activation of specific T cells by CMV infection can contribute to the development of MS pathology. On the other hand, immune evasion of CMV could potentially contribute to minimizing the proinflammatory environment that may, in turn, contribute to the development of MS[100]. Several studies conducted on CIS patients revealed that those with high levels of CMV seropositivity were more prone to experiencing relapses[107] and deep gray-matter atrophy[105]. Consequently, CMV

seropositivity was associated with MS progression[108]. Conversely, CMV infection may trigger anti-inflammatory responses that could reduce the severity of the disease[109]. It has been reported that being infected with the CMV during childhood can reduce the risk of developing CIS or MS in young adults[110]. Therefore, since age is the most common factor that may contribute to the development of progressive MS, several researchers have speculated that changes in the immune system due to aging may be related to the disease[111]. Among the infectious agents that can trigger chronic latent infection is CMV. Therefore, the development of immune senescence combined with the age of CMV infection could explain the dual effects of CMV on MS[111].

- *The increased proinflammatory response to pHERV-W$_{env\ 486\text{-}504}$ exposure compared with syncytin-1$_{env\ 486\text{-}500}$ reinforces the idea of direct involvement to neuroinflammation by pHERV-W in MS*

Further aim of this study was to investigate the T and B-cell-mediated specific responses against endogenous peptides derived from Syncytin-1 and *p*HERV-W antigens. MS has traditionally been regarded as mediated by the T-cells, specifically CD4 T-cells, mainly due to the genetic connection between MS and the Major histocompatibility complex (MCH) class II region[112]. Nevertheless, several studies have revealed that CD8 T-cell and CD19 B-cells lymphocytes play a key role in the humoral response[113,114].

In this study, the main pro-inflammatory cytokines were studied after peptide stimulation. TNF-α is known to be a major mediator of inflammatory and immunological responses in MS, leading to the destruction of oligodendrocyte and myelin in the CNS[115]. Cytometric analyses detected a significant increase of TNF-α in B and T-cells lymphocytes after *p*HERV-W exposure, both in RR-SMS and PP-MS patients. Furthermore, TNF-α levels detected were higher in MS patient than in HCs, primarily attributable to $CD8^+$ T and $CD19^+$ B-cells.

In all population studied, *p*HERV-W triggered an increase IFN-γ response, with the highest level recorded for PP-MS patients. Several studies show that IFN-γ is a significant factor that could contributes to the development of MS, with production increasing before relapse stages[116,117]. In addition, some studies suggest that the presence of IFN-γ, together with other types 1

cytokines, may enhance the function of some $CD4^+$, $CD8^+$ and $CD14^+$ cells in patients undergoing disease reactivation[118]. On the other hand, IFN- γ is one of the most important factors that plays a key role in the treatment of viral infections by activation of cytotoxic T cells and stimulating cytokine production in T lymphocytes[119]. Therefore, the increase in IFN- γ detected in $CD8^+$ T-cells of HCs following *p*HERV-W exposure aligns with viral infection response.

GM-CSF, a cytokine implicated in the development of EAE-associated inflammation, was also examined[120]. Cytometric analyses detected a significant increase of GM-CSF levels in $CD8^+$ T-cells lymphocytes after *p*HERV-W exposure in RR-SMS and PP-MS patients. In addition, GM-CSF levels, primarily attributable to $CD8^+$ T-cells, were found to be higher in all MS patients compare to HCs. Higher GM-CSF levels in *p*HER-W-response was also detected in $CD19^+$ B-cell in RR-MS patients, while GM-CSF were higher in PP-MS patients than HCs. Several studied have reported that the presence of specific subsets of highly pro-inflammatory memory B cells, such as $CD19^+$ and $CD27^+$ [121] which secrete IFN- γ, IL-6 and GM-CSF, in MS patients compared with HCs[122]. The B and T cells that can produce GM-CSF are more common in patients with MS, increasing the proinflammatory responses[123]. Studies on EAE model have shown that treatment with antibodies against the receptor of GM-CSF can prevent or reduce the onset of the disease[124]. According to Rasouli *et al.* (2015), the presence of GM-CSF-producing $CD8^+$ T cells in peripheral blood was higher in patients with MS than in the healthy individuals. Although majority of these cells were co-producing IFN- γ, only GM-CSF-producing $CD8^+$ cells were identified[125].

More controversial is the role of IL-17 in the inflammatory condition. The complex feedback loops produced by IL-17 through its signaling pathways can either amplify or decrease the inflammatory response[126]. In animal models of stroke (brain ischemia) and multiple sclerosis (EAE), IL-17 promotes neurodegeneration and neuroinflammation[127,128]. In 2016, Kumar and colleagues noted that increasing the number of IL-17 T-cells$^+$ during intestinal dysbiosis can exacerbate the development of CNS-targeted autoimmune diseases[129]. Activation of CNS-resident cells by IL-17 could leads to neurons hyperexcitability,[130] accompanied by increased production of cytokines and chemokines by oligodendrocytes and astrocytes,[131] triggering the neuroinflammation. In this study, the intracytoplasmic cytokine expression assay detected a significant increase of IL-17 levels in $CD8^+$ T-cells and $CD19^+$ B-cells lymphocytes in RR-MS,

following *p*HER-W exposure. The role of IL-17A in the pathophysiology of MS has gained attention due to its ability to promote human $CD4^+$ T cell migration through a blood-brain barrier[132]. Therefore, the migration of these cells across the barrier could explain the reduced $CD4^+$ response in the peripheral blood of MS patients detected in this study.

The activation of the Toll-like receptor 4 (TLR4) and the coreceptor CD14 by *p*HERV-W the is the main factor that contributes to the development of the inflammatory response in the blood of MS patients[133,134]. Several types of cells can express TLRs including B-cells[135], monocytes, astrocytes[136], dendritic cells (DCs), and oligodendrocytes[137], which play a role in the body's innate immune response[138,139]. Exposure to *p*HERV-W can activate the TRL-4 pathway, leading to the release of inflammatory cytokines, as IL-6, TNF, IL-12 and GM-CSF[133]. DCs activation by TRL-4 upregulates the antigen presentation, activating specific T-cell and abnormal polyclonal T-cell in which *p*HERV-W act as superantigen resulting in massive cytokine release[133,140]. GM-CSF appears to promote the lipopolysaccharide (LPS) production, an agonist of TRL-4[141], leading to the upregulation of TLR-4 and CD14 expression and activation of an inflammatory phenotype by Th1 cells[142]. In turn, activated Th-cell secreting TNF-α an IFN-γ, which upregulate *p*HERV-W secretion by PBMCS[143]. Finally, the inflammatory state induced by *p*HERV-W activates vascular endothelial cells by overexpression of ICAM-1, a mediator of leukocyte adhesion to endothelial cells, thus enhancing the migration of circulating cells towards the CNS[144]. In accordance with this, Okada *et al.* (2018) showed in a *vitro* study conducted on the peripheral blood samples of MS that there was a crosstalk between TLR-4 and the interleukins produced by microglia to interact with T-cell activation[145].

Therefore, the expression of the *p*HERV-W envelope protein throughout a patient's lifetime can trigger the development of an inflammatory and chronic cascade by TLR-4[146]. These changes in the expression of both *p*HERV-W env genes and specific immune cells and receptors responsible for the development of the innate immune response may contribute to explain the progression of MS[147].

- *Positive correlations were observed between proinflammatory cytokine production from pHERV-$W_{env\ 486\text{-}504}$ and anti-EBV/VCA antibody titers in patients with MS. On the other*

hand, the opposite was the case for the proinflammatory cytokines produced by pHERV-W$_{env\ 486\text{-}504}$ and anti-CMV antibodies.

Stimulation of quiescent sequences of HERV genes can result from both environmental factors and additional viral infections[57,58]. Studies have shown that EBV viral infections can activate the genes associated with MSRV[148], circumventing the inhibitory effects of viral genome methylation[149]. Data obtained from the analysis of PBMCs from MS patients show a strong link between EBV and MS *via* activation of neuropathogenic *p*HERV-W at both mRNA and protein levels[148], furthermore, as discussed above, EBV gp350 protein may trigger the transcription of the MSRV genes in the PBMCs[98]. These results support the strong positive correlation found in this study between increased proinflammatory cytokines, such as IFN-γ and GM-CSF in B-cells, following *p*HERV-W exposure, and levels of humoral IgG response against EBV/VCA in PP-MS patients. Consistent with this, a study on the Spanish MS population reported a positive correlation between the 12-month change in EBV viral load and the 12-month change in *p*HERV-W ENV gene expression in patients with RR-MS[150]. These results support the hypothesis that an EBV infection is an early trigger of future MS and, even years later, may lead to the activation of *p*HERV-W resulting in the triggering of chronic neuroinflammation[148].

The IgG CMV humoral response and $CD8^+$ T-Cell to several cytokines exhibited an opposite trend after *p*HERV-W exposure. Maintaining seropositivity to the CMV virus may reduce the severity of the response to an EBV infection[151]. It has been theorized that immune competition between the two viruses could reduce the risk of developing MS in people who are infected with both EBV and CMV[47]. Beneficial effects on MS disease from exogenous infections through the potential modulation of T-cell-mediated immunity are not uncommon[152]. The therapeutic effects of *Bacillus Calmette-Guérin* (BCG) vaccination can be seen in EAE models by expressing regulatory T-cells, as well as by differentiation of astrocytes and microglia into anti-inflammatory phenotypes[152]. Therefore, early and latent CMV seropositivity can also reduce the chances of both developing MS and increasing the EDSS score[153], by decreasing the adverse immune reaction against EBV[154,155]. Furthermore, significant and similar interactions were also studied between HHV-6A and CMV. This suggests that the EBV-based assumption could be extended to HHV-6A[41]. As discussed, the timing of CMV infection could explain the

protective aspect of the virus with respect to its involvement in MS. Several evidence indicates that breastfeeding by CMV-seropositive mothers may reduce the risk of MS in their offspring[41,156,157].

Therefore, early CMV seropositivity can reduce the severity of the EBV infection. This may, in turn, affect the expression of the *p*HERV-W protein, which may contribute to the reduction of the inflammatory response.

CONCLUSIONS

- IgG Humoral response levels of anti-*p*HERV-$W_{env\ 486-504}$/anti-Syncytin-$1_{env\ 486-500}$ are significantly higher in MS patients, both relapsing and progressive forms, with respect to HC. This fact would support the involvement of pHERV-W ENV/syncytin-1 in MS pathogenesis.

- The increased IgG humoral response against *p*HERV-$W_{env\ 486-504}$ /Syncytin-$1_{env\ 486-500}$ observed in the group of patients with progressive clinical forms and acute phase of relapsing remitting compared with the RR-SMS group could confirm the involvement of *p*HERV-W ENV/syncytin-1 in exacerbation and progression of MS.

- Patients with acute and primary progressive MS showed a major prevalence of antibodies to EBV infection. In addition, patients with relapsing remitting and primary progressive MS had higher IgG antibody titers against HVV-6A/B and EBV infections, supporting the idea that viral elements may contribute to the MS evolution.

- Humoral response against *p*HERV-$W_{env\ 486-504}$/Syncytin-$1_{env\ 486-500}$ combined with clinical and demographic variables allowed the design of mathematical and statistical models aimed at differentiating MS remissive and progressive forms.

- The increased proinflammatory response to *p*HERV-$W_{env\ 486-504}$ exposure compared with syncytin-$1_{env\ 486-500}$ reinforces the idea of direct involvement to neuroinflammation by *p*HERV-W and not by syncytin-1 in MS.

- Positive correlations were observed between proinflammatory cytokine production from *p*HERV-$W_{env\ 486-504}$ and anti-EBV/VCA antibody titers in patients with MS. On the other hand, a negative trend between proinflammatory cytokines produced by *p*HERV-$W_{env\ 486-504}$ exposure in C AAI37382.1D8-T cells and levels of anti-CMV IgG antibody responses was observed.

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ANNEXES

Annex I. Publications Related to the Ph. Thesis Presented

- **Ruberto S**, Cossu D, Sechi LA. **Correlation between antibodies against the pathogenic pHERV-W envelope protein and the inflammatory phase of multiple sclerosis.** Immunology. 2024 Feb;171(2):270-276. doi: 10.1111/imm.13712.

- Cossu D*, **Ruberto S***, Yokoyama K, Hattori N, Sechi LA. **Efficacy of BCG vaccine in animal models of neurological disorders**. Vaccine. 2022 Jan 24;40(3):432-436. doi: 10.1016/j.vaccine.2021.12.005.

Annex II. Publications related to other research work during the Ph.

In the order of time:

- Manca MA, Solinas T, Simula ER, Noli M, **Ruberto S**, Madonia M, Sechi LA. HERV-K and HERV-H Env Proteins Induce a Humoral Response in Prostate Cancer Patients. Pathogens. 2022 Jan 14;11(1):95. doi: 10.3390/pathogens11010095.
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- Simula ER, Manca MA, Noli M, Jasemi S, **Ruberto S**, Uzzau S, Rubino S, Manca P, Sechi LA. Increased Presence of Antibodies against Type I Interferons and Human Endogenous Retrovirus W in Intensive Care Unit COVID-19 Patients. Microbiol Spectr. 2022 Aug 31;10(4): e0128022. doi: 10.1128/spectrum.01280-22.
- **Ruberto S**, Santovito A, Simula ER, Noli M, Manca MA, Sechi LA. Bisphenols induce human genomic damage and modulate HERVs/env expression. Environ Mol Mutagen. 2022 Jul;63(6):275-285. doi: 10.1002/em.22499. Epub 2022 Sep 1. PMID: 36054626; PMCID: PMC9826028.
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- Manca MA, Scarpa F, Cossu D, Simula ER, Sanna D, **Ruberto S**, Noli M, Ashraf H, Solinas T, Madonia M, Cusano R, Sechi LA. A Multigene-Panel Study Identifies Single Nucleotide Polymorphisms Associated with Prostate Cancer Risk. Int J Mol Sci. 2023 Apr 20;24(8):7594. doi: 10.3390/ijms24087594.

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