University of Sassari

Department of Biomedical Sciences

Ph.D. in Life Sciences and Biotechnologies

XXXV cycle

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Investigating the Association Between HERV-K Autoantibodies and Neurodegeneration in Amyotrophic Lateral Sclerosis: A Molecular and Clinical Approach

A Thesis Submitted for The Degree of Doctor of Philosophy

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PhD Thesis

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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This thesis is based on manuscripts published during my Ph.D. and on unpublished data.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 carried out during my enrolment as a Ph.D. student at the Department of Biomedical Sciences, University of Sassari, Italy, in the period from December 1st, 2019 to February 28th, 2023.

The samples analyzed in the present study were collected from subjects referred to the Neurology Unit Clinic of the University Hospital of Sassari, from the Blood Transfusion Center of Sassari, and from the Northeast ALS Consortium of Massachusetts (NEALS-USA).

Statement of authorship

The results described in this thesis have been published in scientific international journals:

1. **Simula ER**, Arru G, Zarbo IR, Solla P, Sechi LA. TDP-43 and HERV-K Envelope-Specific Immunogenic Epitopes Are Recognized in ALS Patients. *Viruses*. 2021; 13(11):2301.

2. Garcia-Montojo, M., **Simula, E.R**., Fathi, S., McMahan, C., Ghosal, A., Berry, J.D., Cudkowicz, M., Elkahloun, A., Johnson, K., Norato, G., Jensen, P., James, T., Sechi, L.A. and Nath, A. (2022), Antibody Response to HML-2 May Be Protective in Amyotrophic Lateral Sclerosis. *Ann Neurol*, 92: 782-792.

3. **Simula ER**, Manca MA, Jasemi S, Uzzau S, Rubino S, Manchia P, Bitti A, Palermo M, Sechi LA. HCoV-NL63 and SARS-CoV-2 Share Recognized Epitopes by the Humoral Response in Sera of People Collected Pre- and during CoV-2 Pandemic. *Microorganisms*. 2020; 8(12):1993.

4. **Simula, E. R.,** Manca, M. A., Noli, M., Jasemi, S., Ruberto, S., Uzzau, S., … Sechi, L. A. (2022). Increased Presence of Antibodies against Type I Interferons and Human Endogenous Retrovirus W in Intensive Care Unit COVID-19 Patients. *Microbiology Spectrum*, *10*(4).

Preface

The thesis submission is in line with the requirements for the Ph.D. degree in Life Sciences and Biotechnologies. The research project was carried out at the Department of Biomedical Sciences, Viale San Pietro 43b, University of Sassari, Italy, and at the National Institute of Neurological Disorders and Stroke (NINDS – NIH) Neurosciences Center, 6001 Executive Blvd. Bethesda, MD 20892-9521, USA.

Abstract

Amyotrophic lateral sclerosis (ALS), is a devastating neurodegenerative disease that affects about 400,000 people worldwide. Despite decades of intensive research, the cause of ALS remains widely unknown. Several studies documented the connection between Human Endogenous Retrovirus K (HERV-K) and the development of ALS.

Given the recent findings and emerging scientific evidence that reinforces this correlation, the purpose of this study is to investigate the relationship between the autoantibodies (AAbs) against the HERV-K envelope and the pathogenesis and progression of ALS. Our study gave particular attention to the humoral response toward the most immunogenic epitopes of the envelope protein. Additionally, since microRNAs (miRNAs) play a crucial role in regulating gene expression, our study investigated their potential involvement in ALS. Although miRNAs dysregulation has been previously associated with ALS, understanding of its potential impact on HERV-K transcriptional levels is still limited. By delving deeper into these topics, our project aimed to contribute to the development of new strategies for the prevention, diagnosis, and treatment of ALS.

During my doctoral research, I had the opportunity to work on a project pertaining to the role of the humoral response against NL-63, HERVs, and Interferons in SARS-CoV-2 patients. The study was conducted during the outbreak of the SARS-CoV-2 pandemic. Its purpose was to better understand the reasons for the extremely diverse immune responses observed in the population through the detection of antibodies (Abs) that could cross-react with SARS-CoV-2 antigens or predict a more severe form of the disease.

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List of abbreviations

AAb: Autoantibody

Ab: Antibody

ACE2: Angiotensin-converting enzyme 2 receptor

AD: Alzheimer's disease

ALS-LS: ALS-Long-Survivors

ALS-ND: ALS-Newly-Diagnosed

ALS: Amyotrophic lateral sclerosis

anti-dsDNA: Anti-double-stranded DNA

BCA: Bicinchoninic acid

CA: Viral Capsid

CNS: Central Nervous System

CT: Chest Computed tomography

CTF: C-terminal fragment

DMEM: Dulbecco’s Modified Eagle’s medium

DMSO: Dimethyl sulfoxide

EBV: Epstein-Barr virus

ELISA: Enzyme-linked immunosorbent assay

EMG: Electromyography

ERV: Endogenous Retrovirus

FTLD: Frontotemporal lobar degeneration

HBV: Hepatitis B virus

HCoV: Human coronavirus

HDs: Healthy Donors

HERV : Human Endogenous Retrovirus

HIV: Human immunodeficiency virus

ICU: Intensive care unit

IEDB: Immune Epitope Database

IFN: Interferon

IFNAR: Type I interferon receptor

Ig: Immunoglobulin

IL: Interleukin

LINE: Long Interspersed Nuclear Element

LTR: Long terminal repeat

MA: Viral Matrix

miRNA: microRNA

MRI: Magnetic resonance imaging

mRNAs: Messenger RNAs

MS: Multiple Sclerosis

NC-miR: Negative Control miRNA

NC: Viral Nucleocapsid

NCS: Nerve conduction studies

NEALS: Northeast ALS Consortium

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

OD: Optical density

ORFs: Open reading frames

PBMCs: Peripheral blood mononuclear cells

PBS: Primer binding site

PPT: Polypurine Tract

PRRs: Pattern recognition receptors

RLRs: RIG-I-like receptors

RT: Reverse Transcriptase

SINE: Short Interspersed Nuclear Element

SLE: Systemic lupus erythematosus

SU: Envelope Surface

TBS: Tris-buffered saline

TDP-43: TAR DNA-binding protein 43

TLRs: Toll-like receptors

TM: Envelope Transmembrane

TMPRSS2: Transmembrane protease serine 2

TNF: Tumor necrosis factor

TPs: Time Points

Chapter I

1 Introduction

1.1 Retroviruses

Retroviruses are a unique group of viruses that are characterized by the ability to reverse-transcribe their RNA genome into DNA and integrate it into the host cell's genome. This particular life cycle has a profound impact on the biology of both the retrovirus and the host. Once inside the cell, the viral RNA genome is reverse-transcribed into DNA by the viral enzyme reverse transcriptase. The resulting DNA is then integrated into the host cell's genome by the viral integrase enzyme. This integration process is a critical step in the retroviral life cycle, as it allows the virus to persist within the host cell for an extended period. Retroviruses work backward from what we expect in biology. In fact, the Central Dogma of Molecular Biology, which states that the genetic information flows only in one direction, from DNA, to RNA, to protein, or RNA directly to protein, had to be revised when the replication of retroviruses was understood.

1.1.1 Retroviruses: genomic structure

Both endogenous and exogenous retroviruses exhibit a basic highly conserved genetic sequence consisting of four major genes: *5'-gag-pro-pol-env-3'*. The *gag* gene encodes structural proteins such as the capsid and matrix proteins. The *pro* gene encodes the protease. *Pol* gene encodes enzymes required for reverse transcription and integration, such as reverse transcriptase and integrase. Lastly, the *env* gene encodes the viral envelope glycoprotein, which facilitates virus entry into host cells.

The genes are flanked by long terminal repeats (LTRs) that serve as viral promoters and enhancers. The LTRs are essential for viral replication and transcription, as they contain binding sites for cellular transcription factors and the viral protein *Tat*, which enhances transcriptional activity.[1]

In addition to the four major genes, retroviruses also encode several accessory genes that are not essential for viral replication but play important roles in virus-host interactions. For example, the human immunodeficiency virus (HIV) encodes six accessory genes that contribute to the virus's ability to evade the host immune system and enhance viral replication.[2]

The integration of retroviruses into the host cell's genome is mediated by the viral integrase enzyme, which catalyzes the insertion of the viral DNA into the host chromosomal DNA. The integrated viral DNA (provirus) becomes a permanent part of the host cell's genome and can be passed on to daughter cells during cell division.[3]

The ability of retroviruses to integrate into the host genome has both beneficial and detrimental effects. On the one hand, it allows the virus to persist within the host for an extended period and provides a mechanism for stable, long-term gene expression.

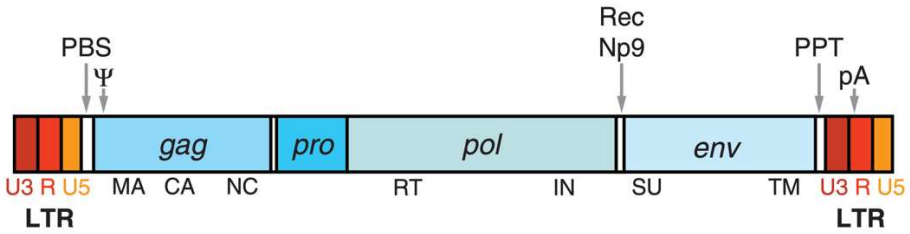

Figure 1. Organization of an integrated HERV (provirus).

The long terminal repeats (LTRs) at the 5′ and 3′ ends consist of the U3, R, and U5 regions. The R region, located within the 5'-LTR, is responsible for the initiation of transcription and the end of the R region in the 3′-LTR represents the polyadenylation signal. Upstream of the *gag* reading frame, is located the primer binding site (PBS). The polyprotein encodes for the viral matrix (MA), capsid (CA), and nucleocapsid (NC). The reverse transcriptase (RT) and integrase (IN) are part of the downstream polymerase. The surface (SU) and transmembrane (TM) units are located in the envelope. Regarding HERV-K(HML-2), it encodes accessory proteins, Rec, Np9, located in the region of the *pol/env* junction.

1.1.2 Retroviral endogenization

The integration of retroviruses into host genomes, known as endogenization, is a remarkable and unique evolutionary process. This process has occurred repeatedly throughout evolution and has

resulted in the proliferation of retroviral sequences in many animal genomes, including humans.[4] The endogenization process occurs when a retrovirus infects a germ cell, and integrates its DNA into the host genome. The resulting progeny inherit the integrated retroviral DNA, which can then be passed on to subsequent generations.[3] Over time, these integrated retroviral sequences can accumulate in the host genome and comprise a significant portion of its genetic material. For example, it has been estimated that up to 8% of the human genome is derived from retroviral sequences.[5]

The process of retroviral endogenization has important implications for the evolution of host organisms. Retroviral sequences can provide a source of genetic variation that can drive adaptive evolution by introducing new genes or regulatory elements into the host genome. It has been suggested that retroviral endogenization may have contributed to the evolution of the mammalian placenta by providing a source of novel regulatory elements.[6]

However, retroviral endogenization can also have deleterious effects on host organisms. The integration of retroviral DNA can disrupt host genes or regulatory elements, leading to the manifestation of disease or abnormalities.[7] In addition, retroviral endogenization can contribute to the development of cancer by activating oncogenes or disrupting tumor suppressor genes.[8]

Despite the potential risks associated with retroviral endogenization, it is clear that this process has been an important driver of genome evolution. In some cases, retroviral endogenization has even led to the formation of new host genes with important biological functions.[9] Thus, while retroviral endogenization can have both positive and negative effects on host organisms, it represents a key mechanism that has shaped the evolution of many animal genomes.

1.1.3 Retroviral proliferation process

The proliferation of integrated retroviral elements, known as endogenous retroviruses (ERVs), resulted in the presence of multiple copies at different chromosomal locations. Each ERV lineage

derived from a single founder virus constitutes an independent monophyletic group, which may include fixed and non-fixed proviruses. The actual proportion of fixed proviruses depends on replication activity, host population dynamics, and selective pressures. Most of the ERVs in the human genome are derived from a small number of germline colonization or selectively amplified elements. Some of these have proliferated with great success, resulting in thousands of progeny sequences that are still identifiable in the human genome. However, the accurate prediction regarding the total number of independently derived lineages and their mutual relationships remains a challenge, despite extensive phylogenetic investigations.

Several well-defined and widely accepted proliferation models and mechanisms have been proposed, although their role in amplifying a particular lineage remains controversial. The complexity of ERV proliferation is due to the interplay between various factors such as host factors, retroviral genomic features, and environmental factors. The understanding of ERV proliferation is important in elucidating their potential roles in host biology and disease.

One model that has gained attention is the *Reinfection model*, where the progeny of an endogenized virus reintegrates into the germ line of the host during a replication-competent phase. This can occur either directly from a germ-line cell or from a somatic cell that has an active provirus or has become infected. The fitness of the virus, its ability to successfully replicate and spread within a host population, may improve in response to selective pressures, resulting in an apparent increase in the preservation level of the lineage. This process is supported by evidence from HERV families with moderate and low copy numbers that have been under continuous purifying selection for more than 30 million years, indicating that their expansion was partly due to reinfection.[10] This implies the prolonged presence of replication-competent lineage members during the periods of chromosomal colonization. Most of these infectious elements were not fixed and were regularly lost from the population's gene pool, but a few have acquired inactivating mutations that increased their chance of being driven to fixation.

Another way that ERVs can proliferate is through *Retrotransposition*. There are two retrotranspositional proliferation hypotheses for ERV phylogenies. Firstly, the *Random template model* proposes that all copies of an ERV lineage can amplify, while the *Master gene model* suggests the presence of only one or a few active elements, known as master genes, that remain functional for a long time and release copies that cannot proliferate.[11] The latter model is more suitable for explaining the expansion of Short Interspersed Nuclear Element (SINE) and Long Interspersed Nuclear Element (LINE) retroelements. This mechanism apparently played a significant role in the amplification of the HERV-K (HML-3) lineage.[12]

Even if multiple genes of a provirus are deactivated, it can still propagate within the genome as long as specific regulatory sequences, such as packaging motifs, the polypurine tract, and the primer binding site, are preserved and the provirus is able to produce transcripts.

Lastly, the missing functional proteins can be replaced by proteins from other viruses or retroelements through a mechanism called *Complementation in trans*. This process does not increase the replication fitness of the provirus itself, but it allows defective viruses to amplify. Homologous recombination, gene conversion, and template switching can also reverse the inactivation of proviruses and create chimeric proviruses that are replication-competent. This can initiate cycles of ERVs' bursts, a period of increased retroviral proliferation and spread within a host population, observed in some retroviral lineages throughout primate evolution.[13] Although the entity of that contribution may be limited, ERV copy number may also increase through chromosomal duplication events and other passive amplification mechanisms.[14]

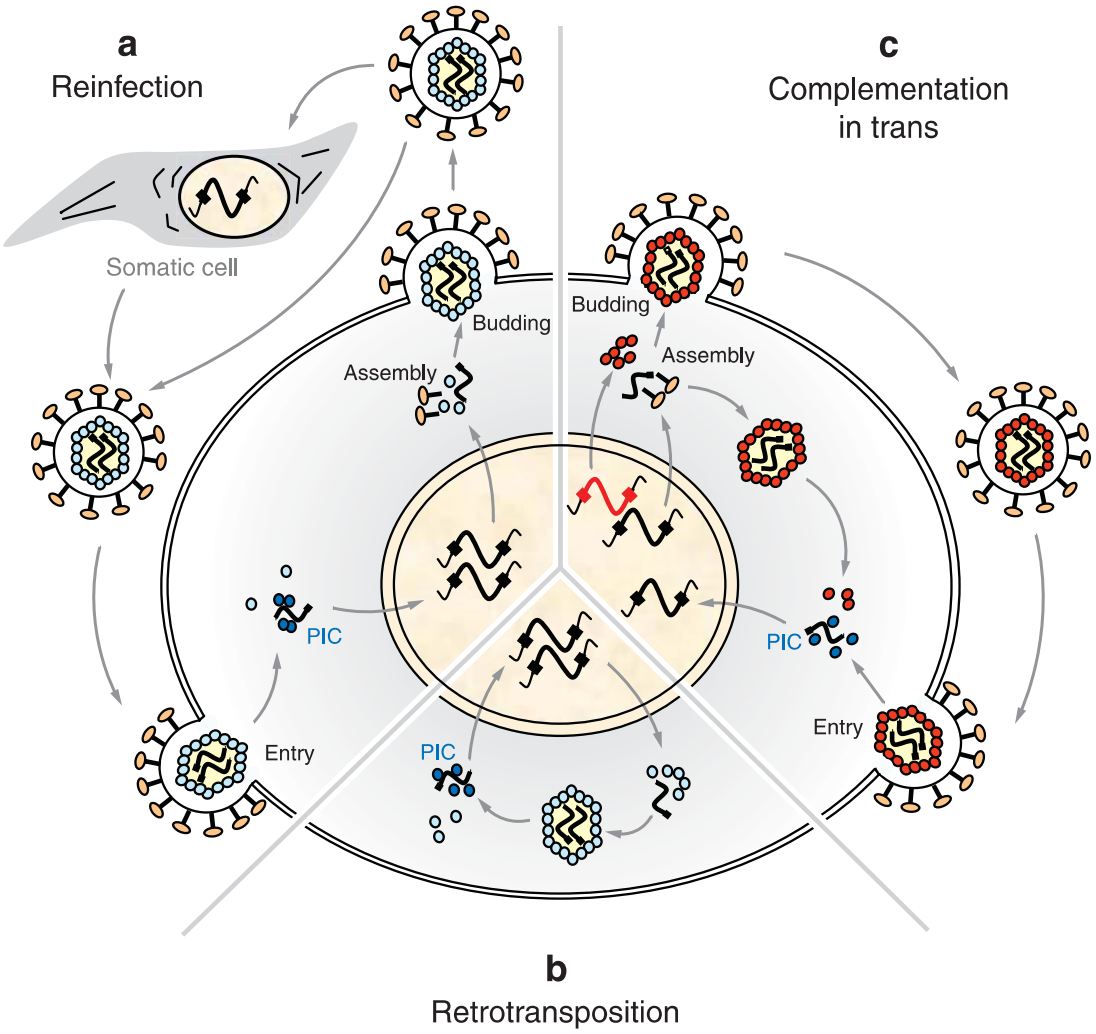

Figure 2. Proliferation modes of ERVs.

To reinfect a host, the virus must possess a functional envelope protein that allows it to re-enter a germ-line cell. Once inside, the virus undergoes a brief period of replication in somatic cells, which leads to adaptation and increases the overall fitness of the lineage.(**a**) In retrotransposition, a poorly understood mechanism, viral RNA contained in core particles are likely formed.(**b**) The presence of a "helper virus" (displayed in *red*) is fundamental fort the Complementation in *trans* mechanism with the goal of forming entities able to proliferate by retrotransposition or reinfection. PIC represents the preintegration complex.(**c**) [14]

1.2 Human Endogenous Retroviruses

As previously mentioned, Human endogenous retroviruses (HERVs) are remnants of ancient retroviral infections that have integrated into the human genome during evolution, accounting for approximately 8% of it.[15] They represent a subset of ERVs and the majority of HERVs are

defective and lack the ability to produce viral particles. However, over the year, some of them have acquired both physiological and disease-related functions. In particular, abnormal expression of HERVs has been implicated in the development and progression of various neurological diseases, including ALS.[16]

Notwithstanding their origin as viral elements, many HERVs have been found to carry out important physiological roles. An emblematic example is syncytin protein, a placenta protein derived from the *env* gene of HERV-W. It plays a critical role in trophoblast fusion and syncytiotrophoblastic layer formation.[17,18] The acquisition of syncytin may have provided a selective advantage to early humans, enabling the development of a more efficient placenta, which in turn may have contributed to the success of the species. Furthermore, HERV-K (HML-2) has been implicated in the control of human embryonic development and pluripotency.[19,20] These findings emphasize the potential physiological importance of HERVs and their more complex role in human biology beyond origins as retroviral debris.

1.2.1 ERVs, classification and integration time

The human genome contains a vast number of ERVs that have integrated into the germ line during evolution. The classification and nomenclature of these retroviruses are complex and constantly evolving. Based on their phylogenetic relatedness to exogenous viruses, ERVs are classified into three classes: class I ERVs are related to gamma- and epsilon-retroviruses, class II comprises viruses that cluster with beta-genera or distantly to delta- and lenti-viruses, and class III comprises elements similar to spuma-viruses.[21]

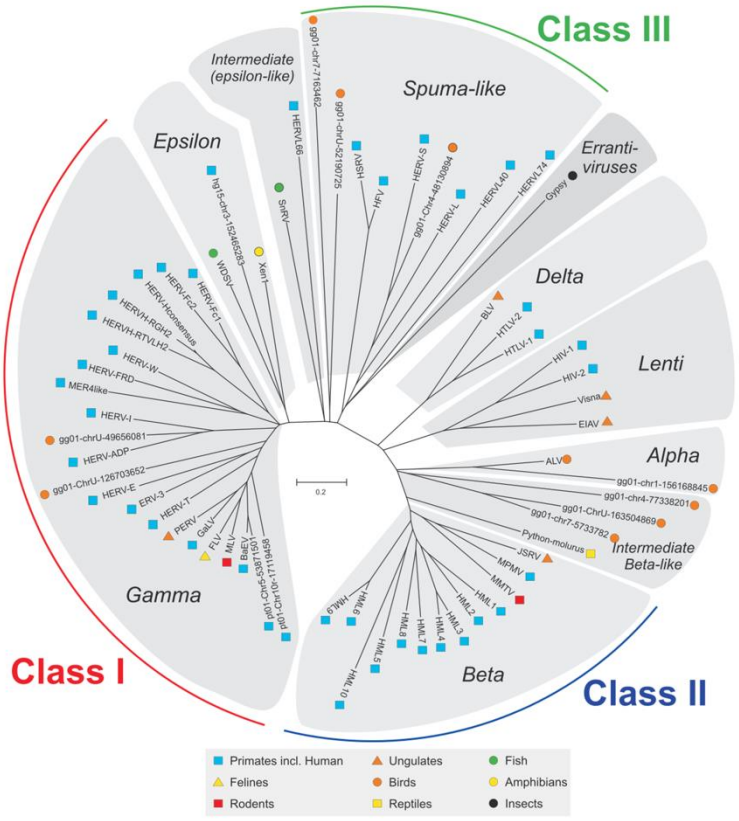

Figure 3. Representative unrooted Pol neighbor-joining NJ dendrogram of retroviral genera.
Unrooted Pol neighbor-joining dendrogram of the retroviral genera: alpha-, beta-, gamma-, delta-, epsilon-, lenti-, and spuma-like retroviruses. The host species are provided with symbols next to each taxonomic unit.[22]

The HERVs have been vertically transmitted to the offspring of the host species. They are grouped into families based on their sequence similarity and genomic organization.[23] The naming of HERV families is based on the sequence of the 18 nucleotides constituting the primer-binding site, which hybridizes with a specific cellular tRNA that initiates the reverse transcription reaction. The HERV families are named by adding a suffix to the acronym HERV, indicating the one-letter code of the amino acid specificity of the most probable tRNA.[24]

Currently, approximately 50 HERV groups have been identified in the human genome, but the number of retroviruses within each HERV group can vary depending on the method of classification used.[25] Most HERV families are ancient and are believed to have entered the human genome through multiple colonization events that occurred over millions of years. The presence

of several HERV families in both Old and New World monkeys suggests that the primary colonization occurred over 35 million years ago.[26]

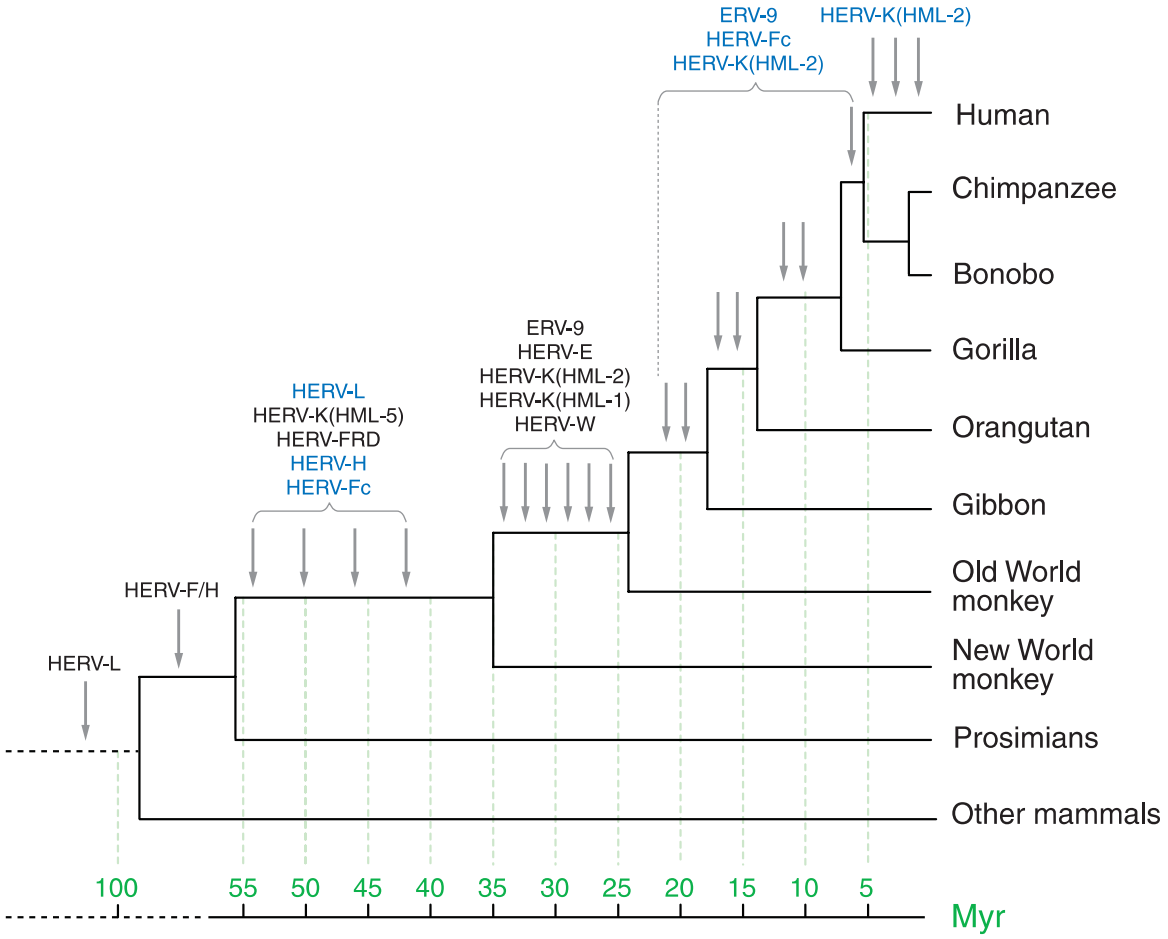

Figure 4. HERVs integration.

Phylogeny of primate species. Important subsequent amplification periods of different families are dispayed in blue color.[14]

1.2.2 Human endogenous retroviruses in physiology and pathology

As has been previously highlighted, HERVs have been implicated in both physiological and pathological processes. In the context of human physiology, studies have suggested that HERVs may play a role in normal cellular processes such as differentiation, development, and immune

response regulation.[27,28] HERVs are transcribed in a systematic and stage-specific manner during early embryogenesis, and their expression serves as a hallmark of cellular identity and potency that characterizes various cell populations in early human embryos.[29] HML-2 has been shown to be transcribed during normal human embryogenesis, with transcription starting at the eight-cell stage during embryonic genome activation and continuing when epiblast cells emerge in preimplantation blastocysts.[19] The presence of proviral RNAs leads to the production of viral-like particles and Gag proteins, suggesting that early human development occurs in the presence of retroviral products. [19] The envelope protein of HERV-K is expressed at high levels on the cell membrane of human pluripotent stem cells, and plays a crucial role in maintaining stemness via the mammalian target of rapamycin pathway.[20] When the HML-2-env is down-regulated or epigenetically silenced, human pluripotent stem cell colonies become dissociated and differentiation along neuronal pathways is enhanced, indicating that regulation of HML-2 is critical for human embryonic development and neural differentiation.[20]

Contrarily, HERVs have also been implicated in the development of various pathological conditions such as cancer, autoimmune diseases, and neurological disorders.[30,31] HERV expression has been detected in several types of cancer including breast, prostate, and ovarian cancer.[32] In addition, its expression has been linked to the pathogenesis of autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus, where they may contribute to the breakdown of self-tolerance and the activation of autoreactive T cells.[33,34] HERVs have also been implicated in the pathogenesis of neurological disorders such as schizophrenia, MS and ALS, where they may contribute to neuroinflammation and neuronal dysfunction.[35]

Given the potential involvement of HERVs in both physiological and pathological processes, there is considerable interest in understanding their regulatory mechanisms and potential therapeutic applications.

1.2.3 Human endogenous retroviruses in neurodegenerative disease

The expression of HERVs within the central nervous system (CNS) is regulated by a variety of factors, including HERV insertional and sequence polymorphisms, epigenetic modifications of HERV proviruses, antiretroviral cellular responses, and environmental triggers. Although the majority of HERV sequences are functionally inactive, some retain the potential for expression, which is suppressed by epigenetic modifications of DNA, such as methylation and supercoiling, that are influenced by both age and disease status.[36–38]

Owing to their evolutionary capacity to infect germ cells, it is comprehensible that the expression of HERVs is increased in human reproductive tissues.[39,40] However, the CNS shows a higher level of HERV expression compared to most other tissue types.[41–43]

The reasons for this heightened expression in the CNS are not yet fully understood. Microarray analysis reveals that not all HERV families are transcribed in the healthy human prefrontal cortex.[41] Some HERVs are universally active in brain tissue samples obtained from patients with systemic diseases but without any known brain injury, while others are transcriptionally silent.[41] The diverse transcriptional profiles observed may potentially indicate individual genetic disparities or similar patterns of gene induction for a particular clinical condition.[41,44,45]

Alternatively, specific HERV-encoded proteins may serve unknown biological functions in the CNS, analogous to the critical role of syncytin-1 in placental and embryonic tissues.[40,46,47]

Currently, HERV expression has been consistently and strongly linked to various neurological disorders, although the underlying mechanisms of how HERVs contribute to these diseases are not yet fully understood.

One possible reason for the higher expression of HERVs observed in diseased human brain samples is related to the tropism of environmental triggers, such as viruses and bacteria, in the CNS.[34] Certain neurotropic pathogens, including herpesviruses, exogenous retroviruses, Toxoplasma gondii, Chlamydia pneumoniae, and some influenza virus strains, can cross the blood-

brain barrier and infect the CNS. Although most of these infections are abortive, their presence in the CNS could activate HERVs expression. For example, multiple sclerosis (MS) has been linked to herpesviruses, such as Epstein-Barr virus (EBV) and human herpesvirus-6, which exhibit an affinity for infecting meningeal B lymphocytes or white matter.[48,49]

Accumulating evidence indicates that the expression of HERV RNA and protein may be triggered in the presence of other retroviral infections, such as HIV. The activation of HERV-K expression has been repeatedly observed in the serum and peripheral blood mononuclear cells (PBMCs) of individuals infected with HIV[50], as well as in the *in vitro* models of HIV infection[51]. In contrast to only 2-3% of healthy adults, HERV-K102 expression in sera occurs in 70-80% of HIV viremic cases, as measured by peptide serology or viral RNA[52]. It has been suggested that HIV's ability to compromise innate defense mechanisms triggers HERV expression by removing or altering cellular control of HERVs activity.[53]

Opportunistic infections are another potential source of HERV expression in the CNS. For instance, Toxoplasma gondii infection can activate HERVs transcriptionally in neuronal cells.[54] It may be worthwhile in future studies to explore additional pathogenic factors that can trigger HERVs expression in the CNS, given the range of viral, bacterial, and parasitic infections associated with HERV-linked diseases.[48,55] This is especially relevant for MS patients who developed progressive multifocal leukoencephalopathy caused by Polyomavirus JC infection while receiving natalizumab treatment. Some of these patients developed a persistent JC virus infection in the brain despite immune system normalization.[56] The possibility that HERVs expression is driving this phenomenon should be taken into account.

Similar to retrotransposons, HERVs can undergo cycles of transposition, recombination, and reintegration.[12,57] Reintegration requires only a subset of viral genes, specifically *gag* and *pol*, since the nascent genomic viral RNA is not packaged into virions but instead is reverse-transcribed and reintegrated into the host cell. Some HERV families, including HERV-K (HML-3), which are

present in high copy numbers within the human genome, are thought to have spread through the reintroduction of an existing provirus.[12] De novo accumulation of HERVs reveals a preference for integration into gene-rich and transcriptionally active sites, as shown by the integration pattern of a reconstituted consensus HERV-K virus.[58]

The ability of integrated proviruses to influence the expression of adjacent or distant host cell genes is another important aspect. Enhancer sequences and transcription factor-responsive elements are present within HERV LTRs, which play a role in regulating host gene expression.[59–64] HERV proviruses also provide alternative transcription initiation sites, splice acceptor/donor sites, and polyadenylation signals to neighboring host sequences.[65,66]

Additionally, HERV-K LTRs can produce regulatory RNA through antisense transcription, which downregulates adjacent genes by reducing their mRNA levels[65]

The transcription and translation of individual HERV-encoded retroviral proteins may have significant effects on various cellular pathways in addition to their effects during retroviral replication. Although a considerable number of intact HERV open reading frames (ORFs) exist, their potential contribution to neurodegeneration remains largely unexplored. HERV proteins, and accessory proteins, may modulate cellular activity. For example, HERV-K encodes the accessory protein Rec, which is similar to the HIV Rev protein and may contribute to oncogenesis by inhibiting the PML zinc finger protein and promoting c-myc expression.[67] HERV-K also encodes Np9, a splice variant of Rec that interacts with the RING-type E3 ubiquitin ligase LNX, which is involved in the Notch signaling pathway.[68] Another HERV-K accessory protein is produced by cleavage of the HML-2 Env precursor, forming the nucleolar localized signal peptide.[69] The exact functions of these regulatory proteins are only beginning to be understood, but it is clear that they may play a key role in neuropathogenesis.

1.2.4 HERV-Related Immune Response in the Nervous System

Continued activation of an innate immune response mediated by HERVs from CNS-resident cells may contribute to neural damage and enhance neuroinflammation together with the adaptive immune response. Retroviral proteins, both endogenous and exogenous, particularly the envelope glycoproteins, have a tendency to modulate the immune system and provoke neuropathogenic responses in glial cells.[70–72]

The expression of the envelope protein (env-W) from HERV-W can result in various immunomodulatory processes that cause damage to the nervous system. The ERVWE1 provirus of HERV-W encodes syncytin-1, overexpressed in glia within MS lesions.[73] Although it is unclear which genomic loci encode the HERV-W envelope protein, evidence suggests that envelope proteins, particularly syncytin-1, can cause neurotoxicity. It triggers an innate immune response by activating TLR4 signaling, which leads to the secretion of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), in human monocytes.[74] *In vitro*, these cytokines can enhance the release of HERV-W by PBMCs from HERV-W-positive individuals, resulting in a positive feedback loop of virus production and inflammation.[75] In addition, inflammatory mediators like TNF-α, IFN-γ, IL-6, and IL-1β activate the ERVWE1 promoter, leading to syncytin expression,[76] which can contribute to an uncontrolled inflammatory cycle. The ERVWE1 promoter is activated by the transcription factor NF-kB, indicating that the recognition of HERV nucleic acids and proteins,[77] or co-infecting viruses,[78] could reactivate the expression of latent HERVs.

An instance of superantigen envelope can be seen in the HERV-K family. The provirus HERV-K18 is located in an antisense orientation within the first intron of the CD48 gene. The env gene is the only intact viral gene of this provirus and encodes a superantigen (ENV-K18), which stimulates Vβ7 TcR CD4+ T cells.[79] Although most ENV-K18 reactive thymocytes are negatively selected in the thymus, a small fraction of Vβ7 thymocytes may escape deletion in certain

individuals, leading to an increased number of circulating Vβ7 TcR CD4+ T cells. These ENV-K18 Super Antigen-reactive T cells can be triggered during viral infection, either by type I IFN or directly by herpesvirus proteins. Strikingly, and unlike other HERV-K proviruses, the expression of ENV-K18 can be induced by type I IFNs in the non-T-cell fraction of PBMC.[80] Specifically, the IFN-α1 isoform elicits robust ENV-K18 transcription, compared with IFN-α2 or IFN-β. The induction of ENV-K18 can also be synergistically boosted by IFN-γ.[80] These findings suggest that acute or chronic viral infections that drive IFN responses may trigger ENV-K18 expression and activate peripheral Vβ7 TcR CD4+ T cells.[81–83]

All types of cells in the CNS can initiate innate and intrinsic immune responses. However, resident antiviral immunity in the brain is mainly mediated by microglia and astrocytes. These cells possess a variety of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors, which allow them to sense retroviral nucleic acids and viral proteins.[84] PRR signaling results in the induction of both IFN and proinflammatory responses, which create a cellular antiviral state and attract effector immune cells. Nevertheless, retroviruses have developed mechanisms to evade the innate immune system. While several examples exist, such as those seen in Cheng et al. and Kirchhoff, [85,86] it is still unclear whether HERVs employ similar strategies. After being recruited to the CNS, immune cells such as T cells may recognize novel antigens derived from HERVs that are presented by CNS cells or antigen-presenting cells that have infiltrated the CNS. It has been reported that during cellular apoptosis, HERV proteins can be cleaved by activated caspases or granzyme B, potentially leading to the formation of T-cell epitopes, similar to the process that generates nuclear autoantigens.[87] These CD4+ T cells and cytotoxic CD8+ T cells can exacerbate ongoing inflammatory responses and directly contribute to demyelination, and tissue injury.

1.2.5 Human Endogenous Retrovirus K and Amyotrophic Lateral Sclerosis

HML-2 is a member of the HERV family. It is one of the most recently integrated, transcriptionally, and biologically active in the human genome with an estimated integration time of 1-5 million years ago. HERV-K has been found to be expressed in a variety of human tissues, including the testis and placenta.[25,88]

It is unique among HERVs in having maintained the ability to produce infectious viral particles, and its expression has been related to several physiological and pathological processes, including immune regulation, embryonic development, neurodegeneration, and cancer.[88] Diverse studies have suggested that HERV-K may have a role in the pathogenesis of ALS. For instance, elevated levels of HERV-K RNA and protein have been measured in the spinal cord tissue of ALS patients compared with healthy controls.[89] In a study by Li et al.[89] the HERV-K presence was detected in cortical and spinal neurons of patients with ALS, but not in neurons from healthy individuals. When HERV-K or its envelope protein was expressed in human neurons, it caused the retraction and beading of neurites. Transgenic animals that expressed the envelope gene exhibited progressive motor dysfunction, accompanied by selective loss of motor cortex volume, decreased synaptic activity in pyramidal neurons, dendritic spine abnormalities, nucleolar dysfunction, and DNA damage. Injury to anterior horn cells in the spinal cord resulted in muscle atrophy and pathological changes consistent with nerve fiber denervation and reinnervation.

Altogether, the multiple expression and functional roles of HERV-K indicate that it may serve an important role in both normal physiology and pathological conditions.

Based on this evidence, different approaches could be adopted for the development of therapies targeting HERV-K in ALS. A potential strategy entails the use of drugs that inhibit the expression of HERV-K or interfere with its activity. Another potential therapeutic approach entails the use of monoclonal Abs or small molecules that target the HERV-K envelope protein and prevent its effect. Therapies that target the downstream effects of HERV-K activity, such as inflammation and

oxidative stress, can be evaluated. However, it should be mentioned that the development of HERV-K-targeted therapies in ALS poses a big challenge, and the potential side effects of HERV-K-targeted therapies are not currently known.

1.3 Amyotrophic Lateral Sclerosis

ALS is a neurodegenerative disease that affects motor neurons in the brain and spinal cord. Initial symptoms of ALS may include muscle weakness or stiffness, often starting in the arms or legs, and difficulty speaking or swallowing. As the disease progresses, people can experience difficulty breathing. Other symptoms can include fasciculations, and muscle wasting or atrophy. In some cases, cognitive and behavioral changes also may occur, including problems with decision-making and problems with memory. It is important to point out that symptoms can vary greatly from person to person and that the disease can progress at different rates.[90] ALS can be classified based on motor neuron involvement: limb-onset ALS, bulbar-onset ALS, and spinal-onset ALS. Each subtype has unique clinical features and disease progression, suggesting that different underlying mechanisms may contribute to the development and progression of the pathology. To date, there is no known cure for ALS, and treatment options are limited to managing symptoms and slowing disease progression. However, there are numerous ongoing research efforts aimed at identifying new therapies and potential targets for ALS treatment.

1.3.1 Amyotrophic Lateral Sclerosis Epidemiology and Risk factors

The global prevalence of ALS is estimated to be 4.1 cases per 100,000 population and it also varies by age and gender, with a higher prevalence in men and in people over 60 years of age.[90]

Several environmental risk factors include exposure to toxic substances such as lead, mercury, and pesticides, as well as smoking and physical trauma.[91] Different studies suggested that exposure to viruses, as enteroviruses and HERVs, may be associated with an increased risk of ALS. Indeed, higher levels of HERV-K expression have been found in the brains of individuals with ALS compared to healthy controls.[89,92]

Approximately 10% of all ALS cases are caused by genetic factors, and over 20 genes have been linked to the disease. Mutations in the SOD1 gene were the first genetic cause identified in ALS. Other genes such as C9orf72, FUS, TARDBP, and UBQLN2 have been associated with ALS.[93] The presence of these mutations has been shown to cause neuronal toxicity and mitochondrial dysfunction, which ultimately leads to motor neuron death. Male gender, increasing age, lower educational level, and occupation in industries such as agriculture and sports represent other factors that have been associated with an increased risk of ALS.[94,95]

1.3.2 Amyotrophic Lateral Sclerosis Diagnosis and Therapy

The diagnosis of ALS is primarily based on clinical symptoms and neurological examination and can be confirmed through electromyography (EMG) and nerve conduction studies (NCS), widely used to gauge muscle and nerve functionality in patients with suspected neuromuscular disorders such as ALS.[96] EMG assesses the electrical activity of muscles at rest and during contraction, while NCS evaluates the speed and strength of nerve signals. In addition, the revised El Escorial criteria provide a standardized framework for the diagnosis of ALS and its subtypes and have been broadly adopted in clinical practice.[97] To exclude other conditions that may mimic ALS, other exams such as magnetic resonance imaging (MRI) and blood tests, can be performed.

ALS therapy involves comprehensive care with a combination of pharmacological and non-pharmacological approaches. Riluzole and Edaravone represent the pharmacological interventions

for ALS treatment. Riluzole acts by reducing the release of glutamate, a neurotransmitter that can cause motor neuron damage, whereas Edaravone is a free radical scavenger that decreases oxidative stress in motor neurons.[98,99]

Non-pharmacological interventions typically include respiratory management strategies, such as ventilation or cough assistance, as well as physical therapy to support strength and mobility.[100]

Ongoing research is currently exploring the potential of gene therapies, immunotherapies, and stem cells as promising approaches for the treatment of ALS. These innovative therapeutic strategies aim to target the mechanisms underlying the disease and foster motor neuron survival. Gene therapies involve therapeutic gene delivery to replace or correct mutated genes, while immunotherapies use the immune system to target toxic proteins and inflammatory pathways associated with the disease. Stem cell therapies, on the other hand, seek to replace lost motor neurons and restore neural function by introducing healthy stem cells into affected regions of the central nervous system. The development of these novel therapies holds great promise for improving the quality of life for people affected by ALS.

1.4 Interplay Between Amyotrophic Lateral Sclerosis and the Immune System

There is emerging evidence that dysregulation of the immune system contributes to the development and progression of ALS. Indeed, anomalies in the innate and adaptive immune defense have been described in ALS patients and in animal models of the disease.[101] Pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, are elevated in ALS patients, suggesting that neuroinflammation plays a role in ALS pathogenesis.[102]

Both cellular and humoral immune responses have been involved in the pathogenesis of ALS. Activated microglia and astrocytes contribute to neuroinflammation and neuronal death in ALS.

Microglia are the resident immune cells of the CNS and are implicated in the immune response to injury and disease. Astrocytes play a key role in the maintenance of homeostasis in the CNS, including the regulation of neurotransmitter levels and the removal of toxins. Microglia and astrocytes have been found to interact with each other, forming a complex network that influences the progression of ALS.[103] The release of proinflammatory cytokines, because of their activation, leads to the recruitment and activation of peripheral immune cells such as T lymphocytes and monocytes.[104] Some reactive oxygen species, such as nitric oxide and proteases, can be released as a consequence of chronic activation of microglia thus leading to neurodegeneration and neuroinflammation.[105] Several studies have shown that activated T cells are present in the spinal cord and brain of ALS patients and contribute to neuroinflammation.[106] The mechanisms by which these immune cells induce damage in ALS are complex and not fully understood, but they likely involve a combination of direct neurotoxicity and indirect effects mediated by the release of proinflammatory cytokines and chemokines.

Increasing evidence points to the role of the humoral immune system in ALS and various mechanisms have been proposed to explain how its activation may contribute to the damage. The humoral immune response triggers the activation of B-cells and the production of antibodies (Abs), leading to inflammation and neurotoxicity. By targeting the activated complement, a cascade of proteins that can incite inflammation and tissue damage, through specific inhibitors, we can potentially devise an innovative therapeutic strategy for treating motor neuron diseases.[107]

However, the exact mechanisms underlying ALS' humoral immune response and contribution to disease progression remain unclear and demand further investigation. In a study conducted by our research group, we investigated the potential involvement of HERV-K in the immune response of patients with ALS. Using different methodological approaches to study the effects of HERV-K in humoral and cell-mediated immune responses, we found that HERV-K can modulate the immune

response in ALS patients by inducing the production of pro-inflammatory cytokines and increasing the expression of immune-related genes.[108]

The role of the immune system in ALS pathogenesis is not fully understood and further studies are needed to elucidate the mechanisms involved. However, the emerging evidence suggests that targeting immune dysregulation may be a promising therapeutic strategy for ALS.

1.5 TAR DNA-binding protein 43 in Amyotrophic Lateral Sclerosis

TAR DNA-binding protein 43 (TDP-43) is a multi-functional nucleic acid-binding protein that has a crucial role in RNA metabolism, involving alternative splicing, mRNA stability, and mRNA transport. It is primarily found in the nucleus; however, under certain conditions, such as cellular stress or neurodegenerative diseases, it can translocate into the cytoplasm and form aggregates. These aggregates are hallmarks of several neurodegenerative disorders, including ALS, frontotemporal lobar degeneration (FTLD), and Alzheimer's disease (AD). Mutations in the TDP-43 gene have been linked to familial ALS and sporadic ALS. TDP-43 pathology has also been observed in other neurological disorders, including Parkinson's disease and Huntington's disease. The presence of these aggregates in neurodegenerative diseases suggests a common mechanism of pathogenesis.

The loss of TDP-43's regulatory effect on RNA combined with its mislocalization and aggregation can have a downstream effect on gene expression and cellular processes and the acquisition of toxic properties by TDP-43 can lead to dysfunction and neuronal death. This dual role of TDP-43 in disease has been observed in several studies and highlights the importance of understanding the mechanisms underlying TDP-43 pathology in neurodegenerative diseases.[109]

The role of TDP-43 in ALS pathogenesis has been investigated by several studies and it has been found to be the primary contributor to ubiquitinated inclusions in motor neurons of ALS patients, hinting at a key role in the pathology.[110]

Mutations in the gene encoding TDP-43 (TARDBP) have been identified in both familial and sporadic cases of ALS.[111] Animal model studies of ALS have proven that either reducing TDP-43 levels or inhibiting its aggregation can retard or ameliorate the disease phenotype.[112,113]

One of the most prominent hallmarks of TDP-43 pathology in ALS is abnormal phosphorylation of the protein, causing it to accumulate in cytoplasmic inclusions. The exact mechanism by which phosphorylation contributes to TDP-43 pathology is not clearly understood, but it is widely considered to play a role in affecting the normal function of the protein. Several kinases have been implicated in the abnormal phosphorylation of TDP-43, with many studies suggesting that their levels are increased in the spinal cord of ALS patients.[114]

The post-transcriptional modification, the mechanisms underlying the aggregation of TDP-43, and its toxicity are still not fully understood. However, several therapeutic approaches targeting TDP-43 are currently under investigation. These include RNA-based therapies that target TDP-43 mRNA or modulate its splicing, small molecule compounds that inhibit TDP-43 aggregation or promote its clearance, and immunotherapy approaches that target TDP-43 protein aggregate.[115] The development of efficacious treatments for TDP-43-associated diseases remains challenging, requiring further investigations to better grasp the pathogenesis of these disorders and identify new targets.

The regulatory role of TDP-43 toward HERV-K is an important aspect to consider since the binding of TDP-43 to HERV-K LTR was confirmed through chromatin immunoprecipitation by Li et al.[116] They observed that the HERV-K expression occurred in human neurons transfected with TDP-43 plasmid. Increased replication of HERV-K, as evidenced by the reverse transcriptase activity in the culture supernatants and increased viral transcripts in the cell extracts, has been documented

following HeLa cells co-transfection with HERV-K and TDP-43. Additionally, HERV-K expression decreased in a knockdown of endogenous TDP-43. The TDP-43 DNA binding capability can lead to the transactivation of HERV-K. This observation is meaningful given that the HERV-K long terminal repeat (LTR) contains five TDP-43 binding sites, suggesting that TDP-43 may function as a key regulator of HERV-K expression and potentially contribute to neurodegeneration.[116]

1.6 microRNA in Amyotrophic Lateral Sclerosis

microRNAs (miRNAs) are small non-coding RNA molecules that exert crucial roles in the regulation of gene expression in biological processes, such as development, differentiation, and disease.[117] They act by binding to specific sequences on target messenger RNAs (mRNAs), resulting in mRNA degradation or translational repression.[118] Dysregulation of miRNA expression or function has been implicated in numerous diseases, including cancer, neurodegenerative disease, and cardiovascular disease.[119] For instance, studies have shown that miRNAs are differentially expressed in various types of cancer and can function as either oncogenes or tumor suppressors.[120] In neurodegenerative diseases, such as ALS, Alzheimer's disease, and Parkinson's disease, the miRNA-altered expression and function have been linked to disease pathogenesis and progression.[121] Thus, miRNAs represent promising therapeutic targets for the treatment of various diseases.

Several studies have suggested that the dysregulation of miRNAs could contribute to the pathogenesis of ALS.[122] MiRNAs have been found to be differentially expressed in ALS patients compared to healthy controls, and some of them are known to regulate axonal transport, neuroinflammation, protein aggregation, and other key pathways involved in neuronal function.[123] For instance, miR-206, a muscle-specific miRNA, has been found to be upregulated in the spinal

cord of ALS patients and in mouse models of the disease, where it contributes to motor neuron degeneration by inhibiting the expression of target genes involved in neuromuscular junction maintenance.[124] MiR-155, involved in the regulation of immune responses, has been shown to be upregulated in the spinal cord of ALS patients and to contribute to neuroinflammation by promoting the activation of microglia and the production of pro-inflammatory cytokines.[125]

Other miRNAs have been found to be dysregulated in ALS and to contribute to the pathogenesis of the disease through different mechanisms, such as by regulating the expression of RNA-binding proteins (e.g., TDP-43 and FUS) involved in ALS. However, the exact roles of miRNAs in ALS pathogenesis are still not completely understood, and further studies are needed to elucidate the underlying mechanisms.

Since miRNAs play a role in regulating gene expression, we speculate whether they may also regulate HERV-K expression to explain the pathological observed retroviral reactivation. In fact, one hypothesis concerns that the downregulation of specific miRNAs may lead to a failure to repress HERV-K, inducing its overexpression. There is a lack of scientific supporting evidence regarding the interaction between specific miRNAs and HERV-K, but, from predictions of in silico binding and moving forward with molecular studies, we aim to further explore this topic.

1.7 Results

1.7.1 TDP-43 and HERV-K Envelope-Specific Immunogenic Epitopes Are Recognized in ALS Patients

First, we aimed to investigate the humoral response directed against a highly immunogenic epitope derived from the envelope of HERV-K and against epitopes identified within the C-terminal portion of the TDP-43 protein. Among the latter epitopes, we also aimed to investigate the one that exhibited phosphorylation. Epitopes' list are displayed in Materials and Methods, Tab. 2.

The Abs levels against HERV-K-env-su$_{(19-37)}$ and three specific epitopes of TDP-43 were assessed in the plasma of the ALS patients and healthy donors (HDs) groups. To compare the quantitative values of the Abs' OD among groups, the Mann–Whitney U test was performed. ROC analysis was used to determine the cut-off values, percentage of positive and negative samples was determined using Fisher's exact test.

Abs targeting HERV-K-env- su$_{(19-37)}$ moiety were present in 40% of the ALS population, versus 8.89% in the healthy control group. (Fig. 5A): Mann–Whitney U test, $p < 0.0001$, HDs median = 0.139, 95% CI [0.107, 0.175], ALS median = 0.371, 95% CI [0.305, 0.45]; cut off value of 0.393; Fisher's exact test, $p = 0.0011$; and AUC = 0.86. Regarding the humoral response against TDP-43 epitopes, we observed that 24.44% of ALS patients exhibited Abs against TDP-43$_{(258–271)}$, versus only 4.44% in the healthy group (Figure 5B): Mann–Whitney U test, $p < 0.0001$, HDs median = 0.157, 95% CI [0.084, 0.191], ALS median = 0.284, 95% CI [0.243, 0.318]; cut off value of 0.381; Fisher's exact test, $p = 0.013$; and AUC = 0.813. An increased Abs presence against the TDP-43$_{(398–411)}$ epitope (Figure 5C) has been detected in ALS patients, with a positivity measured in 51.11% of the ALS group compared to 4.44% of HDs with a Fisher's exact test $p < 0.0001$ (Mann–Whitney U test, $p < 0.0001$, HDs median = 0.11, 95% CI [0.074, 0.168], ALS median = 0.386, 95% CI [0.332, 0.453]; cut off value of 0.385; and AUC = 0.915). Figure 5D shows the Abs levels targeting TDP-43$_{(398–411)}$P; 4.44% of healthy subjects were positive, whereas, we observed an increased percentage of up to 20.00% in ALS patients (Mann–Whitney U test, $p = 0.0006$, HDs median = 0.151, 95% CI [0.103, 0.195], ALS median = 0.226, 95% CI [0.202, 0.255]; cut off value of 0.35; Fisher's exact test, $p = 0.049$; and AUC = 0.706).

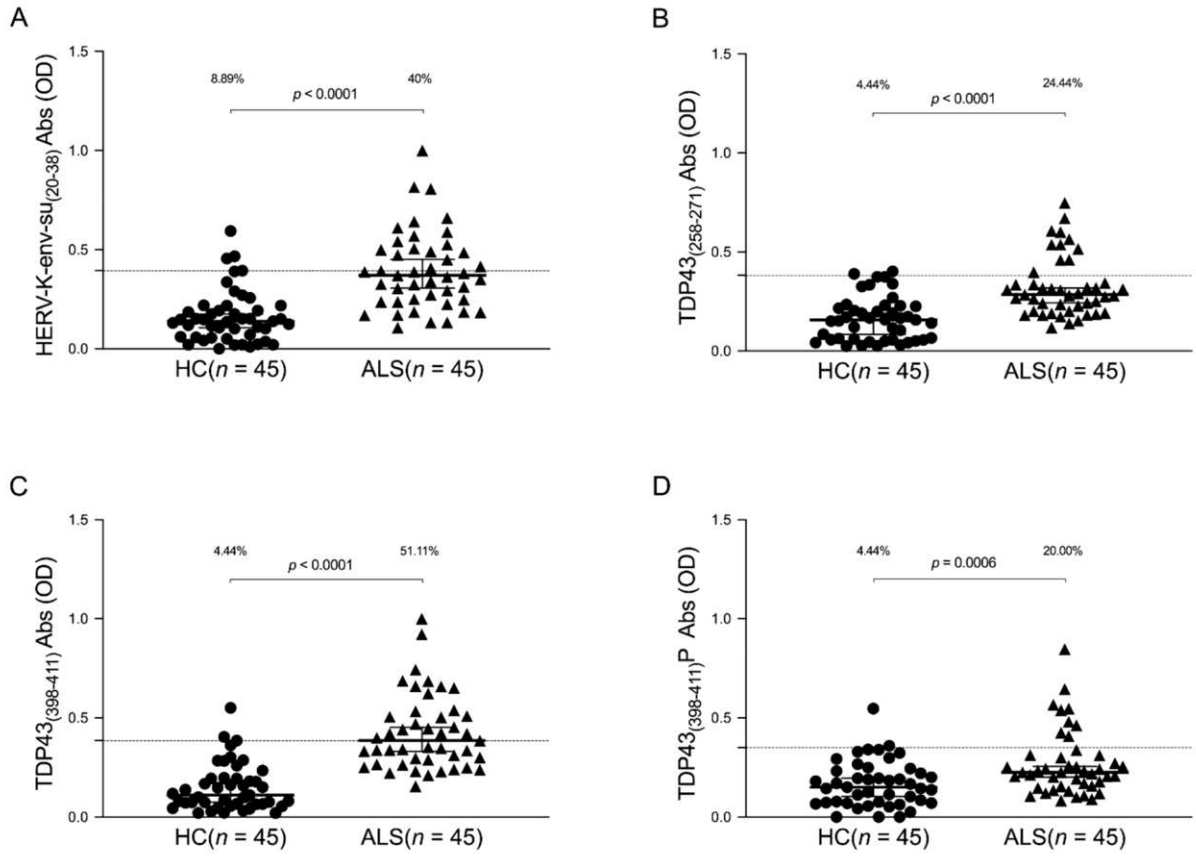

Figure 5. ELISA-based analysis of Abs reactivity against HERV-K- and TDP-43-derived epitopes.

Abs levels targeting HERV-K-env- su$_{(19\text{-}37)}$ (**A**), TDP-43$_{(258–271)}$ (**B**), TDP-43$_{(398–411)}$ (**C**), and TDP-43$_{(398–411)}$P (**D**) fragments, were assessed in plasma samples from ALS patients and HDs subjects. Median and dashed lines depict the thresholds used to interpret the positivity of the samples. The Mann-Whitney p-value and the percentage of positive patients evaluated by Fisher's exact test are indicated at the top of each graph.

To explore a possible variation in the humoral response in the plasma of ALS-Newly-Diagnosed (ALS-ND) and ALS-Long-Survivors (ALS-LS) patients, the Kruskal-Wallis test and Dunn's post hoc analysis were conducted. The limitation of this analysis is that the three groups (HD, ALS-LS, and ALS-ND) are not matched for sex due to the small ALS population; therefore, it would be quite intriguing to understand thoroughly if any sex-related statistical differences exist in a wider population. The obtained results reveal a significant difference between HDs versus ALS-ND/ALS-LS for the epitopes derived from TDP-43 and HERV-K sequence. There was no difference in humoral response observed between ALS-ND and ALS-LS. (Fig. 6)

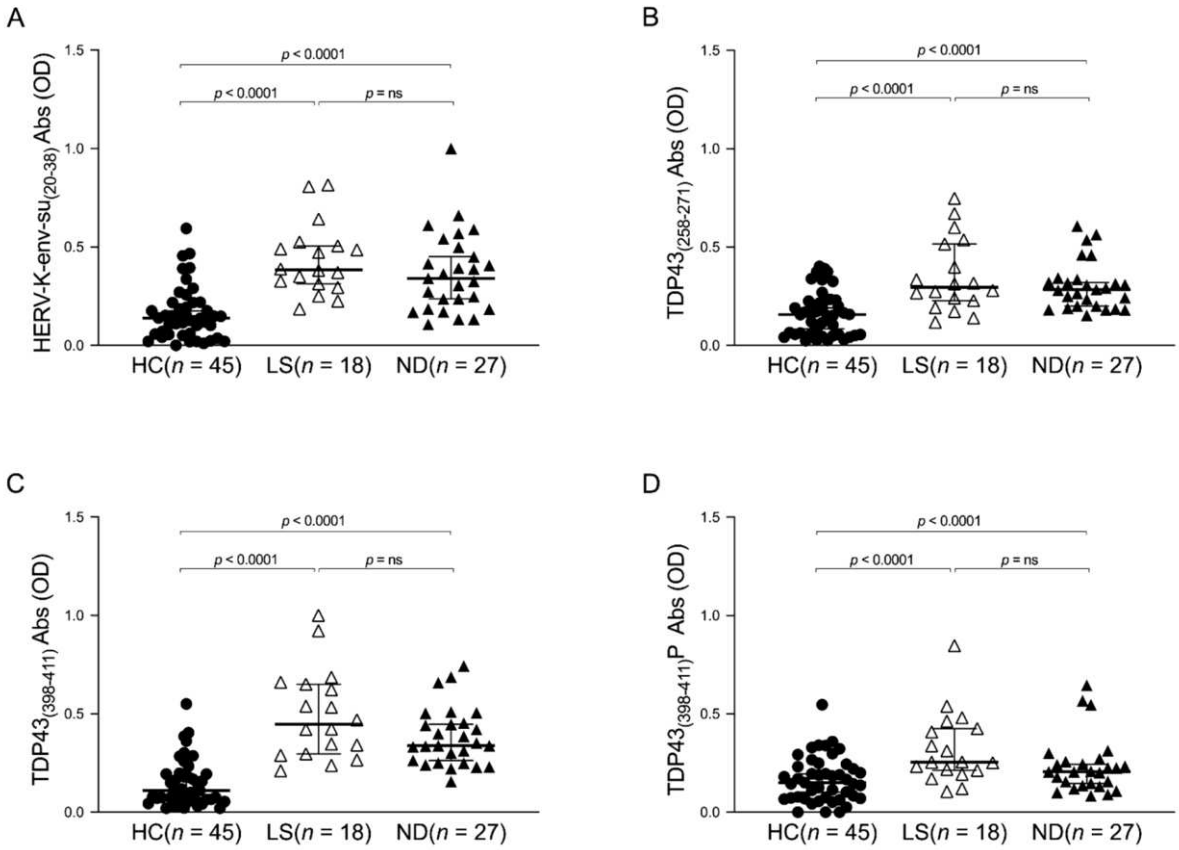

Figure 6. ELISA-based analysis of Abs reactivity against HERV-K- and TDP-43-derived epitopes.
Abs levels targeting HERV-K-env-su$_{(19–37)}$ (**A**), TDP-43$_{(258–271)}$ (**B**), TDP-43$_{(398–411)}$ (**C**), and TDP-43$_{(398–411)}$P (**D**) fragments, were assessed in plasma samples from ALS patients and HDs subjects. A Kruskal–Wallis test and Dunn's post hoc analysis were performed. Scatter plots represent the median, and the *p*-value is indicated in the upper part of each graph.

To investigate the potential correlation between TDP-43 and HERV-K humoral response, additional analysis was performed. We documented a correlation between the Abs levels against HERV-K and TDP-43 epitopes in ALS patients: HERV- K-env-su$_{(19–37)}$ and TDP-43$_{(258–271)}$ (r = 0.297, p = 0.048) (Fig. 7A), HERV-K-env-su$_{(19–37)}$, and TDP-43$_{(398–411)}$ (r = 0.488, p = 0.001) (Fig. 7C), and HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(398–411)}$P (r = 0.435, p = 0.003) (Fig. 7E). The corresponding analysis was carried out in HDs.

No significant correlations were found in HDs; HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(258–271)}$ showed r and p values, respectively, of −0.138 and ns (Fig. 7B), while HERV-K-env-su$_{(19–37)}$, and TDP-

43$_{(398–411)}$ showed an $r = -0.173$ and p = ns, (Fig. 7D), HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(398–411)}$P showed an $r = -0.099$ and p = ns (Fig. 7F).

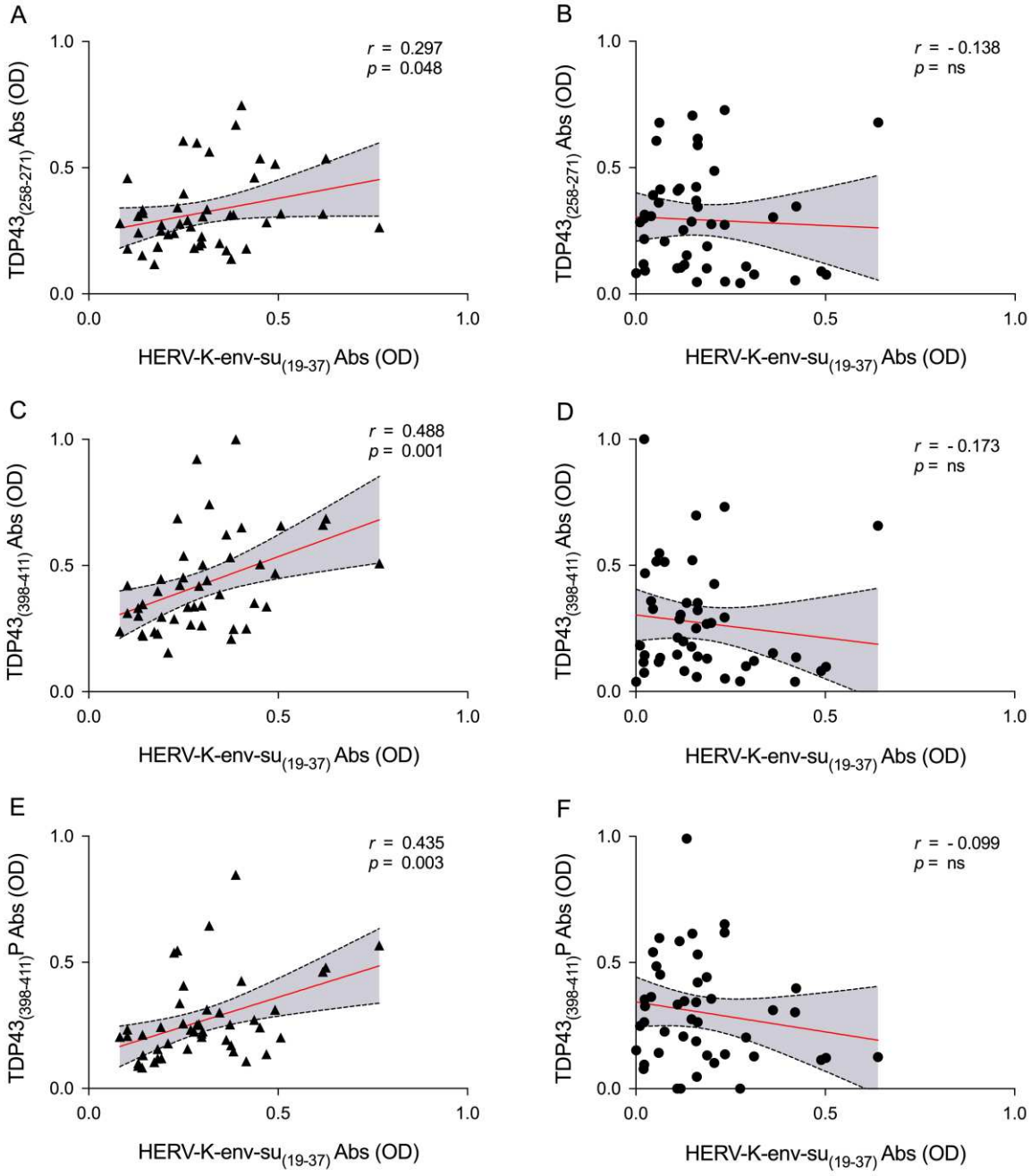

Figure 7. Scatter plots of Abs to TDP-43 derived peptides, and HERV-K env epitope in ALS populations.
The graphs show the correlation between HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(258–271)}$, TDP-43$_{(398–411)}$, and TDP-43$_{(398–411)}$P in ALS patients (**A**,**C**,**E**) and in HDs (**B**,**D**,**F**).

Then, we looked at the correlation between the humoral response against TDP-43 and HERV-K in patients classed in ALS-ND and ALS-LS. Results showed a positive correlation between the epitopes in both groups. Regarding the ALS-ND group: HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(258–271)}$ (r = 0.246, p = ns) (Fig. 8A), HERV-K- env-su$_{(19–37)}$ and TDP-43$_{(398–411)}$ (r = 0.451, p = 0.018) (Fig. 8C), and HERV-K-env- su$_{(19–37)}$ and TDP-43$_{(398–411)}$P (r = 0.357, p = ns) (Fig. 8E). Regarding ALS-LS patients: HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(258–271)}$ (r = 0.395, p = ns) (Fig. 8B), HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(398–411)}$ (r = 0.570, p = 0.013) (Fig. 8D), and finally HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(398–411)}$P (r = 0.473, p = 0.047) (Fig. 8F).

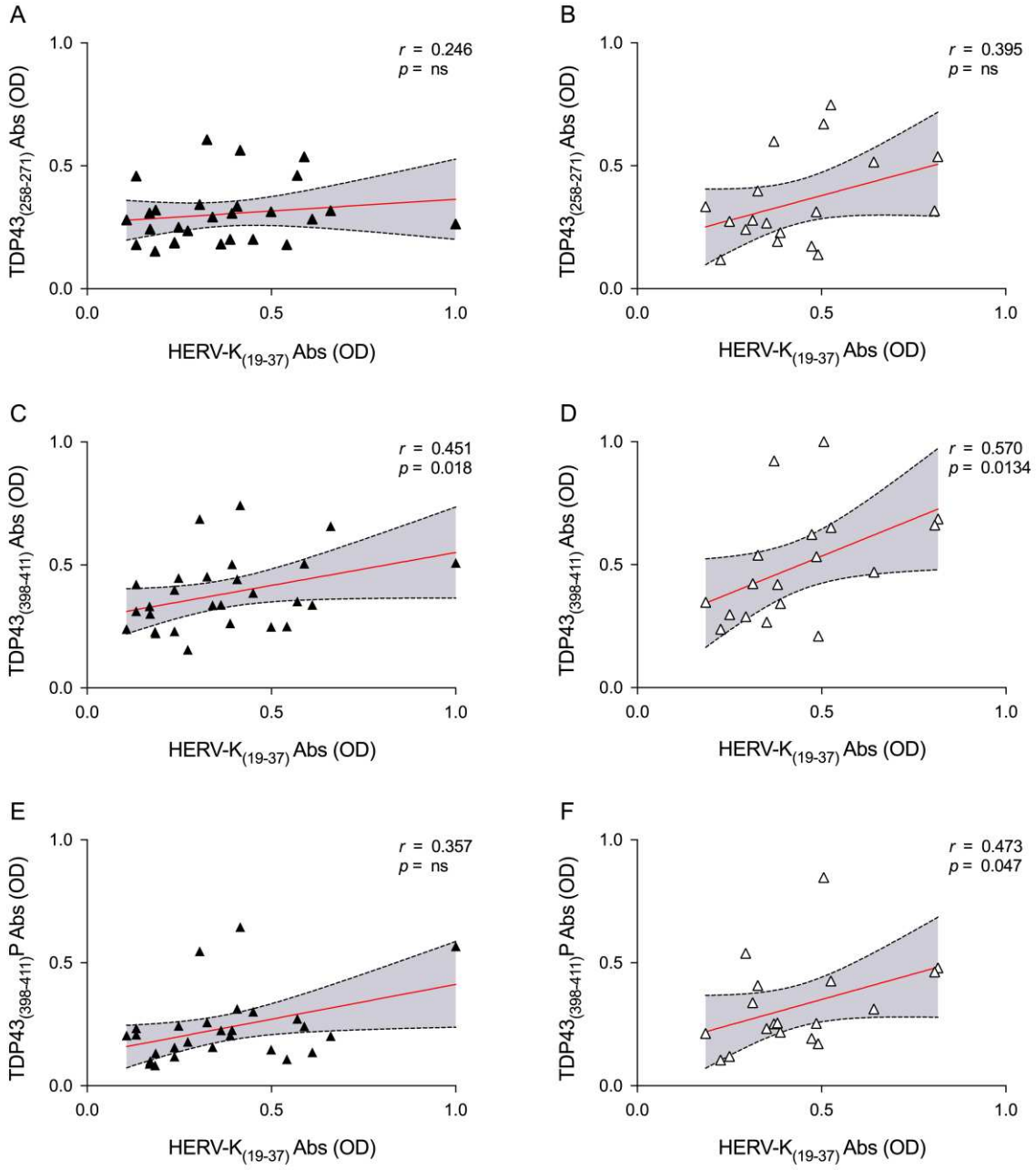

Figure 8. Scatter plots of Abs targeting TDP-43 and HERV-K-env derived epitope in ALS patients. The graphs illustrate the correlation between HERV-K-env-su$_{(19–37)}$ and TDP-43$_{(258–271)}$, TDP-43$_{(398–411)}$, and TDP-43$_{(398–411)}$P in ALS-ND patients (**A**,**C**,**E**) and in ALS-LS (**B**,**D**,**F**).

1.7.2 Antibody Response to HERV-K May Be Protective in Amyotrophic Lateral Sclerosis

In the second study, conducted in collaboration with the National Institute of Neurological Disorders and Stroke, we aimed to better understand the role of the humoral response directed against the envelope protein of HERV-K and whether it correlated with disease progression.

Epitope mapping was carried out on Abs against HERV-K measured in the serum of 10 ALS individuals and 8 healthy controls, employing a peptide array that covers the whole protein sequence. The analysis revealed that ALS subjects presented a significantly higher Abs response toward more regions of the protein than controls (Wilcoxon corrected $p < 0.05$) (Fig. 9A). Notably, statistical significance was only observed for peptides that elicited a stronger response in the ALS group compared with controls subjects.

The reactivity pattern of HERV-K-env Abs was clearly different between ALS individuals and controls. In particular, while healthy control sera exhibited responsiveness against only a limited number of HERV-K sequence-specific peptides, ALS sera showed reactivity against a much wider range of HERV-K-env regions.

Using the median of the HDs as a positivity threshold, 50.14% of peptides elicited a humoral response in controls and 98.26% in ALS sera (Fisher exact test; $p < 0.0001$) (Fig. 9B). Using Phyre2 software for 3D modeling, we were capable of localizing peptides on HERV-K-env subunits. The majority of peptides recognized from the sera of ALS-affected individuals were found to accumulate on the transmembrane subunit of the protein, particularly in the nonextracellular areas (Fig. 9D,E, left and center).

We identified 35 distinct epitopes ranging from 4 to 12 amino acids in length targeted by the Abs in ALS patients through the analysis of adjacent peptides that elicited a higher Ab response in ALS compared to controls.

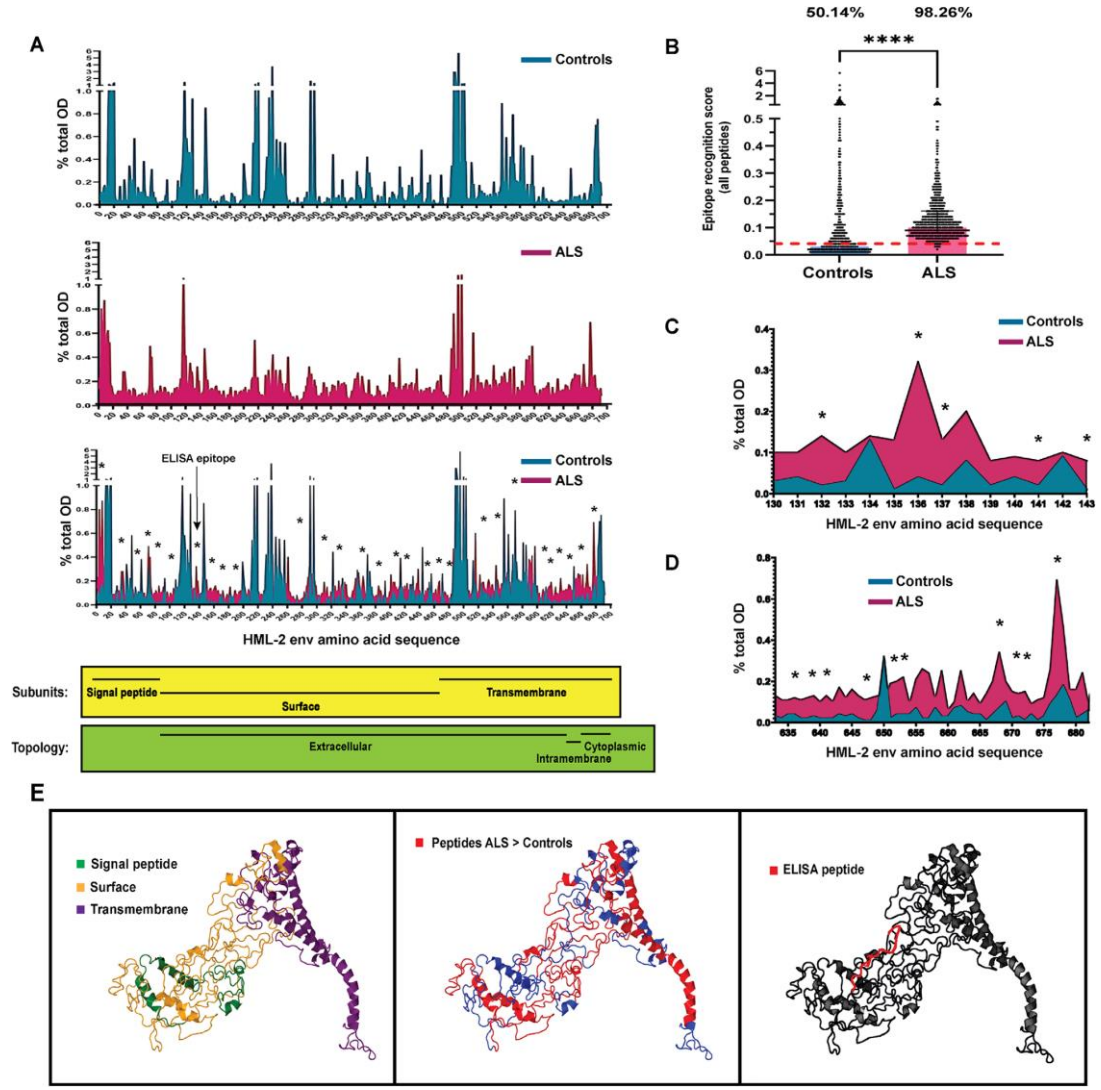

Figure 9: **Differential recognition of HML-2 env peptides in individuals with amyotrophic lateral sclerosis (ALS) and controls.**

Analysis of the percentage of total optical density (OD) per peptide in controls (n = 8) (A, upper part), in individuals with ALS (n = 10) (B, center), and comparison of both groups (A, lower part) (Wilcoxon test; *$p < 0.05$), as determined by HML-2 env peptide array. Location and topology of the protein areas according to the amino acid sequence (lower part).(**A**) Comparison of the values of epitope recognition score of all peptides covering the full sequence of HML-2 env between ALS individuals and controls (Mann–Whitney; ****$p < 0.0001$), and comparison of percentage of peptides eliciting a humoral response. Red dashed line represents the cutoff used to consider a peptide as positive (median of controls) (Fisher's exact test; $p < 0.0001$).(**B**) Percentage of total OD in an area of the protein mapping the peptide used in the enzyme-linked immunosorbent assay (ELISA) assay showing higher reactivity in ALS than in controls' sera (Wilcoxon test; *$p < 0.05$).(**C**) Percentage of total OD in a non-extracellular part of the protein located in the transmembrane subunit, showing higher reactivity in ALS than in controls' sera (Wilcoxon test; *$p < 0.05$).(**D**) The 3D modeling of the HML-2 env protein showing the location of the subunits (left), the peptides differentially recognized by ALS sera (center) and the peptide used in the ELISA assay (right).(**E**)

Afterward, we performed ELISA analysis on sera from 243 ALS patients, 242 HD, and 85 individuals with MS to screen for Abs to the envelope peptide of HERV-K-env$_{(19-37)}$. This epitope had shown higher reactivity in ALS patients in our previous study and aligned with a region exhibiting higher reactivity in ALS patients in the epitope mapping analysis.

The Abs levels were increased in ALS (mean OD ± SEM = 0.667 ± 0.028) than in HDs (0.340 ± 0.021) (Mann–Whitney; $p < 0.0001$) and in MS (0.258 ± 0.027) (Fig. 10A). The optical density (OD) cutoff value of 0.478 indicated the best sensitivity and specificity. According to this value, 55.14% of ALS patients and only 21.16% of HDs and 13.10% of MS individuals presented Abs targeting the HML-2 epitope (AUC = 0.769, $p < 0.0001$, Fisher's exact test $p < 0.0001$) (Fig. 10A, B).

We assayed extracellular DNA levels of HML-2 (expressed as the ratio of HML-2 copies to RPP30 copies) in the serum of a subgroup of individuals aiming to investigate possible differences in HML-2 expression. ALS individuals (n = 60) showed a higher HML-2 ratio (mean ratio ± SEM = 14.75 ± 3.79) compared to HDs (n = 27) (13.11 ± 3.54), even though the difference was not as striking as with Abs, probably because of the smaller sample size (Mann–Whitney; $p = 0.02$) (Fig. 10C). In ALS patients with a disease duration of fewer than 5 years, we found a weak but positive correlation between disease duration and the HML-2 ratio (Spearman's $r = 0.34$; $p = 0.02$) (Fig. 10D).

In individuals with a disease duration <5 years we observed a strong positive correlation between the number of peptides eliciting an immune response and the disease duration (Spearman's $r = 0.81$; $p = 0.02$) (Fig. 10E).

We likewise investigated if the observed disparities in Abs response between ALS patients and HDs were HERV-K specific or were linked to a broader disease-associated immunoglobulin imbalance. To accomplish this, we analyzed total IgG levels in serum samples and found no significant differences between ALS patients (mean ± SEM = 5,585 ± 383.5 μg/ml) and controls

(5,805 ± 228.9 μg/ml) (Mann–Whitney; $p = 0.19$) (Fig. 10F). Besides, no correlation was observed between total IgG levels and anti-HERV-K Ab levels in either healthy donors (Spearman' $r = 0.11$; $p = 0.3$) (Fig. 10G) or ALS subjects (Spearman's $r = 0.13$; $p = 0.2$) (Fig. 10H).

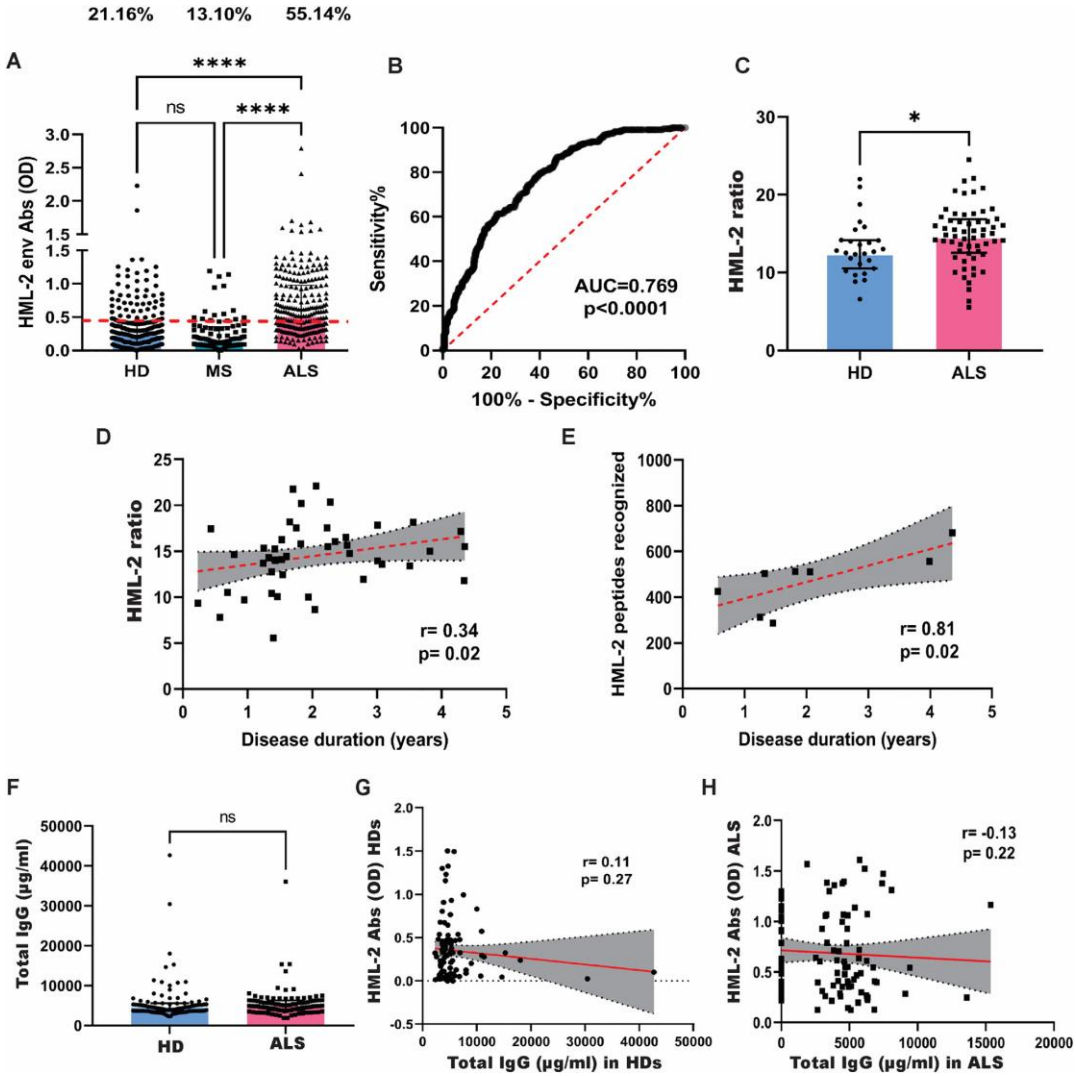

Figure 10: HML-2 levels and antibodies against a select HML-2 env peptide.

Comparison of levels of antibodies to a select HML-2 peptide determined by enzyme-linked immunosorbent assay (ELISA) in healthy donors (HD) (n = 242), individuals with amyotrophic lateral sclerosis (ALS) (n = 243) and in multiple sclerosis (MS) (n = 85) (Kruskal–Wallis test with Dunn post-hoc test; ****p < 0.0001) and comparison of percentage of positive individuals. Red dashed line represents the threshold used to assess the samples' positivity (Fisher exact test; p < 0.0001).(**A**) ROC curve between sensitivity and specificity of the HML-2 antibody ELISA test for every possible cutoff. The area under the ROC curve (AUC) is a measure of the diagnostic test accuracy (AUC = 0.769; p < 0.0001).(**B**) HML-2 ratio (HML-2 env copies/RPP30 copies) in individuals with ALS (n = 60) and HDs (n = 27) as determined by digital PCR (Mann–Whitney; *p = 0.02).(**C**) Correlation between disease duration and HML-2 ratio in ALS patients (with duration <5 years) (n = 54; Spearman's r = 0.34; p = 0.02).(**D**) Correlation between the number of peptides recognized and the disease duration in individuals with ALS (with duration <5 years) (n = 8; Spearman's r = 0.81; p = 0.02).(**E**) Levels of total IgG antibodies as determined by ELISA in HDs (n = 100) and individuals with ALS (n = 100) (Mann–Whitney; p = 0.19).(**F**) Correlation between concentration of total IgG antibodies and levels of HML-2 antibodies in the serum of HDs (n = 99; Spearman r = 0.11; p = 0.27).(**G**) Correlation between concentration of total IgG antibodies and levels of HML-2 antibodies in the serum of individuals with ALS (n = 99; Spearman r = 0.13; p = 0.22).(**H**)

On the classification of ALS subjects according to EL Escorial's criteria, we observed a weak positive correlation between the HML-2 ratio and the degree of diagnosis of ALS (Spearman's $r = 0.24$; $p = 0.03$) (Fig. 11A). Although the levels of HML-2 Abs were higher in all classes of ALS compared to controls (Kruskal–Wallis test; $p < 0.0001$), we found a weak negative correlation between the level of confidence in the ALS diagnosis and the levels of HML-2 Abs in ALS patients (Spearman's $r = 0.15$; $p = 0.02$) (Fig. 11B).

Indeed, patients with definite ALS (unpaired t-test; $p = 0.03$) (Fig. 11C) showed lower Abs levels than patients with a non-definite diagnosis of ALS (suspected, possible, or probable combined). Since the level of confidence in the diagnosis is dependent on the extent to which the disease has spread, the definitive diagnosis of ALS needs upper and lower motor involvement in at least three regions, this finding hints that Abs levels decline in the later stages of the disease. To investigate if Abs levels were associated with disease survival, we categorized individuals with ALS into three groups according to the ENCALS survival prediction model. The results showed that very long survival individuals (≥91 ± 1.84 months) had higher levels of HML-2 env Abs than the patients predicted to have a moderate (from 25.3 ± 0.06 to 43.7 ± 0.21 months) or very short survival (≤17.7 ± 0.20 months) (ANOVA with Sidak' post-hoc; $p = 0.03$ and $p = 0.02$, respectively) (Fig. 11D). When we divided the ALS group into tertiles based on HML-2 env Abs levels, the patients with the lowest Abs presence had lower survival (Tertile 1 median survival = 36 months) versus the subjects with higher levels of Abs (Tertile 2 median survival = 51 months; Tertile 3 median survival = 50 months) (Log-rank Mantel-Cox test; $p = 0.03$) (Fig. 11E).

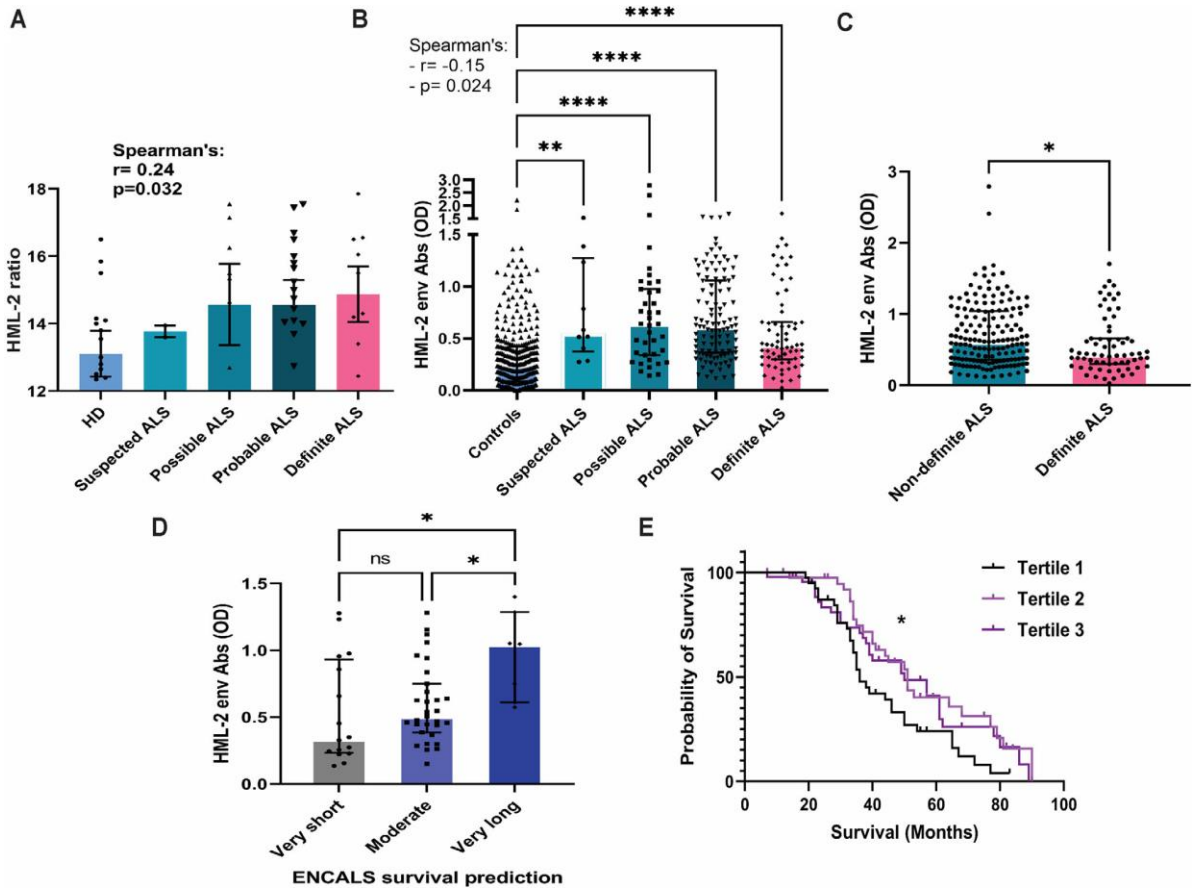

Figure 11: Correlation of HML-2 ratio and antibodies with severity of amyotrophic lateral sclerosis (ALS). (A) HML-2 ratio in individuals with ALS classified by El Escorial criteria (suspected: n = 2; possible: n = 13; probable: n = 29 and definite: n = 11) and in healthy donors (HD) (n = 27) as measured by digital polymerase chain reaction (PCR). Correlation analysis by Spearman's r test (r = 0.24; p = 0.03). (B) Levels of HML-2 env antibodies in individuals with ALS classified by El Escorial criteria (suspected: n = 10; possible: n = 39; probable: n = 112 and definite: n = 67) and in controls (n = 327; 242 HD and 85 multiple sclerosis [MS]), as determined by peptide enzyme-linked immunosorbent assay (ELISA). Comparison between groups (Kruskal–Wallis test with Dunn post-hoc analysis). Correlation analysis by Spearman r test (r = 0.15; p = 0.02). (C) Levels of HML-2 env antibodies in individuals with non-definite and definite ALS according to El Escorial criteria (unpaired t-test; p = 0.03). (D) Levels of HML-2 env antibodies in ALS individuals grouped by predicted survival according to the ENCALS model (analysis of variance [ANOVA] with Sidak post-hoc test; p = 0.02 and p = 0.03). (E) Analysis of observed survival in individuals with ALS according to the levels of antibodies to a select HML-2 env peptide. HML-2 antibodies optical density (ODs): Tertile 1 (n = 78); OD: 0.026–0.380; Tertile 2 (n = 78); OD: 0.381–0.749; Tertile 3 (n = 79); OD: 0.750–2.79. Log-rank Mantel–Cox test; p = 0.03.

1.7.3 Effect of Antiretroviral Therapy on HERV-K Abs Levels in Amyotrophic Lateral Sclerosis Patients

Our purpose aimed to explore whether 24 weeks of antiretroviral therapy, usually used to handle HIV infection, could successfully reduce levels of HERV-K AAbs in ALS patients. We measured Abs levels of HERV-K by ELISA assay, during, and after treatment with the antiretroviral regimen. The ALS patients who had a ratio of HERV-K:RPP30 greater than or equal to 13 were enrolled in the study. About 30% of ALS patients had detectable levels of HERV-K, and about 20% of patients with ALS had a level >1000 copies/ml. We recruited approximately 20% of patients with high levels to determine the antiretroviral effect. Levels of Abs against HERV-K epitopes were measured in 16 out of 28 ALS patients who completed a 24-week antiretroviral therapy regimen consisting of Darunavir, Tenoforvir, and Dolutegravir. Blood samples were collected every 4 weeks up to the 36^{th} week to monitor the trend of the Abs before, during, and after the end of the therapy. The obtained results, expressed as MEAN ± SEM (Fig. 12), did not show significant differences in the levels of Abs directed against the different HERV-K epitopes before, during, and after antiretroviral treatment. The OD values, directly proportional to the Abs presence in the plasma, ranged from 0.758 to 0.902 for HERV-K-$env_{(19\text{-}37)}$ (Fig. 12A), from 0.583 to 0.704 for HERV-K-$env_{(165)}$ (Fig. 12B), from 0.684 to 0.740 for HERV-K-$env_{(648)}$ (Fig. 12C), and from 0.521 to 0.590 for HERV-K-$env_{(668)}$ (Fig. 12D).

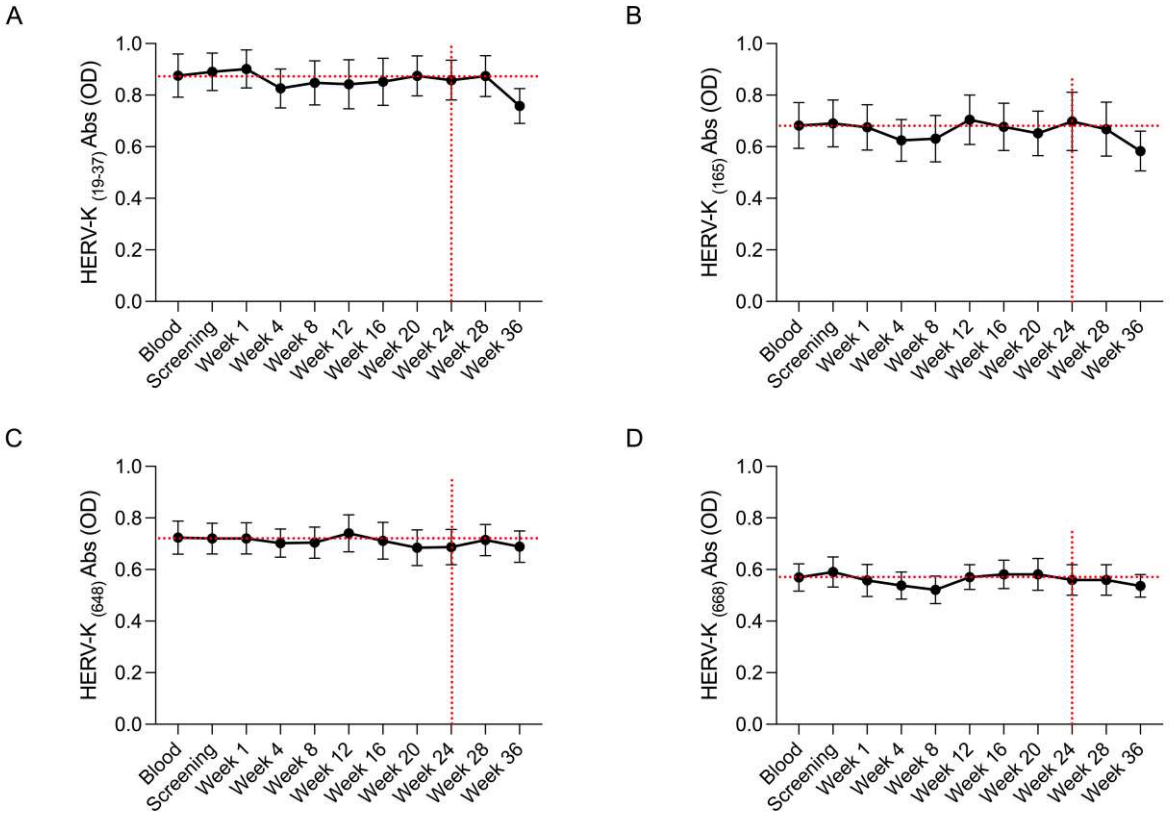

Figure 12. ELISA-based analysis of antibody reactivity against HERV-K-derived peptides in ALS patients undergoing antiretroviral therapy at multiple time points.

Plasma samples were tested against HERV-K-env-su(19-37) (**A**), TDP-43(165) (**B**), TDP-43(648) (**C**), and TDP-43(398–411)P (**D**) peptides. A Kruskal–Wallis test and Dunn's post hoc analysis were performed.

To have a clearer understanding of the tendency of Abs levels during the therapy, we categorized all time points (TPs) into three groups: pre-treatment TP, including blood and screening TPs; post-treatment TP, including those at weeks 28 and 36 TPs; and the end of antiretroviral therapy at week 24 TP. The results, displayed in Fig. 13, show a decreasing trend in Abs levels, although not statistically significant among the three TPs. Briefly, we observed a decrease in the OD levels from 0.883 to 0.823 for HERV-K-env $_{(19-37)}$ (Fig. 13A), from 0.695 to 0.626 for HERV-K-env$_{(165)}$ (Fig. 13B), from 0.733 to 0.694 in HERV-K-env$_{(648)}$ (Fig. 13C) and from 0.592 to 0.539 for HERV-K-env$_{(668)}$ (Fig. 13D). A limitation of this study is that the post-treatment period considered is probably not sufficient to observe the onset of Abs production, as it is necessary to consider the period of HERV-K reactivation after antiretroviral therapy. It would be very interesting to analyze

the long-term Abs trend for the patients involved in the study and correlate these results with the HERV-K-env gene expression levels, as well as with the clinical data.

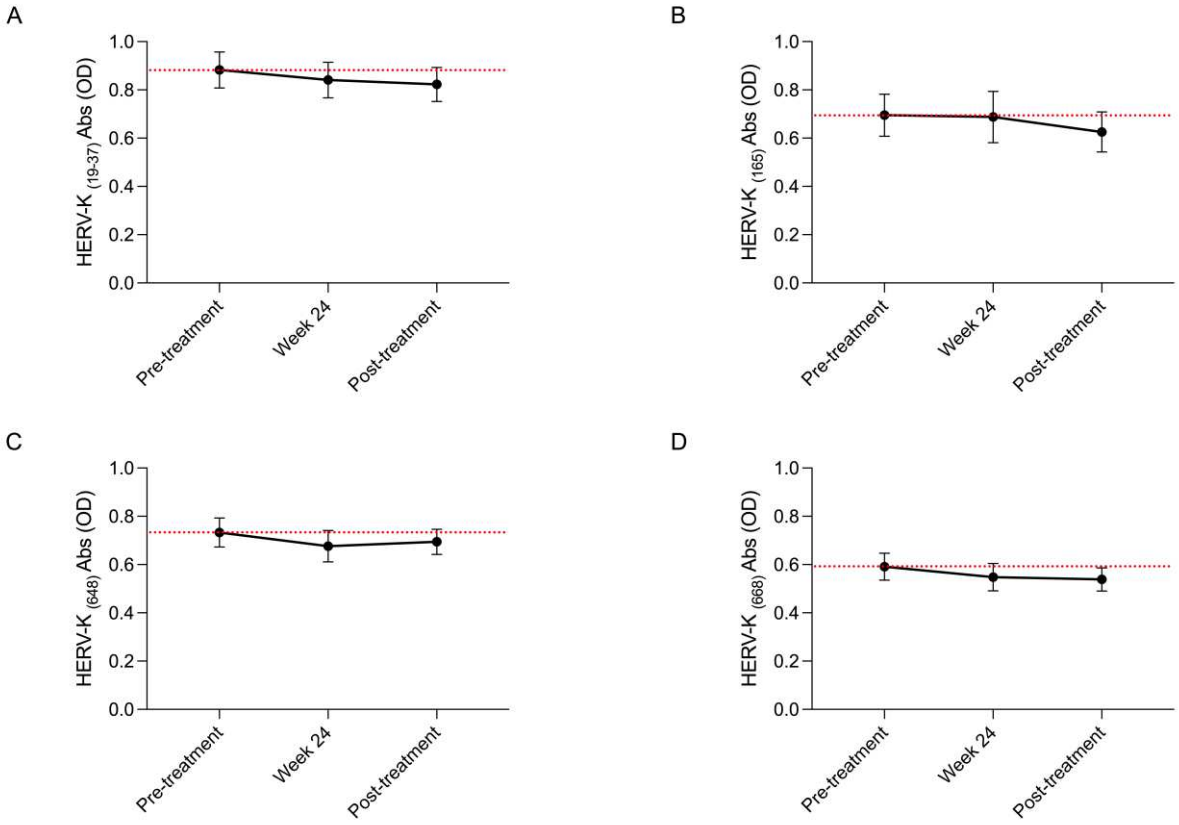

Figure 13. The reactivity of Abs against HERV-K-derived peptides in ALS patients under antiretroviral therapy was analyzed by ELISA at three different time points. The pre-treatment time point represents the average between the blood collection and screening time points, while the post-treatment time point represents the average between the Week 28 and Week 36-time points. Plasma samples were tested against HERV-K-env-su(19-37) (**A**), TDP-43(165) (**B**), TDP-43(648) (**C**), and TDP-43(398–411)P (**D**) peptides. A Kruskal–Wallis test and Dunn's post hoc analysis were performed.

1.7.4 Co-Transfection and Transfection of microRNAs in HEK-293 and TERA-1 Cells

To investigate the regulatory effect of miRNAs on HERV-K expression, we decided to focus on those that are down-regulated in ALS and capable of binding to the consensus sequence of HERV-

K. We identified 5 miRNAs for which the binding with the HERV-K sequence was predicted in silico analysis using the miRDB database.

In this study, HEK-293 cells were co-transfected with the selected miRNAs and HERV-K plasmid using Lipofectamine 3000 instructions (Thermo Fisher).

We conducted co-transfection experiments in HEK-293 cells to identify miRNAs that had a stronger effect on silencing HERV-K expression. Specifically, we transfected single miRNAs, combinations of miRNAs, and varying concentrations of Negative-Control-miRNA (NC-miR) to determine the concentration of NC-miR that could result in non-specific silencing (Fig. 14).

The Western Blot results showed that the co-transfection led to a decrease in HERV-K protein levels for miR-182 and miR-221, as compared to the cells transfected with the NC-miR. Based on these results, we decided to perform a second transfection using only the two selected miRNAs.

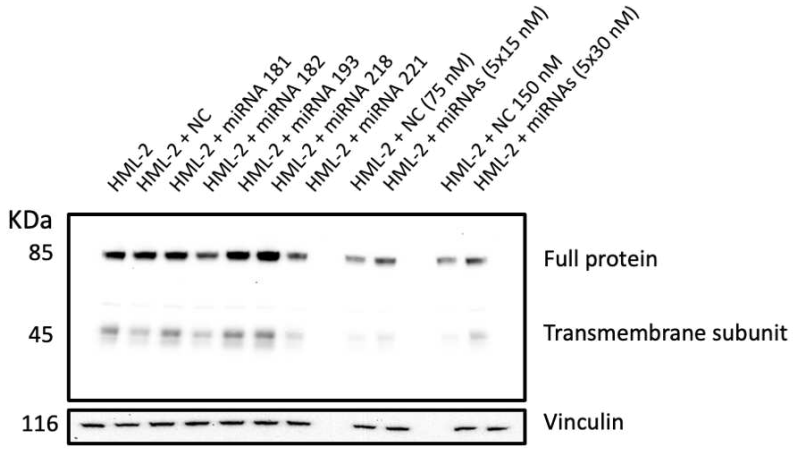

Figure 14. miR-182 and miR-221 modulate protein expression in HEK-293 after transfection.
Proteins purified from the HEK-293 cell line were analyzed by western blot.

We conducted the co-transfection experiment solely using the miR-182 and miR-221 with the aim to investigate their potential role in regulating HERV-K expression. These two microRNAs have been shown to be dysregulated in various neurological diseases, including ALS, and have been implicated in the regulation of multiple cellular processes such as apoptosis, cell proliferation, and differentiation. As shown in Fig.15, we assessed the effect of miRNAs on the gene expression of

HERV-K by considering the -*env* (Fig. 15A), -*gag* (Fig. 15B), and -*pol* (Fig. 15C) genes. The greatest silencing effect was observed at the -*env* gene level, with a significant difference between cells transfected with the NC-miRNA and those transfected with miR-182, miR-221, and the combination of both miRNAs. The same effect has been observed regarding -*gag* gene for the co-transfection with HERV-K and miR-182, HERV-K and miR-221, and HERV-K with the combination of both miRNAs (Fig. 15B). A slightly different result was observed for the silencing of the -*pol* gene. In fact, the only significant difference was observed regarding the co-transfection with HERV-K and miR-182 (Fig. 15C). The results at the protein expression level reflect the data obtained regarding gene expression. It seems that miR-182 acts with a mechanism that does not induce the degradation of the target mRNA but physically inhibits it from proceeding with translation in ribosomes and miR-221 acts with a mechanism that induces the degradation of the target mRNA. Therefore, the combination of the two miRNAs may have a synergistic effect, leading to a significant reduction in protein expression levels of HERV-K (Fig. 15A, B). These results suggest that the use of specific miRNAs or their combination could represent a potential strategy for the regulation of HERV-K expression.

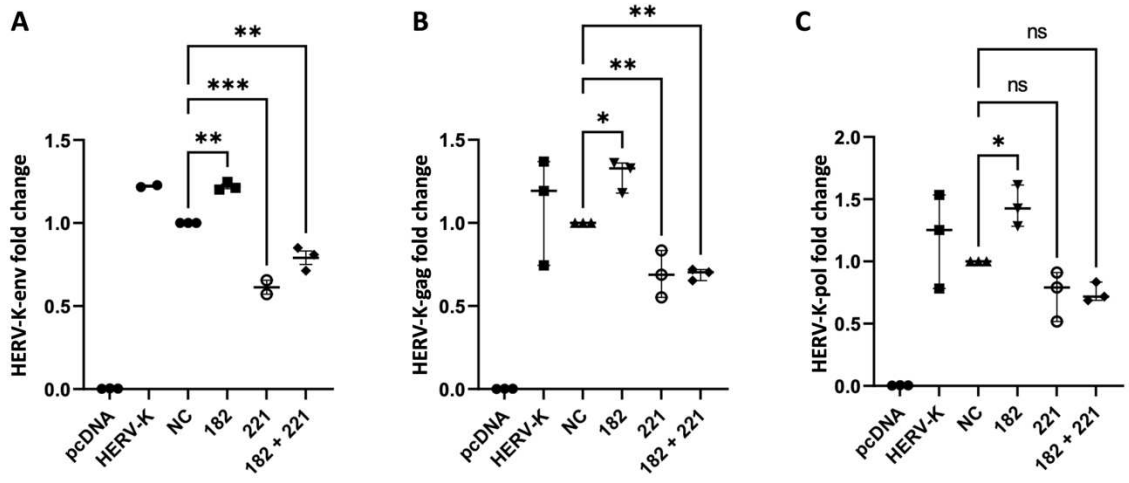

Figure 15. miR-182 and miR-221 modulates env, gag, and pol gene expression in HEK-293 after transfection. After transfection, RNA was collected and used for RT-qPCR to analyze the expression of the env-gene (**A**), gag-gene (**B**), and pol-gene (**C**). $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

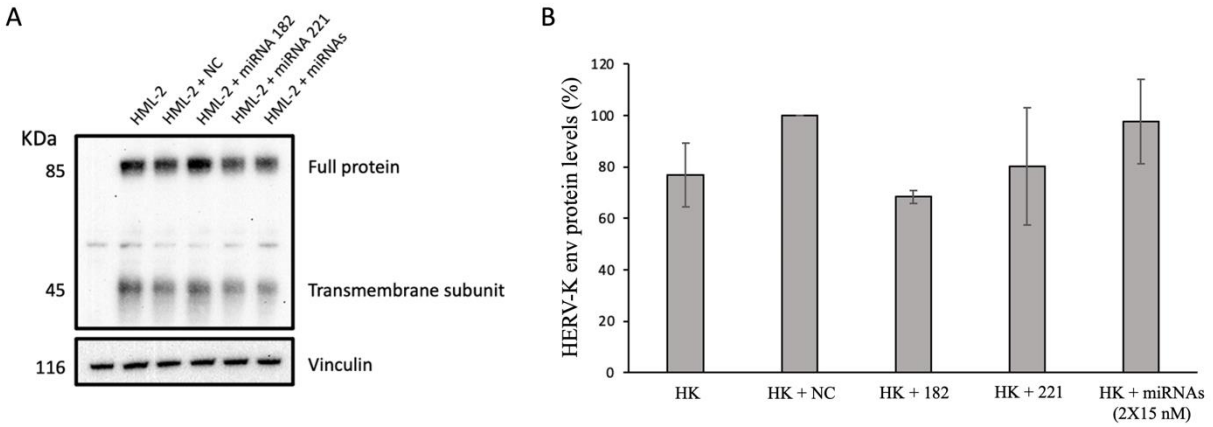

Figure 16. miR-182 and miR-221 modulate HERV-K envelope protein expression in HEK-293 after transfection.

Proteins purified from the HEK-293 cell line were analyzed by western blot. Protein expression levels were assessed by western blot analysis (**A**) and protein signals were quantified using ImageJ and plotted (**B**).

After this evidence, we wanted to further investigate the 5 selected miRNAs' binding property to endogenously expressed HERV-K to get closer to what might happen in pathology. To achieve this, we transfected the TERA-1 cell line, which expresses HERV-K at a relatively high endogenous level. TERA-1 is a human choriocarcinoma cell line that has been widely used as a model system to study the expression and function of HERV-K. Unlike what was observed in the transfection of HEK-293 cells, we did not observe a significant difference in the expression of endogenous HERV-K before and after transfection with miRNAs at the level of the HERV-K-envelope protein (Fig. 17), nor at the level of the pre-processed protein (Fig. 17C) or even at the level of the transmembrane fragment (Fig. 17D).

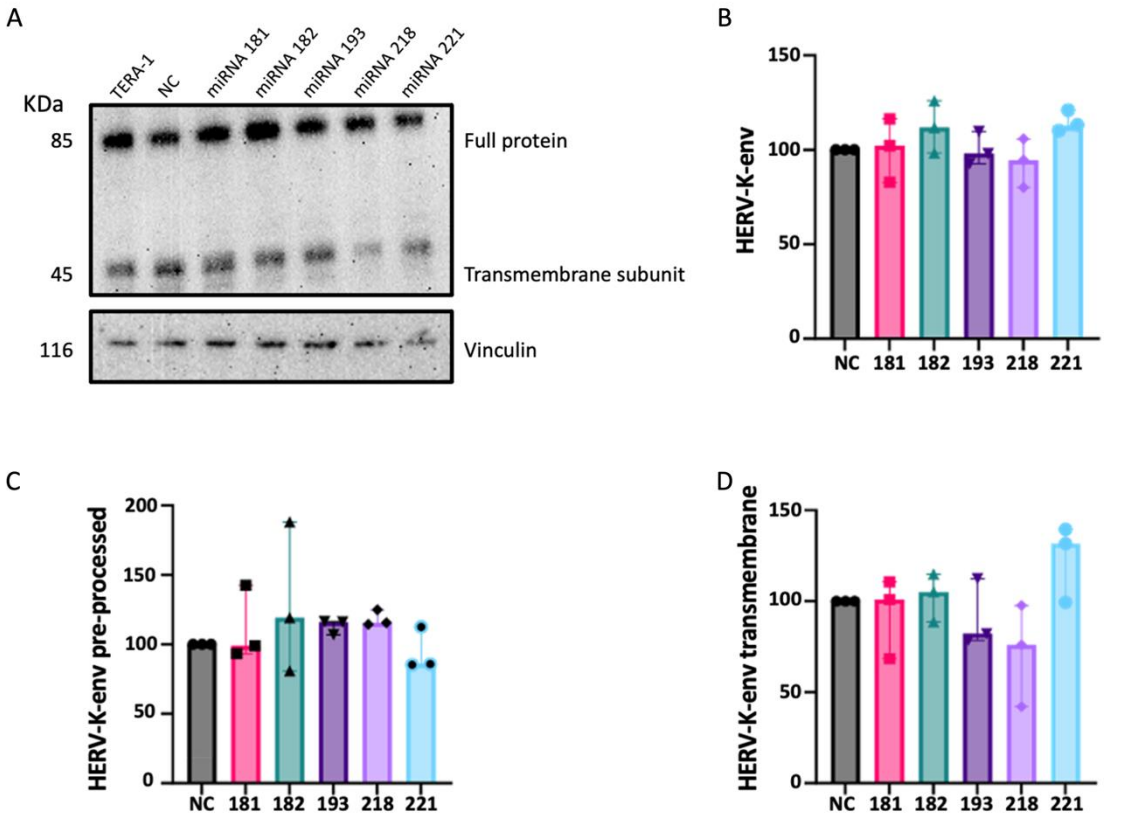

Figure 17. miRNAs transfection in the TERA-1 cell line.

Proteins purified from the TERA-1 cell line were analyzed by western blot (**A**). Protein expression levels were assessed by western blot analysis and HERV-K-env (**B**), HERV-K-env-preprocessed (**C**) and HERV-K-env-transmembrane (**D**) protein signals were quantified using ImageJ and plotted.

1.8 Discussion

Within this investigation, we conducted an assessment of the humoral response elicited by different immunogenic epitopes derived with both HERV-K and TDP-43. Of particular interest has been the hypothesis of its functional role, along with a preliminary analysis of the regulatory miRNAs mechanisms that might govern HERV-K expression.

We observed a noteworthy increase in Abs directed against the C-terminal fragment of TDP-43 and the HERV-K-env$_{(19\text{-}37)}$ epitopes in ALS patients relative to healthy controls. These findings corroborate our prior results and emphasize the significance of HERV-K as a crucial component in ALS pathology.[126]

Conti et al. documented higher levels of both anti-TDP-43 Abs and TDP-43 protein in the serum of patients with ALS compared with healthy controls and those with motor neuron mimic disease, Alzheimer's disease, and frontotemporal lobar degeneration. However, there was a lack of correlation between the levels of Abs and protein in the serum.[127]

On the other hand, the literature has provided clear evidence that the cytoplasmic aggregation of phosphorylated, ubiquitinated, and truncated TDP-43 is a pathological hallmark found throughout the clinical spectrum of ALS.[128,129] The C-terminal region of TDP-43 is a complex domain that plays a crucial role in mediating liquid-liquid phase transitions within the protein. These transitions are essential for the biogenesis of various membrane-less organelles, such as stress granules. However, this region also renders TDP-43 susceptible to misfolding and aggregation.[130] Of note, C-terminal fragments (CTFs) are present within cytoplasmic aggregates alongside the full-length form of TDP-43.[131] Our results emphasize the increased humoral response targeted towards specific C-terminal epitopes, including phosphorylated variants, of TDP-43 in patients with ALS compared to HDs.

A recent study[132] aimed to develop TDP-43-specific immunotherapy that can limit neuronal damage while maintaining physiological TDP-43 function. The study identified the C-terminal domain of TDP-43 as the key targeting domain through *in vitro* and mouse models. Targeting this domain was found to reduce pathology and neurotoxicity while preserving physiological TDP-43 activity. The study also found that monoclonal Abs treatment could enhance phagocytic capacity in ALS patient-derived microglia, enabling clearance of misfolded TDP-43. These results support the clinical strategy of targeting the C-terminal fragment of TDP-43 through immunotherapy.[132]

It would be particularly intriguing to investigate whether exist an association between the plasma Abs presence and the cerebral accumulation of TDP-43, as it might provide valuable insight about the presence of the protein in the brain.

To explore the potential role of Abs as a new biomarker in ALS, we examined their presence in both ALS-ND and ALS-LS patients. The humoral response to the four selected epitopes did not show any significant differences, suggesting that the Abs targeting the selected epitopes may be present in ALS patients at an early stage of diagnosis. Performing a prospective study to investigate the humoral response by searching for AAbs at the onset of symptoms, before a definitive ALS diagnosis is established, would be intriguing.

An aberrant expression of HERV-K pol transcripts has been observed in post-mortem brain samples from ALS patients when compared to individuals with other neurological disorders and healthy controls.[133] Additionally, Arru et al. have reported significantly elevated levels of anti-HERV-K-env-su Abs in the serum and cerebrospinal fluid of ALS patients, suggesting the development of a humoral immune response to HERV-K.[134]

Moreover, HERV-K pol-gene expression was observed to be correlated with TDP-43 mRNA in post-mortem brain tissue of ALS patients[116], and we also identified a correlation between the humoral response against HERV-K-env-su$_{(19-37)}$ and TDP-43 epitopes in ALS patients.[135]

In a recent study, researchers investigated the inter-cellular movement of "prion-like" proteins as a potential explanation for the propagation of neurodegeneration between cells.[49] The study focused on the progression of ALS and FTD, which are not infectious and cannot be caused by the injection of aggregated TDP-43 alone. The study found that ERV expression and TDP-43 proteinopathy are mutually reinforcing and that the expression of certain viral ERVs can stimulate the cytoplasmic aggregation of human TDP-43.[49] This mechanism could potentially explain the neurodegenerative propagation through neuronal tissue caused by TDP-43 proteinopathy.[136]

Unlike the correlation among Abs directed against TDP-43 and HERV-K that we observed in ALS patients, we did not find any correlation in healthy controls, which supports and suggests a specific association between HERV-K and TDP-43 in disease conditions. Taking into account the duration of the disease we found a slightly stronger correlation between TDP-43 and HERV-K Ab levels in

LS-ALS compared to ND-ALS. If future studies validate this finding, this relationship may serve as a potential marker of ALS progression. A prospective study monitoring ALS patients as their condition evolves would be valuable to investigate this field.

It is relevant to point out that in the immune responses, the initial epitopes that trigger the adaptive immune system are usually limited. However, with repeated exposure to an antigen, the T and B cell responses can broaden to include other epitopes of the same antigen. This phenomenon, the intramolecular epitope spreading, refers to the process where the immune response diversifies its specificity from the initial response that targets dominant epitopes on a self or foreign protein to other subdominant or cryptic epitopes on the same protein (intramolecular spreading) or other proteins (intermolecular spreading).[137] An explanation for our results, regarding the multiple epitope recognition of HML-2 env in ALS may be attributed to epitope spreading. Therefore, it remains to be clarified what role these Abs may have in ALS pathology.

We decided to delve deeper into this aspect investigating the Abs response against the viral element HERV-K and its relationship with ALS pathology. Specifically, we sought to evaluate whether intramolecular epitope spreading of the HERV-K virus was involved in the pathogenesis of ALS and whether the Abs response against viral epitopes was associated with disease progression. Additionally, we examined whether anti-HERV-K Abs had a protective effect on ALS. We documented a diminished humoral response against HML-2 in ALS patients in the later stages of the disease. In addition, we observed a correlation between lower levels of anti-HERV-K Abs and poorer predicted and observed survival in ALS patients. Furthermore, consistent with the previous study, we found higher levels of extracellular HERV-K-DNA in the serum of individuals with ALS.[138] Taken together, our results suggest that the reactivation of HERV-K may play a role in the development of ALS and that a humoral response against it could be protective for patients. An alternative hypothesis is that B cell activation may occur during the early stages of the disease, but there could be a subsequent decline in B cell function as the illness progresses. Furthermore,

our study revealed that the serum samples from ALS patients exhibited a distinct reactivity pattern to the envelope protein of HERV-K, with more antigenic regions compared to control samples. We also observed a correlation between the levels of HERV-K in the serum and disease duration, suggesting that some individuals with ALS may experience continuous exposure to HERV-K antigens. The study revealed that there is a correlation between the duration of the disease in ALS patients and the number of HERV-K peptides recognized by their sera. This indicates that epitope spreading may be responsible for the increase in epitope recognition of HERV-K in ALS. Therefore, it is possible that the increase in epitope recognition of HERV-K in ALS patients may be due to epitope spreading resulting from neural death and subsequent release of normally cryptic antigens.

Since HERV-K env is a protein encoded by a human gene, Abs against it would be considered autoantibodies (AAbs). Although AAbs are known to have a pathogenic role, several studies have also suggested that autoimmunity can be protective in certain situations. For instance, in systemic lupus erythematosus (SLE), IgM anti-double-stranded DNA (anti-dsDNA) Abs are negatively correlated with the severity of glomerulonephritis[139,140] and the administration of IgM anti-dsDNA prevents the development of pathology in a SLE mouse model.[141]

There is growing evidence to support that the immune system plays a beneficial role in the progression of ALS. It has been suggested that similar to the situation in cancer immunology, the onset of clinical symptoms in neurodegenerative diseases may reflect immune tolerance towards neurotoxic self-antigens and a loss of immune surveillance. To fight off neurodegenerative conditions, breaking peripheral immune tolerance to the CNS self-antigens may be necessary to boost protective autoimmunity.[142] Protective autoimmunity is believed to be a natural response to CNS trauma. While not yet confirmed, some studies suggest that ALS may be linked to repetitive CNS trauma in certain individuals. For example, ALS rates have been reported to be higher among military veterans and collision-sport athletes.[143–145] It is possible that the expression of HERV-K

may contribute to the increased risk of neurodegeneration after brain trauma. Neural stem cells typically migrate to areas of CNS trauma to facilitate repair, and HERV-K env is highly expressed in neural stem cells but silenced in neurons and differentiated cells. Therefore, it is possible that the reactivation of HERV-K in neural stem cells after CNS trauma may contribute to the development of neurodegenerative diseases such as ALS, Alzheimer's disease, Parkinson's disease, and chronic traumatic encephalopathy. However, more research is needed to test this hypothesis.[20,146] In addition, HERV-K env is known to be neurotoxic, which may lead to degeneration in neurons near areas of brain injury if the expression of HERV-K in differentiating neural stem cells is not silenced quickly enough, or if this process occurs repeatedly due to repetitive brain trauma. However, AAbs against HML-2 env may have a protective effect against neurodegeneration and the development of ALS in individuals with increased expression of HERV-K following brain injury.

In summary, this evidences have important strengths, including large sample size and extensive follow-up of individuals with ALS, allowing for analysis of predicted and observed survival according to Abs levels. The limitations include incomplete information on the date of onset for all patients, and the association between Ab levels and predicted survival may be influenced by additional prognostic factors beyond the progression rate, such as onset type, age at onset, diagnostic criteria, and the presence of genetic mutations. Furthermore, the study only analyzed Abs against HERV-K-env, but there may be other HERV-K proteins or antigens that are also relevant to the development and progression of ALS. Additionally, the study focused on a specific population of European descent, and it is unclear whether these findings can be generalized to other populations with different genetic backgrounds and environmental exposures. Our results suggest that HERV-K Abs may have a protective role in slowing disease progression in ALS, but it is still unclear whether these Abs have a causal effect or are simply a biomarker. Testing these Abs as a potential therapeutic option for ALS could be a promising approach. In fact, a monoclonal

Ab against the envelope of HERV-W has already been developed as a treatment for MS. Further research is needed to investigate the potential therapeutic use of HERV-K Abs in ALS.[147] A preclinical study aimed at investigating the potential therapeutic application of a monoclonal Ab targeting HERV-K env in ALS has demonstrated its ability to prevent neurodegeneration induced by HERV-K-env protein both in vitro and in vivo.[148]

To better understand the role of Abs in ALS, we decided to monitor their levels during the administration of antiretroviral therapy. It has been suggested that HIV infection can lead to the upregulation of HERV-K expression, which may contribute to the chronic immune activation and inflammation seen in HIV-positive individuals.[149] In our study, levels of Abs against HERV-K epitopes were measured before, during, and after a 24-week course of antiretroviral therapy targeting HIV in ALS patients who were negative for HIV. The therapy included Darunavir, Tenofovir, and Dolutegravir. Darunavir is a medication that belongs to a class of drugs called protease inhibitors. It is primarily used in the treatment of HIV infection. Darunavir works by inhibiting the activity of the HIV-1 protease enzyme, which is responsible for cleaving viral proteins into their functional components during the replication of the virus. By inhibiting this enzyme, it prevents the formation of new viral particles, thereby reducing the viral load in the body and slowing the progression of HIV infection. Tenofovir is an antiviral medication that works by inhibiting the reverse transcriptase enzyme of the HIV and the hepatitis B virus (HBV). It blocks the activity of this enzyme and prevents the virus from replicating and spreading in the body. Tenofovir is often used in combination with other antiretroviral drugs to treat HIV infection and to reduce the risk of transmitting HIV to others. It is also used to treat chronic hepatitis B virus infection. Dolutegravir belongs to the class of HIV integrase inhibitors, which blocks the activity of the viral integrase enzyme, preventing the insertion of viral DNA into the host DNA and thus preventing the virus from replicating. In this way, Dolutegravir helps to control HIV infection and prevent the development of AIDS.

Despite the administration of antiretroviral therapy, we did not observe significant changes in the levels of Abs directed against immunogenic epitopes of the HERV-K envelope. The lack of significant changes suggests that antiretroviral therapy against HIV has no effect on the level of Abs directed against HERV-K. The study has some limitations that need to be taken into account. Firstly, the study population is relatively small, with only 16 patients completing the study. Furthermore, the period taken into consideration after the end of antiretroviral therapy is short, since blood samples were collected up to the 36th week. Since we are examining the presence of Abs rather than HERV-K gene expression, it may take more time after the completion of therapy to detect any reactivation of HERV-K, which should result in the production of Abs. Therefore, it is essential to associate the Abs levels data with HERV-K gene expression levels to observe whether these results are directly or indirectly correlated. It is important to note that this type of study is still in its early stages and further research is needed to fully understand the relationship between HERV-K and ALS. However, the detection of Abs against HERV-K in ALS patients may open up new avenues for understanding the pathogenesis of the disease and for developing new therapeutic approaches.

In addition to the role played by the immune system in producing antibodies, we decided to investigate one of the potential roles underlying the regulation of HERV-K gene expression. MicroRNAs predominantly regulate the expression of target genes in a negative manner. Our hypothesis is that a dysregulation at the level of microRNAs may lead to a failure in the silencing of HERV-K and its consequent overexpression. We decided to focus our attention on the two microRNAs that, following a preliminary experiment, showed the greatest effect in regulating HERV-K, miR-182 and miR-221.

MicroRNA 182 (miR-182) is a small non-coding RNA molecule that plays a critical role in various biological processes. It has been shown to be involved in the regulation of neuronal differentiation, synaptic plasticity, and cancer development. Studies have demonstrated that miR-182 can promote

the proliferation and migration of cancer cells in different types of cancers, including breast, lung, liver, and colon cancer. In addition to its role in cancer, miR-182 has also been implicated in neuronal development and function.[150,151]

MicroRNA 221 (miR-221) is a small non-coding RNA molecule that has been shown to play a crucial role in various biological processes, including cell proliferation, apoptosis, and differentiation. It is highly expressed in several types of cancer, and studies have suggested that miR-221 may serve as a potential target for cancer therapy.

Research has demonstrated that miR-221 is upregulated in various types of cancer, including breast, liver, and pancreatic cancer.[152] Moreover, miR-221 has also been implicated in cardiovascular disease. Studies have suggested that miR-221 may play a role in the pathogenesis of atherosclerosis and vascular smooth muscle cell proliferation.[153]

In summary, miR-182 and miR-221 are multifunctional molecules that play critical roles in various biological processes, including cancer and cardiovascular disease. Understanding their molecular mechanisms of action and downstream targets may provide insights into the development of new therapies for ALS.

However, the findings from TERA 1 cell line transfection, which involved cells expressing HERV-K endogenously, did not reveal a significant impact of the microRNAs on HERV-K expression post-transfection. This suggests that, despite their ability to bind and regulate the HERV-K sequence, as we demonstrated in HEK-293 cell line co-transfection, their actual physiological impact may differ. HERV-K expression varies across different cell types, and it would be advisable to confirm this result using cell lines that are directly implicated in the pathology to obtain a more accurate representation. The most appropriate cell lines to study HERV-K regulation in ALS would be those derived from motor neurons or other cell types directly involved in the disease pathogenesis. Therefore, further studies are needed to investigate the expression of HERV-K in

different cell types, particularly those involved in the pathology, to better understand the role of HERV-K-targeting microRNAs in regulating HERV-K expression.

1.9 Conclusions and Future Directions

Our study aimed to achieve a greater understanding of the role of the immune system in the pathology of ALS. There are still many points to clarify, and certainly, one of our next goals will be to continue the analysis aimed at elucidating the function of the immune system and the role of microRNAs in this disease. We also aim to expand the study population by examining not only patients but also close relatives, in order to determine if they exhibit any particular characteristics. Currently, there are no biomarkers available for the diagnosis of ALS, and the disease is typically diagnosed based on clinical presentation and exclusion of other conditions. This lack of biomarkers and early diagnostic tools poses a major challenge in the management and treatment of ALS, as the disease progression can vary greatly between patients. Additionally, many patients may not receive a diagnosis until the disease has already advanced significantly, further limiting the effectiveness of available treatments. There is a critical need for the development of reliable biomarkers and early diagnostic tools in order to improve the diagnosis and management of ALS.

Chapter II

2 Introduction

2.1 SARS-CoV-2 and Human Endogenous Retrovirus

SARS-CoV-2 belongs to the family of coronaviruses, which also includes SARS-CoV and MERS-CoV, both of which caused previous outbreaks. SARS-CoV-2 is a single-stranded RNA virus and it is responsible for the COVID-19 pandemic, a respiratory illness that emerged in Wuhan, China in December 2019, which has affected millions of people around the world and caused significant morbidity and mortality.[154] The virus is highly contagious and spreads through respiratory droplets and contact with contaminated surfaces. The virus can cause a range of symptoms, from mild to severe, and can lead to hospitalization and death in some cases.[155] Some of the most common symptoms include fever, cough, and difficulty breathing, while more severe cases can lead to acute respiratory distress syndrome, septic shock, and multi-organ failure. Recent studies have suggested that a significant proportion of patients with severe COVID-19 disease develop AAbs against a range of self-antigens, which could contribute to the pathogenesis of the disease.[156] AAbs against various targets, including type I interferons and multiple cytokines, have been reported in patients with severe COVID-19.[157,158] In some cases, these AAbs have been associated with an impaired immune response and a worse clinical outcome. While the exact mechanisms underlying the production of AAbs in COVID-19 patients are not yet fully understood, it is believed that the virus may trigger a dysregulated immune response leading to the production of AAbs. Their presence in COVID-19 patients highlights the need for further research to understand the role of autoimmunity in the disease and to develop potential strategies for treatment. Several studies have explored the possible link between HERVs and SARS-CoV-2. A recent *in vitro* study demonstrated that the envelope protein of SARS-CoV-2 can induce the expression of HERV-K.[159] Balestrieri et al.[160] documented the increased expression of HERV-W-envelope protein in COVID-19 leukocytes compared to healthy individuals. The envelope expression was positively correlated

with markers of T-cell differentiation and exhaustion as well as blood cytokine levels. The percentage of lymphocytes positive for HERV-W ENV was also found to be positively correlated with inflammatory markers and the severity of pneumonia in COVID-19 subjects. Interestingly, the expression of HERV-W ENV was found to be indicative of the respiratory outcome of patients during their hospitalization. [160]

In addition, has been shown that HERV-K expression is upregulated in COVID-19 patients, particularly in those with severe disease. The authors suggested that the expression of HERVs may contribute to the pathophysiology of local inflammatory pathways in SARS-CoV-2-infected lungs and that targeting their expression could be a potential therapeutic strategy. [161]

Since the activation of HERVs has been implicated in the pathogenesis of COVID-19, targeting these elements could be a potential therapeutic strategy for the treatment of this disease.

2.2 SARS-COV-2 Epidemiology and Risk factors

The COVID-19 pandemic has rapidly spread worldwide since its emergence in late 2019. Epidemiological data have shown that COVID-19 affects all age groups and both genders, with higher morbidity and mortality rates observed in elderly and male patients, respectively. The virus is mainly transmitted via respiratory droplets, close contact, and fomites. Several risk factors have been identified to be associated with severe COVID-19, including pre-existing comorbidities such as hypertension, diabetes, obesity, and cardiovascular disease, as well as immunocompromised status. In addition, environmental and socio-economic factors have also been shown to play a role in COVID-19 morbidity and mortality.[162] Current prevention measures include vaccination, social distancing, wearing masks, and frequent hand washing. The etiology of SARS-CoV-2 infection is complex and not yet fully understood. The virus is believed to have originated from bats and may have been transmitted to humans through an intermediate host, such as a pangolin.[163] SARS-CoV-

2 enters human cells through the angiotensin-converting enzyme 2 (ACE2) receptor and employs the transmembrane protease serine 2 (TMPRSS2) for viral spike protein priming and membrane fusion.[164] Once inside the host, the virus can cause damage to various organs, including the respiratory system, cardiovascular system, and gastrointestinal system, among others.[165,166] The pathogenesis of SARS-CoV-2 infection involves a dysregulated immune response that can lead to a cytokine storm and acute respiratory distress syndrome in severe cases.[167]

2.3 SARS-COV-2 Diagnosis and Therapy

The diagnosis of COVID-19 is primarily based on RT-PCR assays targeting SARS-CoV-2 nucleic acid.[168] However, chest computed tomography (CT) is also used to diagnose and monitor the disease. CT can reveal the presence of ground-glass opacities and consolidations in the lungs, which are typical radiological features of COVID-19.[169] Other diagnostic tests for COVID-19 include serological assays that detect IgM and IgG Abs against SARS-CoV-2.[170]

Treatment for COVID-19 is mainly supportive and includes symptomatic management, oxygen therapy, and mechanical ventilation for severe cases (WHO, 2020). Several drugs, such as antivirals, immunomodulators, and monoclonal antibodies, have been used in clinical trials to treat COVID-19, but none have been proven to be universally effective.[171] Some of the drugs that have been used include Remdesivir[172], Hydroxychloroquine[173], Tocilizumab[174], and convalescent plasma.[175] While treatments for COVID-19 have improved over time, the best course of action remains prevention through vaccination. Vaccines are a safe and effective tool for preventing COVID-19, reducing the likelihood of infection and severe disease. With the emergence of new variants, vaccination remains critical for maintaining immunity and reducing transmission. While some individuals may still contract COVID-19 even after being vaccinated, the risk of severe

illness and hospitalization is greatly reduced. Additionally, widespread vaccination has been shown to slow the spread of the virus and decrease the likelihood of new variants emerging.

2.4 Results

2.4.1 HCoV-NL63 and SARS-CoV-2 Share Recognized Epitopes by the Humoral Response in Sera of People Collected Pre- and during CoV-2 Pandemic

In our first study, we pursued an explanation for the heterogeneity among responses to SARS-CoV-2 infection.

Despite the lack of structural homology in RBD cores or RBM between HCoV-NL63 and SARS-CoV, both viruses recognize common regions on ACE2. We attempted to identify possible common binding hotspots on ACE2 using the IEDB site by analyzing the S-proteins of both CoVs. We wondered if a serologic cross-reactivity may be found between HCoV-NL63 and SARS-CoV-2. Since HCoV-NL63 is known to infect children and is thus widely distributed in the population, we investigated whether a previous NL-63 infection could elicit a rapid immune response to SARS-CoV-2 infection, thus providing a first line of protection.

We evaluated the humoral response against five selected epitopes of HCoV-NL63 and two epitopes of SARS-CoV-2 in plasma samples from pre-pandemic (samples collected before the pandemic period), mid-pandemic (samples collected during the pandemic period), and SARS-CoV-2 positive patients groups. The NL63-RBM1 epitope showed a statistically significant difference in Abs response between the pre-pandemic and mid-pandemic populations ($p = 0.0055$), as determined by the Mann-Whitney U test. Cut-off values were established using ROC analysis, and Fisher's exact test was used to determine the percentage of positive and negative samples. We

found that 63.1% of the pre-pandemic population (101 out of 160 patients) and 48.9% of the mid-pandemic population (69 out of 141 patients) were seropositive ($p = 0.01$, Fisher's exact test) with a cut-off value of 0.100 and AUC = 0.593 (Fig.18A).

The humoral response against NL63-DISC-like and NL63-RBM2_1 epitopes showed a significant difference between pre-pandemic and mid-pandemic populations, with *p-values* of 0.0001 for both. Of the pre-pandemic patients, 112 out of 160 (70%) were positive for NL63-DISC-like, while 69 out of 141 mid-pandemic patients (48.9%) were positive (p = 0.0002, Fisher's exact test). The cut-off value was determined to be 0.087, with an AUC of 0.627 and *p-value* of 0.0001 (Figure 1F). Similarly, for NL63-RBM2_1, 120 out of 160 pre-pandemic patients (75%) and 69 out of 141 mid-pandemic patients (48.9%) were positive ($p < 0.0001$, Fisher's exact test) with a cut-off value of 0.149, AUC of 0.630 and *p-value* of 0.0001 (Fig. 18B,F).

The Abs response to COV2-SPIKE$_{(421–434)}$ and COV2-SPIKE$_{(742–759)}$ showed a significant difference between the pre-pandemic and mid-pandemic populations, both with $p < 0.0001$ (Fig. 18G,H). The analysis of COV2-SPIKE$_{(421–434)}$ peptide showed that 128 out of 160 patients (80%) in the pre-pandemic population and 60 out of 141 patients (42.5%) in the mid-pandemic population were positive ($p < 0.0001$, Fisher's exact test) with a cut-off value of 0.157, AUC = 0.736, and $p < 0.0001$. In regards to COV2-SPIKE$_{(742–759)}$, 111 out of 160 patients (69.4%) and 72 out of 141 patients (51.1%) in the pre-pandemic and mid-pandemic populations respectively were positive ($p = 0.001$, Fisher's exact test) with a cut-off value of 0.093, AUC = 0.633 and $p < 0.0001$ (Fig. 18G,H). However, the Ab response against NL63-RBM2_2, NL63-RBM3, and NL63-SPIKE$_{(541–554)}$ were not significantly different between the two populations (Fig. 18C,D,E).

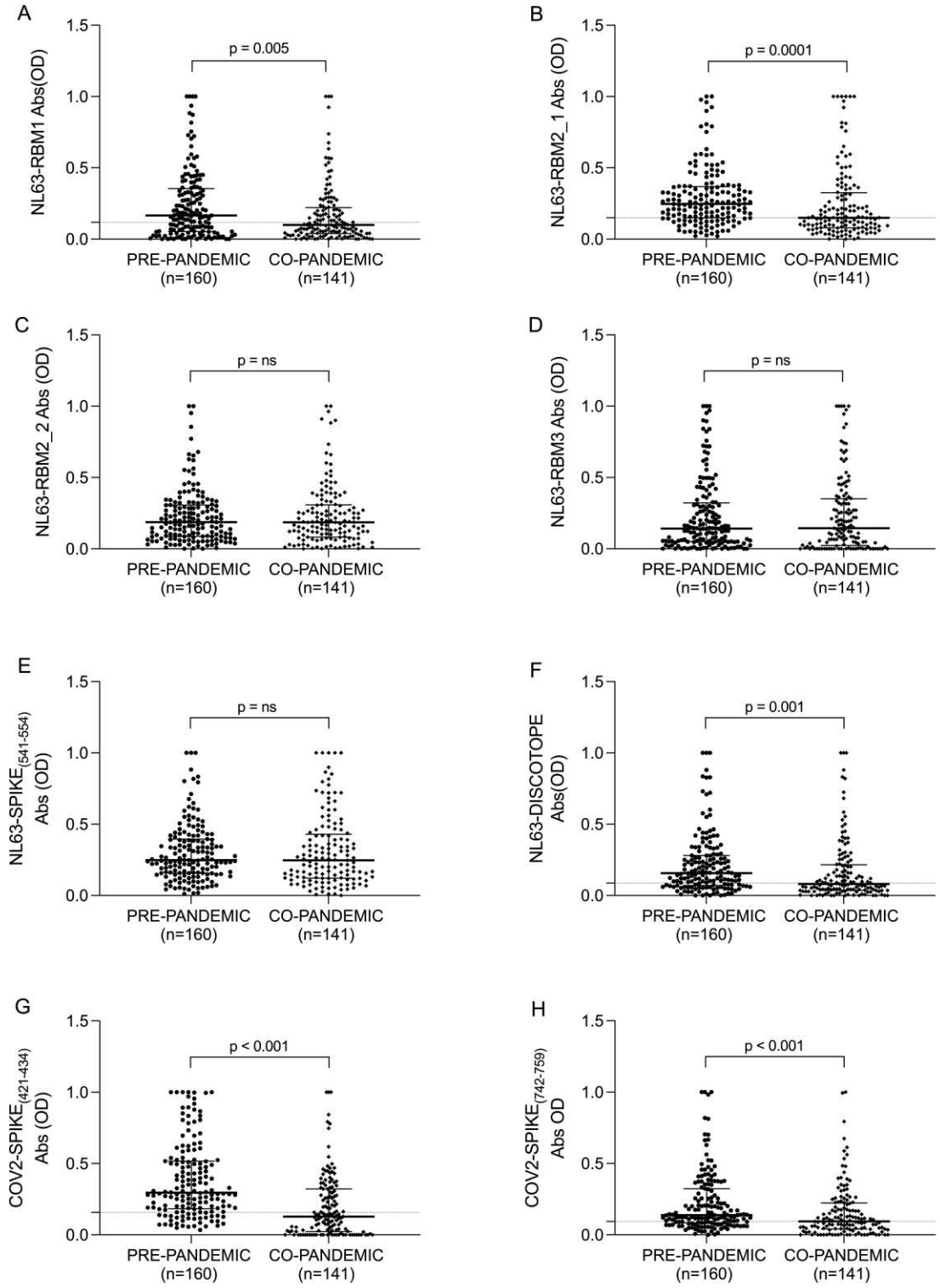

Figure 18. ELISA-based analysis of Abs reactivity against SARS-CoV-2 and HCoV-NL63 derived peptides. Plasma samples were tested against NL63-RBM1 (**A**), NL63-RBM2_1 (**B**), NL63-RBM2_2 (**C**), NL53-RBM3 (**D**), NL63-SPIKE$_{(541-554)}$ (**E**), NL63-DISC-like (**F**), COV-SPIKE$_{(421-434)}$ (**G**) and COV2-SPIKE$_{(742-759)}$ (**H**) peptides. Scatter plots represent the median with the interquartile range. Dashed lines represent thresholds used to assess the sample positivity.

Correlation analyses were conducted using OD values obtained from assessing humoral responses to HCoV-NL63 and SARS-CoV-2 epitopes. Remarkably, in the pre-pandemic COVID-19 group, the Abs response to SARS-CoV-2 epitopes showed a positive correlation with the Abs response to NL63-derived peptides. Specifically, COV2-SPIKE$_{(742–759)}$ showed a moderate correlation with NL63-RBM1 (r = 0.601, p < 0.0001) (Fig. 19A), NL63-RBM2_2 (r = 0.592, p < 0.0001) (Fig. 19C), NL63-RBM3 (r = 0.626, p < 0.0001) (Fig. 19D). Moderate correlations were also observed between COV2-SPIKE$_{(421–434)}$ and NL63-RBM1 (r = 0.661, p < 0.0001) (Fig. 20A), NL63-RBM2_1 (r = 0.5, p < 0.0001) (Fig. 20B), NL63-RBM2_2 (r = 0.571, p < 0.0001) (Fig 20C), NL63-RBM3 (r = 0.582, p < 0.0001) (Fig 20D), and NL63-DISC-like (r = 0.529, p < 0.0001) (Fig 20F); a low correlation was noted between COV2-SPIKE$_{(421–434)}$ and NL63-SPIKE$_{(541–554)}$ (r = 0.414, p < 0.0001) (Fig 20E).

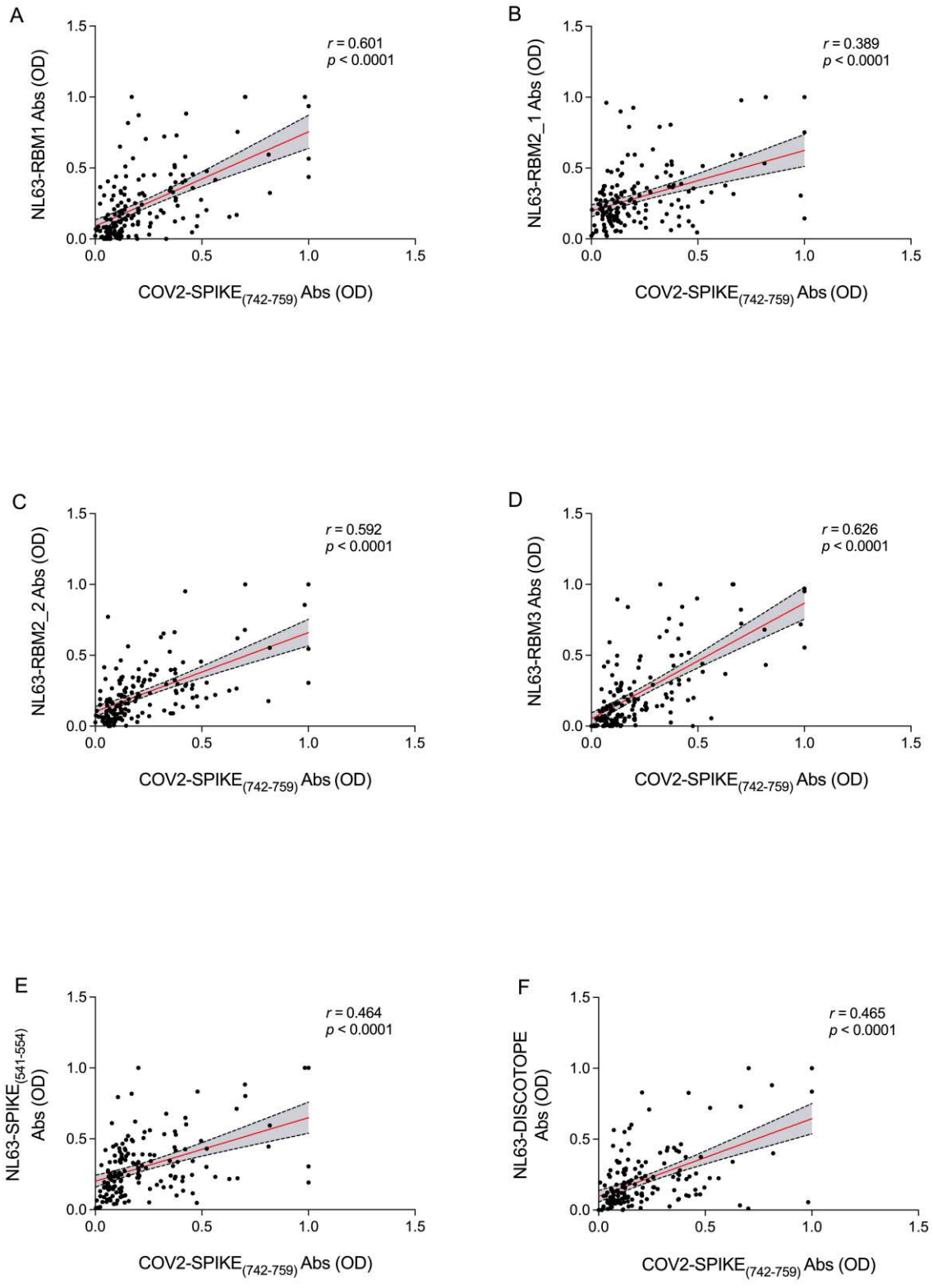

Figure 19. Scatter plot expressing correlation between SARS-CoV-2 and HCoV-NL63 derived peptides in pre-pandemic COVID19 cohort.

The graphs show the correlation between COV2-SPIKE$_{(742–759)}$ and NL63-RBM1 (**A**), NL63-RBM2_1 (**B**), NL63-RBM2_2 (**C**), NL63-RBM3 (**D**), NL63-SPIKE$_{(541–554)}$ (**E**), NL63-DISC-like (**F**).

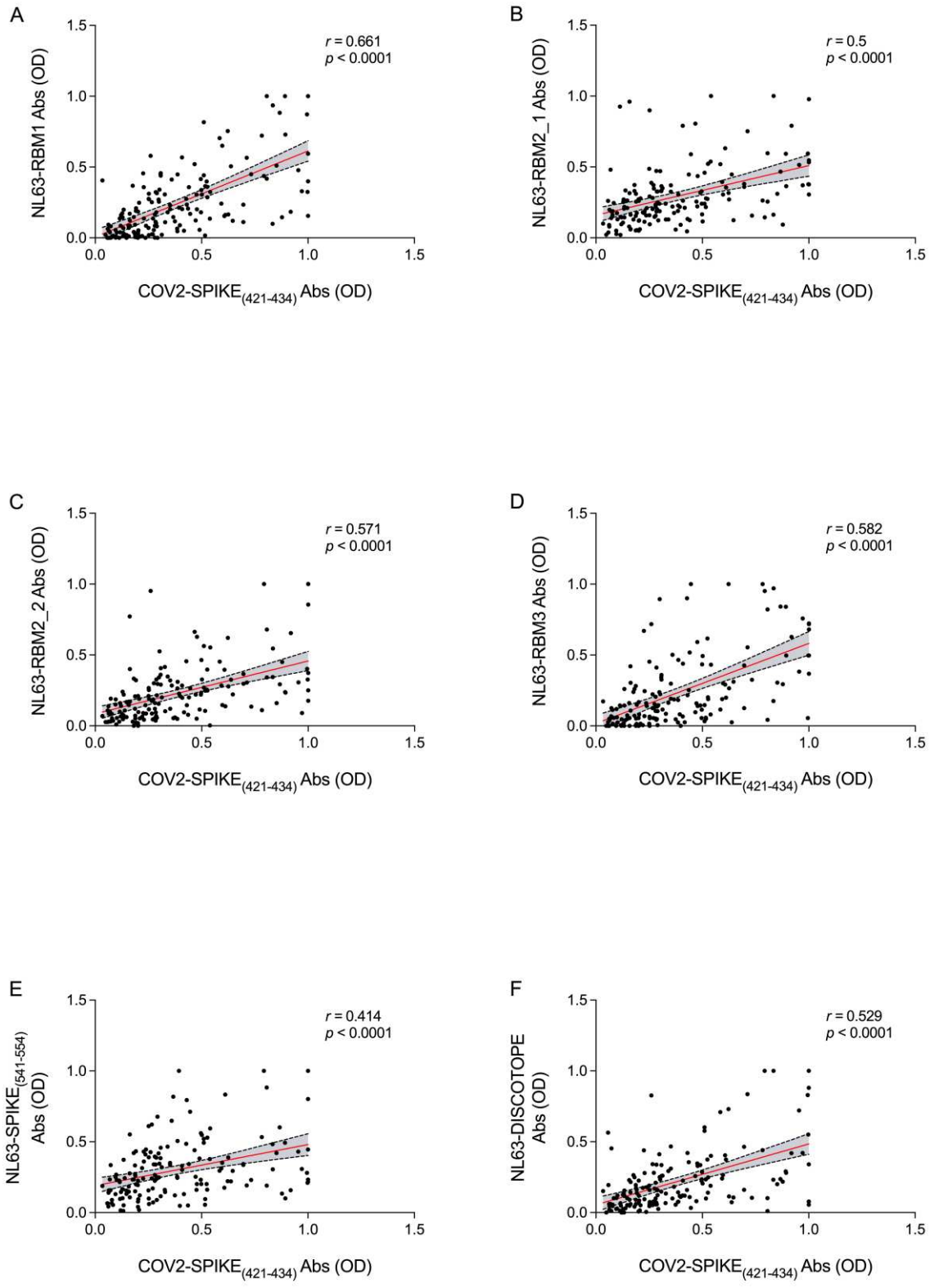

Figure 20. Scatter plot expressing correlation between SARS-CoV-2 and HCoV-NL63 derived peptides in pre-pandemic COVID19 cohort.

The graphs show the correlation between COV2-SPIKE$_{(421–434)}$ and NL63-RBM1 (**A**), NL63-RBM2_1 (**B**), NL63-RBM2_2 (**C**), NL63-RBM3 (**D**), NL63-SPIKE$_{(541–554)}$ (**E**), and NL63-DISC-like (**F**).

In addition, positive correlations were found between SARS-CoV-2 epitopes and NL63-derived peptides in the mid-pandemic group's antibody response. COV2-SPIKE$_{(742-759)}$ showed a low correlation with NL63-RBM1 (r = 0.423, p < 0.0001) (Fig 21A), NL63-RBM2_1 (r = 0.391, p < 0.0001) (Fig 21B), NL63-RBM2_2 (r = 0.395, p < 0.0001) (Fig 21C), NL63-RBM3 (r = 0.342, p < 0.0001) (Fig. 21D), NL63-DISC-like (r = 0.324, p = 0.0001) (Fig. 21F). Meanwhile, COV2-SPIKE$_{(421-434)}$ showed a moderate correlation with NL63-RBM1 (r = 0.589, p < 0.0001) (Fig. 22A), NL63-RBM2_1 (r = 0.564, p < 0.0001) (Fig. 22B), NL63-RBM2_2 (r = 0.551, p < 0.0001) (Fig. 22C), NL63-SPIKE$_{(541-554)}$ (r = 0.502, p < 0.0001) (Fig. 22E). Interestingly, a remarkably high correlation was observed between COV2-SPIKE$_{(421-434)}$ and NL63-RBM3 (r = 0.797, p < 0.0001) (Fig. 22D).

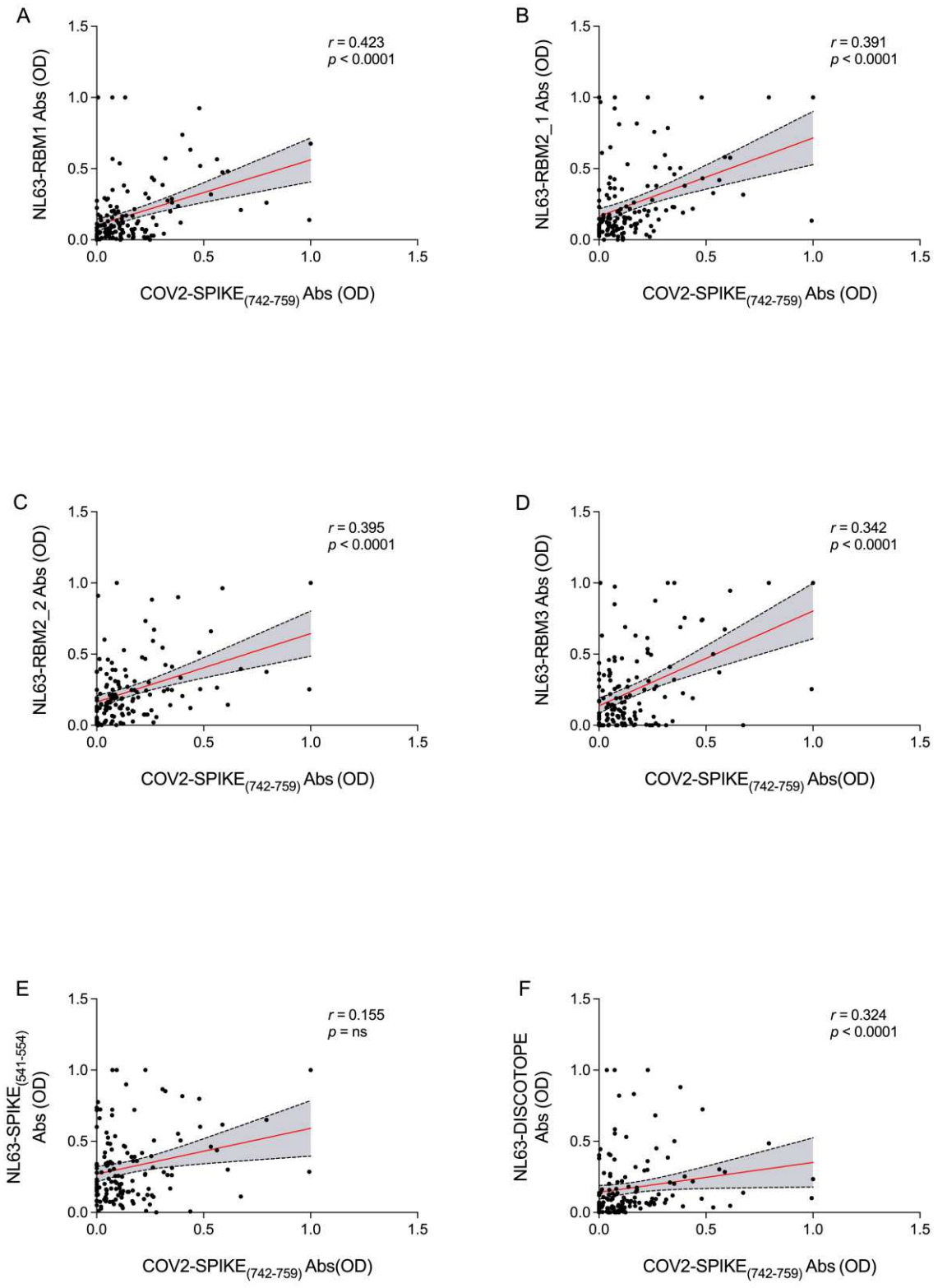

Figure 21. Scatter plot expressing correlation between SARS-CoV-2 and HCoV-NL63 derived peptides in mid-pandemic COVID-19 cohort.

The graphs show the correlation between COV2-SPIKE$_{(742–759)}$ and NL63-RBM1 (**A**), NL63-RBM2_1 (**B**), NL63-RBM2_2 (**C**), NL63-RBM3 (**D**), NL63-SPIKE$_{(541–554)}$ (**E**) and NL63-DISC-like (**F**).

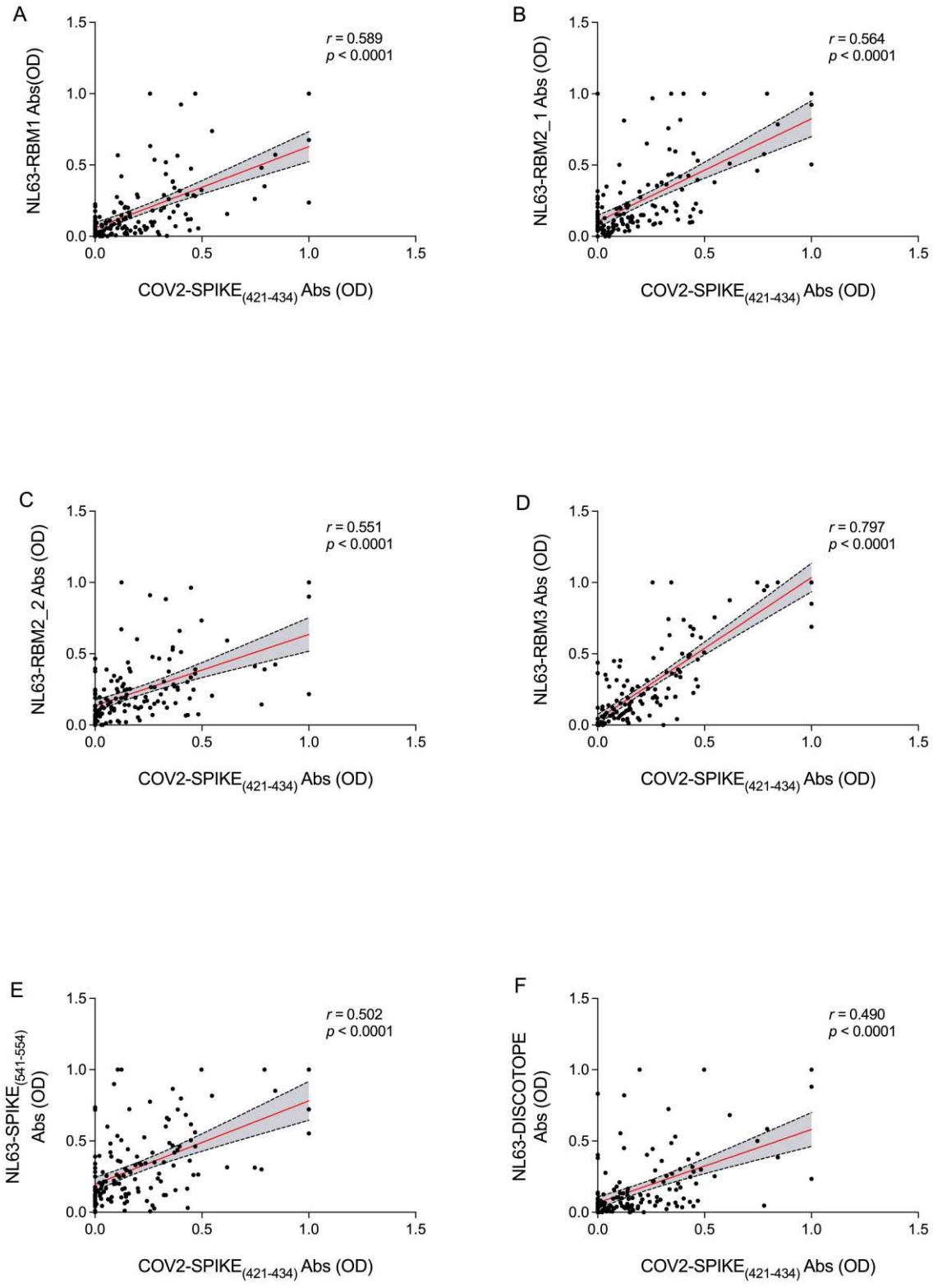

Figure 22. Scatter plot expressing correlation between SARS-CoV-2 and HCoV-NL63 derived peptides in mid-pandemic COVID19 cohort.

The graphs show the correlation between COV2-SPIKE$_{(421–434)}$ and NL63-RBM1 (**A**), NL63-RBM2_1 (**B**), NL63-RBM2_2 (**C**), NL63-RBM3 (**D**), NL63-SPIKE$_{(541–554)}$ (**E**), NL63-DISC-like (**F**).

To investigate age-related differences in the pre-pandemic population, we conducted a Spearman correlation analysis between HCoV-NL63 and SARS-CoV-2 epitopes. The resulting correlation coefficients were used to generate heatmaps (Fig. 23) depicting the strength of the correlations between pairs of epitopes in the pre-pandemic population, stratified by age group as detailed in Table 1.

Table 1. Details of the pre-pandemic population divided by age groups (years old, y/o)

Age Groups	*Number of Patients*
5-10 y/o	14
11-20 y/o	33
21-40 y/o	25
41-60 y/o	50
61-80 y/o	35

In the age group of 5-10 y/o (Fig. 23A), several high correlations were observed between NL63-RBM1 and NL63-RBM2_1 ($r = 0.735$, $p = 0.004$), NL63-RBM2_1 and COV2-SPIKE$_{(421–434)}$ ($r = 0.859$, $p < 0.0001$), NL63-RBM3 and COV2-SPIKE$_{(421–434)}$ ($r = 0.724$, $p = 0.005$), NL63-RBM3 and COV2-SPIKE$_{(742–759)}$ ($r = 0.745$, $p = 0.003$), COV2-SPIKE$_{(421–434)}$ and COV2-SPIKE$_{(742–759)}$ ($r = 0.79$, $p = 0.001$).

In the 11-20 y/o group (Fig. 23B), moderate correlations were observed among several epitopes including NL63-RBM3 and NL63-RBM1 ($r = 0.525$, $p = 0.002$), NL63-DISC-like and NL63-RBM1 ($r = 0.511$, $p = 0.002$), COV2-SPIKE$_{(421–434)}$ and NL63-RBM1 ($r = 0.552$, $p = 0.0009$), NL63-DISC-like and NL63-RBM2_1 ($r = 0.529$, $p = 0.002$), COV2-SPIKE$_{(421–434)}$ and the epitopes NL63-RBM2_1 ($r = 0.678$, $p < 0.0001$), NL63-RBM2_2 ($r = 0.580$, $p = 0.0004$), NL63-RBM3 ($r = 0.560$, $p = 0.0007$), NL63-DISC-like ($r = 0.573$, $p < 0.0001$); a moderate correlation

was also found between COV2-SPIKE$_{(742–759)}$ and the epitopes RBM2_2 (r = 0.520, p = 0.002), NL63-RBM3 (r = 0.579, p < 0.0001) and NL63-DISC-like (r = 0.553, p= 0.001).

Moving to the age group of 21-40 y/o (Fig. 23C), high correlations were found between NL63-RBM1 and COV2-SPIKE$_{(742–759)}$ (r = 0.759, p < 0.0001), COV2-SPIKE$_{(742–759)}$ and COV2-SPIKE$_{(421–434)}$ (r = 0.720, p < 0.0001).

In regard to the 41–60 age group (Fig. 23D), a high correlation was obtained between COV2-SPIKE$_{(421–434)}$ and NL63-RBM1 (r = 0.720, p < 0.0001). Finally, in the 61-80 years age group (Fig. 23E), high correlations were observed between NL63-RBM2_2 and NL63-RBM1 (r = 0.725, p < 0.0001), COV2-SPIKE$_{(742–759)}$ and the epitopes NL63-RBM1 (r = 0.704, p < 0.0001), NL63-RBM2_2 (r = 0.824, p < 0.0001), COV2-SPIKE$_{(421–434)}$ (r = 0.713, p < 0.0001); also, NL63-SPIKE$_{(541–554)}$ and NL63-RBM3 showed a high correlation (r = 0.702, p < 0.0001).

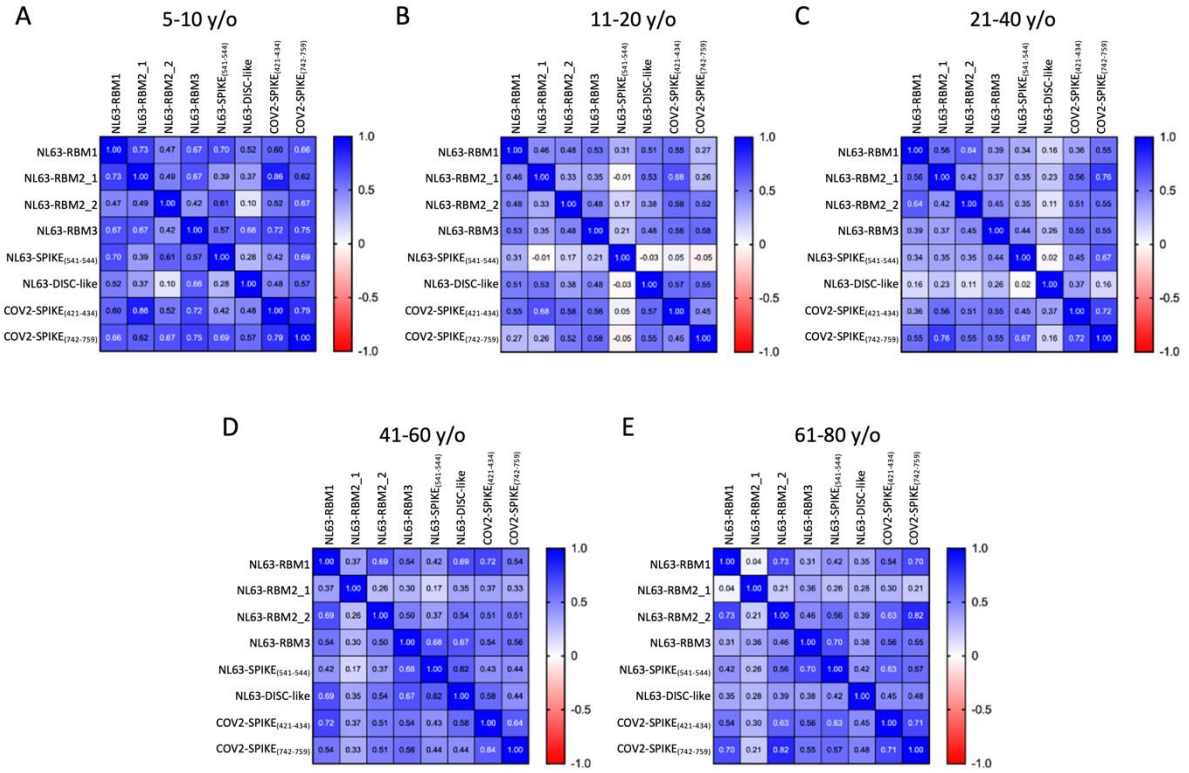

Figure 23. Heatmap displaying the Spearman correlation analysis performed among HCoV-NL63 and SARS-CoV-2 derived peptides.

Heatmap displaying the *r* values obtained from Spearman correlation analysis performed among HCoV-NL63 and SARS-CoV-2 derived peptides used in the previous experiments in the pre-pandemic cohort divided by age groups (**A**) (5–10 years old), (**B**) (11–20 years old), (**C**) (21–40 years old), (**D**) 41–60 years/old) and (**E**) (61–80 years old).

A group of 46 patients diagnosed with SARS-CoV-2 were examined for the presence of Abs against COV2-SPIKE$_{(742–759)}$ and COV2-SPIKE$_{(421–434)}$. To compare the COVID-19 group with the pre-pandemic and mid-pandemic cohorts, a Kruskal-Wallis test was performed. The results showed a statistically significant difference between the pre-pandemic and mid-pandemic populations ($p < 0.0001$) and between the mid-pandemic and COVID-19 populations ($p < 0.0001$). However, no significant difference was found between the COVID-19 group and the pre-pandemic population, as demonstrated in Fig. 24.

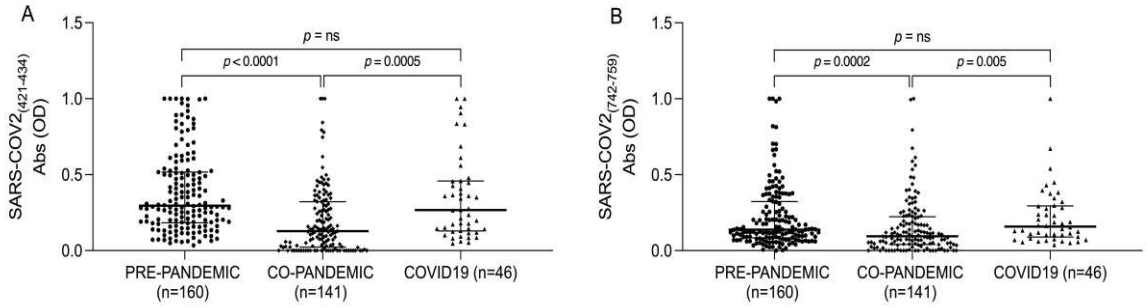

Figure 24. ELISA-based analysis of Abs reactivity against Spike SARS-CoV-2 derived epitopes.

ELISA-based analysis of Abs reactivity against Spike SARS-CoV-2 derived peptides in pre-pandemic, mid-pandemic and COVID19 populations. Plasma samples were tested against COV2-SPIKE$_{(742–759)}$ (**A**) and COV2-SPIKE$_{(421–434)}$ (**B**) peptides. Kruskal–Wallis test was used for the analyses. Scatter plots represent median with interquartile range.

Based on these findings, 11 COVID-19 patients were selected for further analysis, based on their plasma Abs response to the COV2-SPIKE$_{(421–434)}$ peptide. Of these, five patients showed a strong response, while six showed a medium response. Notably, all strongly responsive patients demonstrated significant Abs cross-reactivity, which was markedly reduced following incubation with the NL63-RBM3 peptide. Results from the competition ELISA revealed a reduction of 51.2%, 64.8%, 65.4%, 43.7%, and 78.6% for COVID-19#1 through COVID-19#5, respectively. Meanwhile, medium-responsive patients showed a reduction of 73.9%, 70.1%, 38.6%, 47.6%, 35.1%, and 50.9% for COVID-19#6 through COVID-19#11, respectively (Fig. 25).

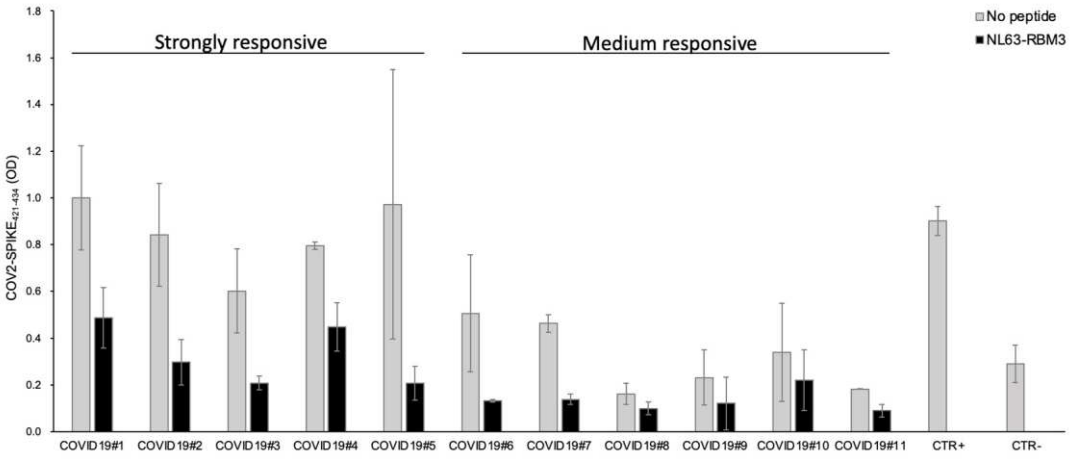

Figure 25. Competition assay with COV2-SPIKE$_{(421–434)}$ coated ELISA plate.

Plasma from 11 COVID19 patients, with strong and medium antibody response against COV2-SPIKE$_{(421–434)}$ epitope, were pre-incubated overnight with NL63-RBM3. The grey and black bars represent plasma reactivity against COV2-SPIKE$_{(421–434)}$ under normal conditions and after incubation with NL63-RBM3, respectively. Positive and negative controls were included in the assay.

2.4.2 Increased Presence of Antibodies against Type I Interferons and Human Endogenous Retrovirus W in Intensive Care Unit COVID-19 Patients

The aim of our study was to investigate the presence of Abs against type I interferons and HERV-W in COVID-19 patients in the intensive care unit (ICU) and compare them with non-ICU COVID-19 patients and healthy controls, in order to understand their potential role in disease severity and outcomes. The study investigated the humoral response in plasma samples of HD subjects , COVID-19 and ICU patients matched by age and sex against selected peptides. The Kruskal-Wallis test and Dunn's post hoc analysis were conducted to examine differences in the humoral response. A significant difference in Abs responses has been observed between HDs and ICU patients considering the presence of Abs against IFN-α peptide and the epitope derived from the envelope portion of HERV-W (HERV- W$_{(248–262)}$), with *p values* of 0.003 and, 0.0001, respectively (Fig. 26B,C). Statistically significant differences were found in the humoral responses against IFN-ω and HERV-W$_{(248–262)}$ between COVID-19 and ICU patient populations,

with *p values* of 0.011 and 0.018, respectively (Fig. 26A,C). The presence of HERV-W$_{(248-262)}$ and IFN Abs were evaluated for their relationship between COVID-19 and ICU patient populations using Spearman's correlation. Positive correlations were also observed. Briefly, as shown in Fig 27A, in HD subjects, positive correlations were found for HERV-W$_{(248–262)}$ and IFN-ω ($r = 0.35$; $p = 0.017$) and HERV-W$_{(248–262)}$ and IFN-α ($r = 0.489$; $p = 0.0006$) (Fig. 27D); in COVID-19 patients, positive correlations were found for HERV-W$_{(248–262)}$ and IFN-ω ($r = 0.6$; $p < 0.0001$) (Fig. 27B) and HERV-W$_{(248–262)}$ and IFN-α ($r = 0.573$; $p < 0.0001$) (Fig. 27E); and in ICU patients, positive correlations were found for HERV-W$_{(248–262)}$ and IFN-ω ($r = 0.49$; $p = 0.02$) (Fig. 27C) and HERV-W$_{(248–262)}$ and IFN-α ($r = 0.848$; $p < 0.0001$) (Fig. 27F).

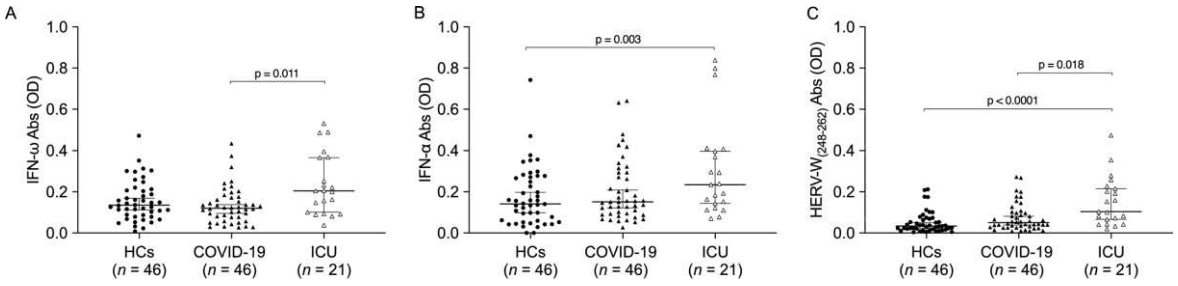

Figure 26. Analysis of humoral responses against IFNs and HERV-W in the HD, COVID-19, and ICU groups. Analysis of humoral responses against IFN-ω (**A**)-, IFN-α (**B**)-, and HERV-W(248–262) (**C**)-derived epitopes in the HD, COVID-19, and ICU groups. A Kruskal-Wallis test and Dunn's post hoc analysis were performed. Scatterplots represent the medians with 95% confidence intervals (CIs), and the P value is indicated in the upper part of each graph. OD, optical density.

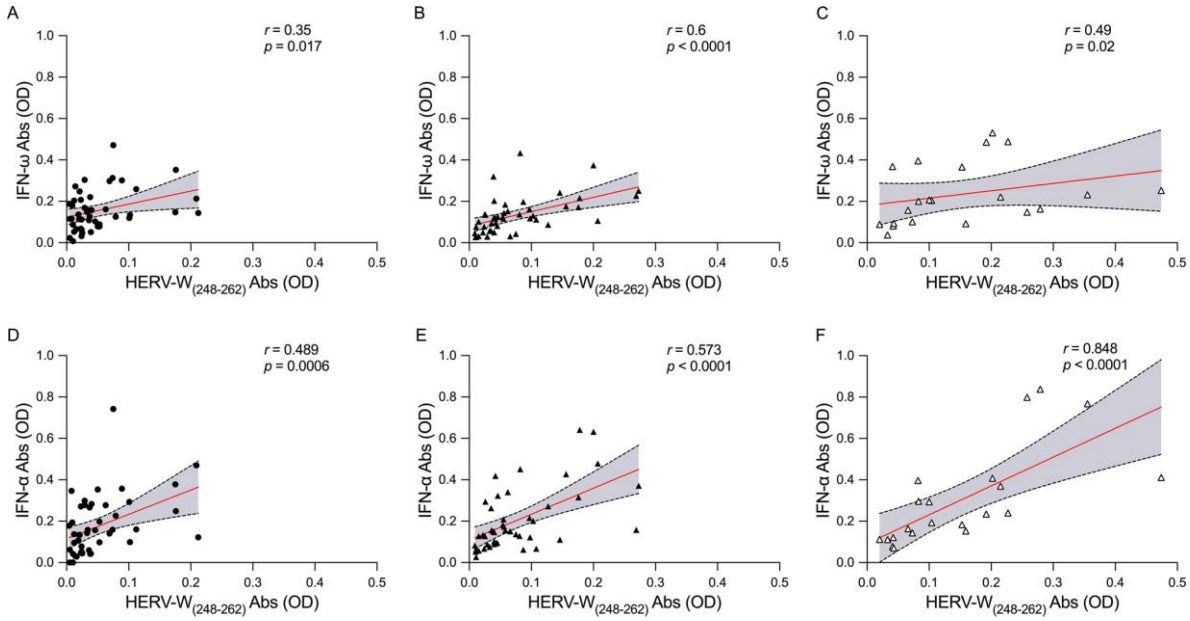

Figure 27. Scatterplots of humoral responses among HERV-W and IFN-derived epitopes in the HD and patients groups.

The graphs show the correlations between HERV-$W_{(248–262)}$ and IFN-ω in the HD (**A**), COVID-19 (**B**), and ICU (**C**) populations and the correlations between HERV-$W_{(248–262)}$ and IFN-α in the HD (**D**), COVID-19 (**E**), and ICU (**F**) populations.

2.5 Discussion

The theory of cross-reactivity between HCoVs providing temporary protection against infection with other CoVs has been widely proposed.[176,177] Sagar et al. have reported that patients with previously detected human coronavirus (HCoVs) infections have experienced less severe coronavirus disease 2019 illness. They suggested that pre-existing immune responses against HCoVs can alleviate disease manifestations from SARS-CoV-2 infection.[178]

There are three known antigenic groups of coronaviruses that are associated with diseases in animals and humans. The known HCoVs, including HCoV-229E and HCoV-NL63 in the alpha-group, and HCoV-OC43 in the beta-group, are typically known to cause mild upper respiratory tract diseases and, in rare cases, lower respiratory tract diseases. Among them, HCoV-NL63 is the

only coronavirus responsible for mild infections that use the ACE2 receptor as a target for its S protein.[179]

The S-protein of HCoV-NL63 has been extensively studied. Despite the lack of structural homology in RBD cores or RBM between HCoV-NL63 and SARS-CoV, both viruses recognize common regions on ACE2, largely due to a "virus-binding hotspot" on ACE2. We attempted to identify possible common binding hotspots on ACE2 using the IEDB site by analyzing the S-proteins of both CoVs. Additionally, structural and phylogenetic analysis of the S-proteins revealed low homology in the amino acid sequence between SARS-CoV and SARS-CoV-2. However, both S-protein RBDs exhibited the same mode of binding to ACE2.[180]

With regard to what was expressed above, we wondered if a serologic cross-reactivity may be found between HCoV-NL63 and SARS-CoV-2. Previous studies have examined Ab cross-reaction between alpha- and beta-coronaviruses, but the findings have been limited. Loos et al.[181] reported some positive relationships between IgG response to common CoVs and SARS-CoV-2 RBD-specific immunity. However, they used the HCoV-NL63 RBD without examining selected highly immunogenic B cell epitopes. Our study aimed to analyze the immunogenicity of these epitopes and determine if they exhibit any cross-reactivity between HCoV-NL63 and SARS-CoV-2. Recently, three studies were published in Nature, Science, and Cell journals, demonstrating evidence of cross-reactive T-cell immunity between human coronaviruses (229E, NL63, OC43, and HKU1), and SARS-CoV-2. These studies provided compelling evidence for the existence of such cross-reactive immunity.[182–184]

Mateus et al.[184] presented three cases where human coronaviruses (HCoVs) analogs were better antigens than the SARS-CoV-2 peptide, suggesting that they could be the corresponding immunogen, including one analog from HCoV-NL63. These results demonstrate cross-reactive HCoV T-cell specificities, which are in contrast to HCoV-neutralizing Abs that are specific to each HCoV species and do not exhibit cross-reactivity against SARS-CoV-2 RBD, as reported by

Premkumar et al.[184–186] Here, a study tested archived human samples collected before the emergence of SARS-CoV-2, including samples from 20 American adults and individuals in South Asia, the Caribbean, and Central America, for binding against RBD spike antigens from HCoVα (NL63) and HCoVβ (HKU1), but no reaction was observed. On the other hand, Sotgia et al. have suggested that epitopes from mild pathogenic coronaviruses could be used for a vaccine.[187]

Ng et al.[188] have reported the identification of several epitopes that were recognized by cross-reactive Abs in uninfected individuals, providing evidence for the presence of preexisting Abs recognizing SARS-CoV-2. These findings and the discovery of preexisting T cell memory against seasonal HCoVs and SARS-CoV-2 may shed light on natural SARS-CoV-2 infection. However, it is important to note that this protective cross-immunity is not long-lasting and likely not sterilizing.[188]

Our study initially evaluated the Ab response to five HCoV-NL63 peptides and two SARS-CoV-2 S-protein epitopes in both a pre-pandemic and a mid-pandemic group. The pre-pandemic group has a high likelihood of recent HCoV infections, including NL63, as it comprises approximately one-third of patients under 18 years old, who are more likely to be exposed to these viruses. The use of short peptides instead of expressed proteins is a study limitation, but it also presents an opportunity to reveal previously hidden epitopes.[189]

The population before the COVID-19 pandemic showed a robust humoral response against HCoV-NL63 compared to the mid-pandemic population, specifically for NL63-RBM1 and NL63-DISC-like. Interestingly, we also observed a surprisingly strong humoral response to SARS-CoV-2 S-protein epitopes in the pre-pandemic population. It should be noted that this response cannot be attributed to SARS-CoV-2 since the samples were collected prior to 2019. We confirmed these findings by conducting a rapid test on one of the most reactive pre-pandemic samples for SARS-CoV-2 epitopes, which yielded a positive result. Our data indicate that 72.5% and 68.7% of the pre-pandemic population are seropositive against COV2-SPIKE$_{(421-434)}$ and COV2-SPIKE$_{(742-759)}$,

respectively. We hypothesize that this heterologous cross-reactivity between HCoV-NL63 Abs and SARS-CoV-2 epitopes may be responsible for the observed response. The correlations observed between HCoV-NL63 and SARS-CoV-2 peptides in both cohorts support the theory of Ab cross-reactivity between the two viruses, indicating that HCoV-NL63 may provide some level of protection against SARS-CoV-2. Additionally, we examined the correlations among the epitopes utilized in this study within the pre-pandemic population, stratified by age groups. The most significant correlation was observed in the 5-10 years and 40-60 years age groups. While further research is necessary to fully comprehend this correlation, one possibility is that NL63 Abs may aid in managing SARS-CoV-2 infections more effectively. In order to validate these findings, a cELISA was conducted on individuals diagnosed with SARS-CoV-2, which revealed a significant discrepancy in Ab response against SARS-CoV-2 epitopes between the pre-pandemic and mid-pandemic cohorts, as well as between the COVID19 and mid-pandemic groups. It is worth noting that HCoV-NL63 infections were mostly observed during the winter season, which is when our pre-pandemic samples were obtained. Additionally, 30.6% of the children under 18 years old were the most commonly affected by HCoV-NL63 during this period, which could explain the comparable Ab response between the pre-pandemic and COVID-19 groups. To further support this notion, Canducci et al. discovered that out of 322 infants with acute respiratory disease, 8.7% of the cases were caused by coronaviruses, with HCoV-NL63 accounting for 21.4% of those cases.[189,190]

Our initial hypothesis that Abs targeted at HCoV-NL63 S-protein may recognize SARS-CoV-2 S-protein epitopes is reinforced by these findings. These results are particularly significant in patients with both strong and moderate Ab responses, as we observed a significant decrease in Abs against COV2-SPIKE$_{(421–434)}$, with the exception of two patients (COVID-19#8 and COVID-19#10) who exhibited a reduction of less than 40%. Given the increased seroprevalence of HCoV-NL63 infections and the lower incidence of SARS-CoV-2 in children, it is possible that Abs

directed against HCoV-NL63 may provide some degree of protection for certain individuals impacted by SARS-CoV-2. A recent report suggests that SARS-CoV-2 exhibits a stronger molecular interaction energy with the human ACE2 receptor compared to SARS-CoV and HCoV-NL63, providing an explanation for SARS-CoV-2's heightened pathogenicity. Additionally, a greater understanding is needed in order to investigate the potential contribution of pre-existing immunity provided by other coronaviruses, such as HCoV-OC43, which may be mediated by different proteins than Spike.[191,192]

We have also wondered whether the individual differences in the response to SARS-CoV-2 infection could be somehow related to the presence of AAbs that weaken the humoral response. Our findings reveal that individuals with severe COVID-19 requiring intensive care exhibit elevated levels of AAbs against IFN-α and IFN-ω compared to those with mild COVID-19 and healthy controls. Additionally, ICU patients demonstrated heightened levels of Abs against HERV-W-env$_{(248–262)}$. These neutralizing AAbs against IFN-I may interfere with the binding of IFN-I to the type I interferon receptor (IFNAR), thereby impeding the activation of the antiviral response.[193–195]

The first evidence linking AAbs against type I interferons and severe forms of COVID-19 disease was published in 2020.[193] These authors found that at least 10% of patients with life-threatening COVID-19 pneumonia had AAbs against type I interferons.[193] Later, the same group reported that nearly 4% of uninfected individuals over the age of 70 also had neutralizing AAbs against type I interferons.[194] These AAbs could account for nearly 20% of COVID-19 deaths, and their levels increased with age.[194] Additionally, Chang et al. reported AAbs against IFN- α in 45% of COVID-19 patients.[195] Recent research by Manry et al.[196] has suggested that AAbs against type I interferons are strong predictors of death in COVID-19 patients. The authors evaluated the association between infection fatality rate (IFR) and the relative risk of death (RRD) and neutralizing AAbs against type I interferons across age groups. It was found that carriers of AAbs

against both IFN-α and IFN-ω had a higher RRD across age groups, particularly those less than 70 years old. These findings further confirm the importance of type I IFN-neutralizing AAbs as predictors of life-threatening COVID-19 disease. The presence of AAbs appears to be a unique feature of SARS-CoV-2 infection, as multiple studies have reported the presence of AAbs typically found in autoimmune diseases, such as vasculitis and rheumatic diseases, in patients with severe COVID-19, rather than in those with milder disease.[156,197,198] In the context of susceptibility to severe COVID-19 and the presence of AAbs, the type I IFN defect provides a partial explanation, which could be confirmed through functional models and assays. It is intriguing that levels of AAbs against the HERV-W-env$_{(248–262)}$ epitope were significantly higher in ICU patients compared to those with mild COVID-19 and healthy controls, and showed a strong correlation with anti-IFN-I AAbs, especially anti-IFN-α. It is well known that HERV expression can be triggered by various factors, including infectious agents. The strong correlation between HERV-W-env and IFN-α Ab levels suggests that SARS-CoV-2 may induce the reactivation of HERV-W sequences, which could contribute to the heightened adaptive response observed in ICU patients. The presence of AAbs against HERV-W-env in ICU patients with life-threatening COVID-19 has been shown to be strongly correlated with anti-IFN-α AAbs, indicating a potential relationship between these two factors. This association has also been observed in a mouse model with anti-myelin oligodendrocyte glycoprotein autoimmune responses. HERV-W envelope has been found to be highly expressed in T lymphocytes of COVID-19 patients, and its transcript levels have been positively associated with clinical parameters and disease severity.[160] The increased presence of Abs against HERV-W-env supports its abnormal expression. Furthermore, HERV-W envelope mRNA levels were strongly correlated with pro-inflammatory cytokines, such as IL-6, IL-17, CXCL6, and MCP1. Studies have demonstrated positive correlations between the mRNA levels of HERVs and those of IFN-I and IFN-II in children with COVID-19. Children with severe COVID-19 had decreased expression levels of the IFN-I and IFN-III genes, along with reduced

levels of HERV-W transcripts, compared to those with mild symptoms. Although the presence of AAbs against HERV-W-env in COVID-19 patients suggests a heightened adaptive response, further research is needed to understand the exact mechanisms of HERV-W expression in COVID-19 and its relationship with the immune response.[199] In conclusion, patients with life-threatening COVID-19 in the ICU display elevated levels of anti-IFN-I AAbs and HERV-W-env$_{(248–262)}$ Abs, in comparison to both healthy controls and COVID-19 patients with mild/moderate disease. Furthermore, the humoral responses against HERV-W-env$_{(248–262)}$ and IFN-α are strongly linked in ICU patients. Further research is necessary to gain a better understanding of the role of HERV-W in COVID-19 and the impact of SARS-CoV-2 on HERV-W activation and the presence of AAbs against IFN-I. It is worth noting that type I IFNs are part of a complex cross-regulatory network, which in rare cases can lead to harm to the host rather than protection against infectious diseases.[200] Additionally, evaluating the levels of anti-IFN-I Abs in a population of individuals before and after SARS-CoV-2 infection could help clarify the impact of SARS-CoV-2 on AAbs levels, HERV activation, the clinical course of the disease, and the potential prognostic significance of these AAbs.

Chapter III

3 Materials and methods

3.1 Samples - TDP-43 and HERV-K Envelope-Specific Immunogenic Epitopes Are Recognized in ALS Patients

We conducted a study to evaluate a group of 45 patients with ALS consisting of 17 females and 28 males with a mean age of 64.67 ± 9.11 years. We also included an age- and gender-matched healthy control group (HDs) of 17 females and 28 males with a mean age of 63.87 ± 4.82 years. The ALS group included newly diagnosed (ALS-ND) ALS patients (7 females and 20 males; mean age ± SD: 64 ± 7.8 years) who were hospitalized at the Neurology Unit Clinic of the University Hospital of Sassari and long-surviving (ALS-LS) ALS patients (10 females and 8 males; mean age ± SD: 65.8 ± 10.4 years) reported by primary doctors and doctors in the Sassari local district.

All participants provided informed consent prior to inclusion in the study, which was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of ALS 1 Sassari (2149/CE).

3.2 Samples - Antibody Response to HML-2 May Be Protective in Amyotrophic Lateral Sclerosis

We examined serum samples from 243 individuals with ALS who were part of the Northeast ALS Consortium (NEALS) (99 females, 140 males, four unknown sex; mean age ± SD = 58.89 ± 10.61 years). We also collected 242 serum samples from age- and sex-matched healthy donors (HD) from the Blood Transfusion Centre of Sassari (84 females and 158 males; age ± SD = 53.29 ± 6.45 years) and 85 samples from individuals with multiple sclerosis (MS) (54 females and 31 males; mean age ± SD = 57.35 ± 9.6 years). ALS individuals were classified based on the EL Escorial

criteria21 or predicted survival by the ENCALS model22 into suspected, possible, probable, and definite ALS. The ALS population included newly diagnosed patients as well as those with long, moderate, or short survival. MS patients were diagnosed based on McDonald's criteria23 and included those treated with immunomodulators or untreated. All samples were stored at -80°C until used, and this study was approved by the local ethics committees on human experimentation. All individuals provided written informed consent.

3.3 Samples - Effect of Antiretroviral Therapy on HERV-K Abs Levels in Amyotrophic Lateral Sclerosis Patients

Serum samples from 16 ALS patients under antiretroviral therapy were analyzed through ELISA assay. For each patient, the presence of autoantibodies against 4 epitopes of HERV-K-envelope was assessed during a 36-week period, with blood sample collection every 4 weeks.

3.4 Blood samples collection

Peripheral venous blood samples were obtained from the participants using K2-EDTA tubes. The collected whole blood was gently layered over an equal volume of Ficoll (Sigma-Aldrich, St. Louis, MO, USA) in a 15 mL tube and centrifuged for 20 minutes at 1800 RPM without brake. The plasma located in the uppermost layer was collected by pipetting and tested for the presence of antibodies against TDP-43- and HERV-K-env-derived epitopes. The PBMCs were collected and stored at -80°C in FBS with 10% dimethyl sulfoxide (DMSO) for further analysis.

3.5 IEDB – epitope prediction

The epitopes utilized in this study were designed using the Immune Epitope Database and analysis resource (IEDB) and synthesized with high purity (>95%) by LifeTein, located in South Plainfield, NJ, USA. The IEDB software was used to predict regions of proteins that are likely to be recognized as epitopes in the context of a B-cell response. All peptides were dissolved in DFM or DMSO, in relation to the chemical-physical characteristics, and stored in single-use aliquots (10 mM) at -80°C. The selected immunogenic sequence are displayed in Tab.2.

Table 2. Immunogenic sequences of epitopes used for ELISA assay

Name	*Epitope sequence*
HERV-K-env(19–37)	VWVPGPTDDRCPAKPEEEG
HERV-K-env(165)	LGRAPGCLMPAVQNW
HERV-K-env(648)	LFCLLLVCRCTQQLR
HERV-K-env(668)	RERAMMTMAVLSKRK
TDP-43(258–271)	SNAEPKHNSNRQLE
TDP-43(398–411)	NGGFGSSMDSKSSG
TDP-43(398–411)P	NGGFGSSMDSK-(PSer)-(PSer)-G
NL63-RBM1	FGGSCYVCKPHQVNI
NL63-RBM2_1	NRVKSGSPGDSSWH
NL63-RBM2_2	VKSGSPGDSSW
NL63-RBM3	WHYTSYTIVGALYVT
NL63-SPIKE(541–554)	SEGNSITGVPYPVS
NL63-DISC-like	SGGRGSGRGGNLTYLNLSSEL
COV2-SPIKE(421–434)	FSQILPDPSKPSKRSFIE
COV2-SPIKE(742–759)	CNGVEGFNCYFPLQS
Annexin-A2	LEGDHSTPPSAYGSVKAYTNFDAER

3.6 Epitope mapping

Using an enzyme-linked immunosorbent assay (ELISA) to detect antibodies against a recombinant HML-2 envelope protein (MyBioSource, catalog number MBS1391552_a0), we screened serum samples from 46 healthy controls (HDs) and 66 individuals with ALS. We selected serum samples from 10 ALS patients and 8 age- and sex-matched HDs that showed reactivity to the protein. Using peptide microarrays covering the complete sequence of the protein (Uniprot ID: Q69384) (PEPperCHIP Immunoassay, PepperPrint, Heidelberg, Germany), epitope mapping of the antibodies to HML-2 envelope was performed. The assay involved converting the elongated 699 amino acids antigen sequence into 15 amino acids peptides with a peptide-peptide overlap of 14 amino acids, in duplicate. The peptide microarrays were incubated in phosphate-buffered saline (PBS) with 0.05% Tween20 and blocking buffer (Rockland Blocking Buffer MB-070), followed by incubation with secondary Ab, washing, and drying before being scanned (GenePix 4300A, Molecular Devices LLC, CA, USA). The serum samples were diluted 1:200 in staining buffer, added to each array, and incubated overnight at 4°C on an orbital shaker. The same process was performed with an anti-hemagglutinin control antibody. The scanned images were analyzed using MAPIX analyzer.

3.7 ELISA

An indirect enzyme-linked immunosorbent assay (ELISA) was carried out to detect specific antibodies against HERV-K-env epitopes. Nunc immuno-plates with 96 wells were incubated overnight at 4°C in a solution of 0.05 M carbonate-bicarbonate (pH 9.5) from Sigma-Aldrich (St. Louis, MO, USA), along with the corresponding peptides at a concentration of 10 μg/mL. The plates were blocked with a solution of 5% non-fat dried milk from Sigma-Aldrich and Tris-buffered saline (TBS) and incubated at room temperature for 1 hour. The plates were washed

twice with TBS-T (TBS with 0.05% Tween-20) and plasma samples were added at a concentration of 1:100, followed by incubation for 2 hours. After washing the plates five times in PBS-T, they were incubated at room temperature for 1 hour with 100 µL of PBS and alkaline phosphate-conjugated goat anti-human IgG polyclonal antibody (1:1000, Sigma-Aldrich, St. Louis, MO, USA). After another washing step in TBS-T, plates were incubated for 8 to 10 minutes in a dark environment with milli-Q water and p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA), and the absorbance at 405 nm was measured using a SpectraMax Plus 384 microplate reader from Molecular Devices (Sunnyvale, CA, USA). Each sample was run in duplicate and normalization was performed with the positive control included in each assay. The background activity was calculated as the mean signal of an immobilized peptide with secondary antibody. The results were expressed as the means of duplicate values of 405 nm OD.

Regarding the competitive ELISA carried out in the field of SARS-CoV-2 experiments, to assess the specificity of binding for each peptide, a competitive ELISA was performed using IFN-α, IFN-ω, and a different peptide, annexin A2, to which the patient was positive.

3.8 Determination of Total IgG in Serum by ELISA.

Levels of total immunoglobulin (Ig)G were measured in serum samples with Human ELISA Kits (Invitrogen) following manufacturer's instructions.

3.9 Analysis of HERV-K Levels in Serum

HML-2 was detected in serum using digital polymerase chain reaction (PCR). To remove cells and debris, serum samples were centrifuged 10 minutes at 300g. Using an EZ1 Advance XL device (Qiagen) and the EZ1 Virus Mini Kit v2.0 (Qiagen), total nucleic acids were extracted from 400

μL of the supernatant, following the manufacturer's instructions. The extracted nucleic acids were eluted in 60 μL of AVE buffer, and the remaining magnetic beads were removed. The digital PCR reaction was set in duplicate in a 96-well plate using an AutoDG Droplet Digital PCR System (Bio-Rad) with a set of primers and a probe (FAM labeled) to detect HML-2 env (forward primer: 5'-ATTTGGTGCCAGGAACTGAG-3'; reverse primer: 5'-GCTGTCTCTTCGGAGCTGTT-3'; probe: 5'-6-FAM-AGGAGTTGCTGATGGCCTCG-Iowa Black FQ-3'). A pre-made assay of primers and probes targeting a cellular DNA (RPP30 gene, HEX-tagged) was included (Bio-Rad) to confirm the extracellular origin of HML-2 in serum. The master mix consisted of 12.5 μL of ddPCR Supermix (no dUTP) (Bio Rad), 1.25 μL of a mix of HML-2 env primers (900 nm) and probe (250 nm) (Bio-Rad), 1.25 μL of RPP30 assay (Bio-Rad), 2.5 μL of nucleic acids, and 7.5 μL of RNAse-free water. After preparing the droplets, the PCR was conducted in a T100 Thermal cycler (Bio-Rad) with the following cycling conditions: 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds and 60°C for 1 minute, and 95°C for 10 minutes. The number of copies was determined using a QX200 Digital PCR reader (Bio-Rad), and results were expressed as a ratio of HML-2 env copies to RPP30 copies.

3.10 Cell culture

HEK293 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) + GlutaMAX. [+] 4.5 g/L D-Glucose. [+] 110mg/L Sodyum Pyruvate supplemented with 10% fetal bovine serum with 5% antibiotics and maintained in a tissue culture incubator at 37°C with 5% CO2.

Tera-1 cells were grown in DMEM + GlutaMAX. [+] 4.5 g/L D-Glucose. [+] 110mg/L Sodyum Pyruvate supplemented with 15% fetal bovine serum with 5% antibiotics and maintained in a tissue culture incubator at 37°C with 5% CO2.

3.11 microRNA identification

The identification of microRNAs capable of binding to the consensus sequence of HERV-K was performed using the online software miRDB. miRDB is an online database designed for predicting miRNA targets and providing functional annotations. The targets within miRDB are predicted using a bioinformatics tool called MirTarget, which has been developed by analyzing thousands of miRNA-target interactions obtained from high-throughput sequencing experiments. Machine learning techniques have been employed using identified common features associated with miRNA binding and target downregulation to predict miRNA targets. Table 3 displayed the list of selected miRNAs.

Table 3: List of downregulated miRNAs, able to bind the consensus sequence of HERV-K in ALS, selected for transfection into cell lines.

Accession Number	*Name*	*Sequence*	*miRNA target*
MIMAT0000259	has-miR-182-5p	UUUGGCAAUGGUAGAACUCACACU	4537 (POL) 5918 (POL) 6876 (ENV)
MIMAT0004614	has-miR-193-5p	UGGUCUUUGCGGGCGAGAUGA	1718 (GAG) 2403 (GAG)
MIMAT0000275	has-miR-218-5p	UUGUGCUUGAUCUAACCAUGU	658 (LTR) 5650 (POL) 9162 (LTR)
MIMAT0000278	has-miR-221-3p	AGCUACAUUGUCUGCUGGGUUUC	1512 (GAG) 7905 (ENV)
MIMAT0004558	has-miR-181a-2-3p	ACCACUGACCGUUGACUGUACC	3558 (PRO) 3989 (POL) 4346 (POL) 6053 (POL)

3.12 Transient co-transfection and transfection

HEK-293 and TERA-1 cells were grown in the respective medium and maintained in a tissue culture incubator at 37°C with 5% CO. Different transfection protocols were followed for the cells. After 24 hours of passaging and achieving approximately 80% confluence, HEK-293 cells were co-transfected with the HERV-K plasmid (kindly provided by the Dr. Avindra Nath Laboratory) and microRNAs, while TERA-1 cells were transfected only with microRNAs.

Briefly, 1.5 ug of HERV-K plasmid, 1.5 ug of pcDNA, and two different concentrations of micorRNAs, 15nM and 30nM, were mixed with Lipofectamine 3000, P3000 and Opti MEM reduced serum medium following the guidelines provided by the Lipofectamine 3000 manufacturer (Thermo Fisher). After 15 minutes of incubation at room temperature, the respective mixture was added to the cells. After 48 hours of incubation, the cells were lysed, and the proteins and RNA were extracted.

3.13 Proteins purification and quantification

For protein extraction cells were lysed in RIPA buffer supplemented with a mammalian protease inhibitor cocktail (QIAGEN), and incubated 10 min in ice. Subsequently, lysates were centrifuged at 10000g for 10 min at 4°C. Supernatant, containing proteins, was transferred to new tubes and proteins were quantified using Pierce BCA Protein Assay Kit (Thermo Fisher) according with manufacturer instructions. Briefly, the assay is based on two reactions, the chelation of copper ions with protein in an alkaline environment, and the reduction of copper ions to form a purple-colored chelate complex with bicinchoninic acid (BCA) in the presence of proteins. The intensity of the purple color produced is directly proportional to the concentration of protein in the sample, allowing for a precise determination of protein concentration. This kit is commonly used in biochemistry and molecular biology research for protein quantification purposes.

3.14 RNA isolation

Total RNA was isolated and purified using RNeasy Mini Kit (QIAGEN) according to the manufacturer's specifications. Briefly, samples were first lysed and homogenized, and ethanol was added to provide optimal binding conditions. The lysate was then loaded onto the RNeasy silica membrane, where RNA was selectively bound to the membrane and all contaminants were efficiently washed away. The purified and concentrated RNA was eluted in water.

3.15 mRNA reverse-transcription

mRNA was reverse transcribed in cDNA using First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's suggested protocol. Briefly, 5 µl of RNA were mixed with 4 µl of RT Buffer, 1 µl of dNTPs, 1 µl of Random hexamers, 0.5 µl of RiboLock RNAse inhibitor, 1 µl of Reverse Transcriptase enzyme and 7.5 µl of RNAse free water. The reaction tubes were incubated for 10 min at 25°C followed by 30 min at 50°C; final step was at 85°C for 5 min. The resulting cDNA was diluted 1:20 and stored at temperature −20°C until use.

3.16 Real Time PCR

cDNA was analysed by quantitative RT-(q)PCR analysis using SYBR Green mix (Kapa Biosystems). cDNA samples were mixed with SYBR green mix and with respective primers. Plate was incubated in CFX Biorad PCR System at 95°C for 20 seconds, then at 95°C for 3 seconds and 65° C for 30 seconds, this step has been repeated for 40 cycles, finally at 95°C for 15 seconds, following 1 minute at 60°C and 15 seconds at 95°C. Relative changes in gene expression,

determined from real-time quantitative PCR experiments, have been calculated using the 2-ΔΔCT method.

3.17 Western Blot

20 µg of protein samples were diluted in Laemmli and β-mercaptoethanol and boiled at 95°C for 5 minutes. Proteins were separated by electrophoresis on precast gels (4– 20% Mini-PROTEAN® TGXTM Precast Protein Gels, Bio-Rad) using MOPS buffer (Sigma Aldrich) at 180V. Proteins were separated and protein size was determined using SpectraTM Multicolor Broad Range Protein Ladder (Thermo Scientific). Proteins were then transferred to a nitrocellulose membrane using Trans-Blot Turbo RTA Transfer Kit, Nitrocellulose (Bio-Rad) according to the manufacturer's guidelines. The membrane was incubated with 5% milk for 30 minutes, then washed and incubated overnight in BSA blocking buffer + Sodium Azide 5% added with primary antibodies, HERV-K-envelope or Vinculin (1:1000). After overnight incubation, the membrane was washed 3 times in PBS-T for 5 min and incubated for 1h with the secondary antibody diluted 1:2500. Afterwards, the membrane was washed 3 times in TBS-T and the signal detected by enhanced chemiluminescent reaction using SuperSignal West Femto (Thermo Scientific). Images were acquired with the Bio-Rad Universal Hood II Gel Doc System.

3.18 Statistical analysis

Descriptive statistics, t-tests, ANOVA, or other appropriate methods were used for statistical analyses based on the study design and data characteristics. The selection of the appropriate statistical test was determined by the type of data obtained and research question, with the goal of

ensuring the validity and reliability of study conclusions. Additional information on the statistical analyses conducted can be found in their respective sections.

Chapter IV

4 References

1. Laspia, M. F., Rice, A. P. & Mathews, M. B. HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* **59**, 283–292 (1989).
2. Malim, M. H. & Emerman, M. HIV-1 accessory proteins--ensuring viral survival in a hostile environment. *Cell Host Microbe* **3**, 388–398 (2008).
3. Miller AD. *Retroviruses*. *Retroviruses* (1997).
4. Johnson, W. E. Endogenous Retroviruses in the Genomics Era. *Annu. Rev. Virol.* **2**, 135–159 (2015).
5. Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921 (2001).
6. Balvay, L., Lastra, M. L., Sargueil, B., Darlix, J.-L. & Ohlmann, T. Translational control of retroviruses. *Nat. Rev. Microbiol.* **5**, 128–140 (2007).
7. Mager, D. L. & Stoye, J. P. Mammalian Endogenous Retroviruses. *Microbiol. Spectr.* **3**, MDNA3-0009–2014 (2015).
8. Cegolon, L. *et al.* Human endogenous retroviruses and cancer prevention: Evidence and prospects. *BMC Cancer* **13**, 4 (2013).
9. Chuong, E. B., Elde, N. C. & Feschotte, C. Regulatory evolution of innate immunity through co-option of endogenous retroviruses. *Science* **351**, 1083–1087 (2016).
10. Belshaw, R. *et al.* Genomewide screening reveals high levels of insertional polymorphism in the human endogenous retrovirus family HERV-K(HML2): implications for present-day activity. *J. Virol.* **79**, 12507–12514 (2005).
11. Deininger, P. L. & Batzer, M. A. Mammalian retroelements. *Genome Res.* **12**, 1455–1465 (2002).
12. Belshaw, R., Katzourakis, A., Paces, J., Burt, A. & Tristem, M. High copy number in human endogenous retrovirus families is associated with copying mechanisms in addition to reinfection. *Molecular biology and evolution* vol. **22** 814–817 (2005).
13. Mang, R., Maas, J., van der Kuyl, A. C. & Goudsmit, J. Papio cynocephalus Endogenous Retrovirus among Old World Monkeys: Evidence for Coevolution and Ancient Cross-Species Transmissions . *J. Virol.* **74**, 1578–1586 (2000).
14. Bannert, N. & Kurth, R. The evolutionary dynamics of human endogenous retroviral families. *Annu. Rev. Genomics Hum. Genet.* **7**, 149–173 (2006).
15. Bannert, N. & Kurth, R. Retroelements and the human genome: New perspectives on an old relation. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 14572-14579 (2004)

16. Mortelmans, K., Wang-Johanning, F. & Johanning, G. L. The role of human endogenous retroviruses in brain development and function. *Apmis* vol. **124** 105–115 at https://doi.org/10.1111/apm.12495 (2016).
17. Sha, M. *et al.* Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature **403**, 785-789* (2000)
18. Blond, J.-L. *et al.* An Envelope Glycoprotein of the Human Endogenous Retrovirus HERV-W Is Expressed in the Human Placenta and Fuses Cells Expressing the Type D Mammalian Retrovirus Receptor. *J. Virol.* **74**, 3321–3329 (2000).
19. Grow, E. J. *et al.* Intrinsic retroviral reactivation in human preimplantation embryos and pluripotent cells. *Nature* **522**, 221–246 (2015).
20. Wang, T. *et al.* Regulation of stem cell function and neuronal differentiation by HERV-K via mTOR pathway. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 17842–17853 (2020).
21. Murant, A. F. Taxonomy and nomenclature of viruses. *Microbiological Sciences* vol. **2** 218–220 at (1985).
22. Jern, P., Sperber, G. O. & Blomberg, J. Use of Endogenous Retroviral Sequences (ERVs) and structural markers for retroviral phylogenetic inference and taxonomy. *Retrovirology* **2**, 50 (2005).
23. Blomberg, J., Benachenhou, F., Blikstad, V., Sperber, G. & Mayer, J. Classification and nomenclature of endogenous retroviral sequences (ERVs): problems and recommendations. *Gene* **448**, 115–123 (2009).
24. Gifford, R. & Tristem, M. The evolution, distribution and diversity of endogenous retroviruses. *Virus Genes* vol. **26** 291–315, (2003)
25. Mayer, J., Blomberg, J. & Seal, R. L. A revised nomenclature for transcribed human endogenous retroviral loci. *Mob. DNA* **2**, (2011).
26. Belyi, V. A., Levine, A. J. & Skalka, A. M. Unexpected inheritance: Multiple integrations of ancient bornavirus and ebolavirus/marburgvirus sequences in vertebrate Genomes. *PLoS Pathog.* **6**, 1–13 (2010).
27. Larsson, E. & Andersson, G. Beneficial role of human endogenous retroviruses: Facts and hypotheses. *Scandinavian Journal of Immunology* vol. **48** 329–338 (1998).
28. Löwer, R., Löwer, J. & Kurth, R. The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5177–5184 (1996).
29. Göke, J. *et al.* Dynamic Transcription of Distinct Classes of Endogenous Retroviral Elements Marks Specific Populations of Early Human Embryonic Cells. *Cell Stem Cell* **16**,

135–141 (2015).

30. Balada, E., Ordi-Ros, J. & Vilardell-Tarrés, M. Molecular mechanisms mediated by Human Endogenous Retroviruses (HERVs) in autoimmunity. *Rev. Med. Virol.* **19**, 273–286 (2009).
31. Perron, H. *et al.* Endogenous retroviral genes, Herpesviruses and gender in Multiple Sclerosis. *J. Neurol. Sci.* **286**, 65–72 (2009).
32. Manghera, M. & Douville, R. N. Endogenous retrovirus-K promoter: A landing strip for inflammatory transcription factors? *Retrovirology* **10**, 16 (2013).
33. Morandi, E., Tarlinton, R. E., Tanasescu, R. & Gran, B. Human endogenous retroviruses and multiple sclerosis: Causation, association, or after-effect? *Mult. Scler.* **23**, 1050–1055 (2017).
34. Perron, H. & Lang, A. The human endogenous retrovirus link between genes and environment in multiple sclerosis and in multifactorial diseases associating neuroinflammation. *Clin. Rev. Allergy Immunol.* **39**, 51–61 (2010).
35. Küry, P. *et al.* Human Endogenous Retroviruses in Neurological Diseases. *Trends in Molecular Medicine* **24**, 379-394 (2018).
36. Schulz, W. A., Steinhoff, C. & Florl, A. R. Methylation of endogenous human retroelements in health and disease. *Curr. Top. Microbiol. Immunol.* **310**, 211–250 (2006).
37. Jintaridth, P. & Mutirangura, A. Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. *Physiol. Genomics* **41**, 194–200 (2010).
38. Stengel, S., Fiebig, U., Kurth, R. & Denner, J. Regulation of human endogenous retrovirus-K expression in melanomas by CpG methylation. *Genes Chromosom. Cancer* **49**, 401–411 (2010).
39. Prudhomme, S., Bonnaud, B. & Mallet, F. Endogenous retroviruses and animal reproduction. *Cytogenet. Genome Res.* **110**, 353–364 (2005).
40. Sugimoto, J. & Schust, D. J. Review: Human endogenous retroviruses and the placenta. *Reprod. Sci.* **16**, 1023–1033 (2009).
41. Frank, O. *et al.* Human endogenous retrovirus expression profiles in samples from brains of patients with schizophrenia and bipolar disorders. *J. Virol.* **79**, 10890–10901 (2005).
42. Seifarth, W. *et al.* Comprehensive analysis of human endogenous retrovirus transcriptional activity in human tissues with a retrovirus-specific microarray. *J. Virol.* **79**, 341–352 (2005).
43. Kim, H.-S., Ahn, K. & Kim, D.-S. Quantitative expression of the HERV-W env gene in human tissues. *Arch. Virol.* **153**, 1587–1591 (2008).
44. Ahn, K. & Kim, H.-S. Structural and quantitative expression analyses of HERV gene family in human tissues. *Mol. Cells* **28**, 99–103 (2009).

45. Krone, B. & Grange, J. M. Melanoma, Darwinian medicine and the inner world. *J. Cancer Res. Clin. Oncol.* **136**, 1787–1794 (2010).
46. Sha, M. *et al.* Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* **403**, 785–789 (2000).
47. Bonnaud, B. *et al.* Evidence of selection on the domesticated ERVWE1 env retroviral element involved in placentation. *Mol. Biol. Evol.* **21**, 1895–1901 (2004).
48. Steiner, I., Nisipianu, P. & Wirguin, I. Infection and the etiology and pathogenesis of multiple sclerosis. *Curr. Neurol. Neurosci. Rep.* **1**, 271–276 (2001).
49. Serafini, B. *et al.* Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *J. Exp. Med.* **204**, 2899–2912 (2007).
50. Contreras-Galindo, R., Kaplan, M. H., Markovitz, D. M., Lorenzo, E. & Yamamura, Y. Detection of HERV-K(HML-2) viral RNA in plasma of HIV type 1-infected individuals. *AIDS Res. Hum. Retroviruses* **22**, 979–984 (2006).
51. Contreras-Galindo, R., López, P., Vélez, R. & Yamamura, Y. HIV-1 infection increases the expression of human endogenous retroviruses type K (HERV-K) in vitro. *AIDS Res. Hum. Retroviruses* **23**, 116–122 (2007).
52. Laderoute, M. P. *et al.* The replicative activity of human endogenous retrovirus K102 (HERV-K102) with HIV viremia. *AIDS* **21**, 2417–2424 (2007).
53. Garrison, K. E. *et al.* T cell responses to human endogenous retroviruses in HIV-1 infection. *PLoS Pathog.* **3**, 1617–1627 (2007).
54. Frank, O., Jones-Brando, L., Leib-Mosch, C., Yolken, R. & Seifarth, W. Altered transcriptional activity of human endogenous retroviruses in neuroepithelial cells after infection with Toxoplasma gondii. *J. Infect. Dis.* **194**, 1447–1449 (2006).
55. Brown, A. S. & Derkits, E. J. Prenatal infection and schizophrenia: a review of epidemiologic and translational studies. *Am. J. Psychiatry* **167**, 261–280 (2010).
56. Ryschkewitsch, C. F., Jensen, P. N., Monaco, M. C. & Major, E. O. JC virus persistence following progressive multifocal leukoencephalopathy in multiple sclerosis patients treated with natalizumab. *Ann. Neurol.* **68**, 384–391 (2010).
57. Mills, R. E., Bennett, E. A., Iskow, R. C. & Devine, S. E. Which transposable elements are active in the human genome? *Trends Genet.* **23**, 183–191 (2007).
58. Brady, T. *et al.* Integration target site selection by a resurrected human endogenous retrovirus. *Genes Dev.* **23**, 633–642 (2009).
59. Sjøttem, E., Anderssen, S. & Johansen, T. The promoter activity of long terminal repeats of the HERV-H family of human retrovirus-like elements is critically dependent on Sp1 family

proteins interacting with a GC/GT box located immediately 3' to the TATA box. *J. Virol.* **70**, 188–198 (1996).

60. Schulte, A. M. *et al.* Influence of the human endogenous retrovirus-like element HERV-E.PTN on the expression of growth factor pleiotrophin: a critical role of a retroviral Sp1-binding site. *Oncogene* **19**, 3988–3998 (2000).
61. Landry, J.-R., Rouhi, A., Medstrand, P. & Mager, D. L. The Opitz syndrome gene Mid1 is transcribed from a human endogenous retroviral promoter. *Mol. Biol. Evol.* **19**, 1934–1942 (2002).
62. Lee, W. J., Kwun, H. J. & Jang, K. L. Analysis of transcriptional regulatory sequences in the human endogenous retrovirus W long terminal repeat. *J. Gen. Virol.* **84**, 2229–2235 (2003).
63. Ruda, V. M. *et al.* Tissue specificity of enhancer and promoter activities of a HERV-K(HML-2) LTR. *Virus Res.* **104**, 11–16 (2004).
64. Leupin, O. *et al.* Transcriptional activation by bidirectional RNA polymerase II elongation over a silent promoter. *EMBO Rep.* **6**, 956–960 (2005).
65. Gogvadze, E., Stukacheva, E., Buzdin, A. & Sverdlov, E. Human-specific modulation of transcriptional activity provided by endogenous retroviral insertions. *J. Virol.* **83**, 6098–6105 (2009).
66. Herai, R. H. & Yamagishi, M. E. B. Detection of human interchromosomal trans-splicing in sequence databanks. *Brief. Bioinform.* **11**, 198–209 (2010).
67. Denne, M. *et al.* Physical and functional interactions of human endogenous retrovirus proteins Np9 and rec with the promyelocytic leukemia zinc finger protein. *J. Virol.* **81**, 5607–5616 (2007).
68. Armbruester, V. *et al.* Np9 protein of human endogenous retrovirus K interacts with ligand of numb protein X. *J. Virol.* **78**, 10310–10319 (2004).
69. Ruggieri, A. *et al.* Human endogenous retrovirus HERV-K(HML-2) encodes a stable signal peptide with biological properties distinct from Rec. *Retrovirology* **6**, 17 (2009).
70. Mangeney, M., de Parseval, N., Thomas, G. & Heidmann, T. The full-length envelope of an HERV-H human endogenous retrovirus has immunosuppressive properties. *J. Gen. Virol.* **82**, 2515–2518 (2001).
71. Nath, A. *et al.* Molecular basis for interactions of HIV and drugs of abuse. *J. Acquir. Immune Defic. Syndr.* **31 Suppl 2**, S62-9 (2002).
72. Medders, K. E., Sejbuk, N. E., Maung, R., Desai, M. K. & Kaul, M. Activation of p38 MAPK is required in monocytic and neuronal cells for HIV glycoprotein 120-induced

neurotoxicity. *J. Immunol.* **185**, 4883–4895 (2010).

73. Antony, J. M. *et al.* The Human Endogenous Retrovirus Envelope Glycoprotein, Syncytin-1, Regulates Neuroinflammation and Its Receptor Expression in Multiple Sclerosis: A Role for Endoplasmic Reticulum Chaperones in Astrocytes1. *J. Immunol.* **179**, 1210–1224 (2007).
74. Rolland, A. *et al.* The envelope protein of a human endogenous retrovirus-W family activates innate immunity through CD14/TLR4 and promotes Th1-like responses. *J. Immunol.* **176**, 7636–7644 (2006).
75. Serra, C. *et al.* In vitro modulation of the multiple sclerosis (MS)-associated retrovirus by cytokines: implications for MS pathogenesis. *J. Neurovirol.* **9**, 637–643 (2003).
76. Mameli, G. *et al.* Regulation of the syncytin-1 promoter in human astrocytes by multiple sclerosis-related cytokines. *Virology* **362**, 120–130 (2007).
77. O'Neill, L. A. J. & Bowie, A. G. Sensing and signaling in antiviral innate immunity. *Curr. Biol.* **20**, R328-33 (2010).
78. Christensen, T. Association of human endogenous retroviruses with multiple sclerosis and possible interactions with herpes viruses. *Rev. Med. Virol.* **15**, 179–211 (2005).
79. Meylan, F. *et al.* Negative thymocyte selection to HERV-K18 superantigens in humans. *Blood* **105**, 4377–4382 (2005).
80. Stauffer, Y. *et al.* Interferon-alpha-induced endogenous superantigen. a model linking environment and autoimmunity. *Immunity* **15**, 591–601 (2001).
81. Hsiao, F. C., Lin, M., Tai, A., Chen, G. & Huber, B. T. Cutting Edge: Epstein-Barr Virus Transactivates the HERV-K18 Superantigen by Docking to the Human Complement Receptor 2 (CD21) on Primary B Cells1. *J. Immunol.* **177**, 2056–2060 (2006).
82. Hsiao, F. C. *et al.* EBV LMP-2A employs a novel mechanism to transactivate the HERV-K18 superantigen through its ITAM. *Virology* **385**, 261–266 (2009).
83. Turcanova, V. L., Bundgaard, B. & Höllsberg, P. Human herpesvirus-6B induces expression of the human endogenous retrovirus K18-encoded superantigen. *J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol.* **46**, 15–19 (2009).
84. Lewis, S. D. *et al.* Toll-like receptor 7 is not necessary for retroviral neuropathogenesis but does contribute to virus-induced neuroinflammation. *J. Neurovirol.* **14**, 492–502 (2008).
85. Cheng, S. M. *et al.* HIV-1 transactivator protein induction of suppressor of cytokine signaling-2 contributes to dysregulation of IFN{gamma} signaling. *Blood* **113**, 5192–5201 (2009).
86. Kirchhoff, F. Immune Evasion and Counteraction of Restriction Factors by HIV-1 and Other

Primate Lentiviruses. *Cell Host Microbe* **8**, 55–67 (2010).

87. Beyer, T. D. *et al.* Apoptosis of the teratocarcinoma cell line Tera-1 leads to the cleavage of HERV-K10gag proteins by caspases and/or granzyme B. *Scand. J. Immunol.* **56**, 303–309 (2002).
88. Grandi, N. & Tramontano, E. HERV envelope proteins: Physiological role and pathogenic potential in cancer and autoimmunity. *Frontiers in Microbiology* vol. **9,** 462 (2018).
89. Li, W. *et al.* Human endogenous retrovirus-K contributes to motor neuron disease. *Sci. Transl. Med.* **7**, (2015).
90. Chiò, A. *et al.* Global epidemiology of amyotrophic lateral sclerosis: A systematic review of the published literature. *Neuroepidemiology* **41**, 118-130 (2013).
91. Sutedja, N. A. *et al.* Exposure to chemicals and metals and risk of amyotrophic lateral sclerosis: A systematic review. *Amyotrophic Lateral Sclerosis* vol. **10** 302–309 at
92. Douville, R. N. & Nath, A. Human endogenous retroviruses and the nervous system. in *Handbook of Clinical Neurology* **123**, 465-485 (2014)
93. Renton, A. E., Chiò, A. & Traynor, B. J. State of play in amyotrophic lateral sclerosis genetics. *Nature Neuroscience* **17**, 17-23 (2014).
94. McGuire, V. *et al.* Occupational exposures and amyotrophic lateral sclerosis: A population-based case-control study. *Am. J. Epidemiol.* **145**, 1076-1088 (1997)
95. Fang, F. *et al.* Workplace exposures and the risk of amyotrophic lateral sclerosis. *Environ. Health Perspect.* **117,** 1387-1392 (2009)
96. Shefner, J. M. *et al.* A proposal for new diagnostic criteria for ALS. *Clinical Neurophysiology* **131,** 1975-1978 (2020).
97. Brooks, B., Miller, R., Swash, M. & Munsat, T. El Escorial revisited: Revised criteria for the diagnosis of amyotrophic lateral sclerosis. World Federation of Neurology Research Group on Motor Neuron Diseases. *Amyotroph. Lateral Scler. Other Mot. Neuron Disord.* **1,** 293-299 (2000).
98. Bensimon, G., Lacomblez, L. & Meininger, V. A Controlled Trial of Riluzole in Amyotrophic Lateral Sclerosis. *N. Engl. J. Med.* **330,** 585-591 (1994)
99. Abe, K. *et al.* Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol.* **16**, 505–512 (2017).
100. Bourke, S. C. & Steer, J. Practical respiratory management in amyotrophic lateral sclerosis: evidence, controversies and recent advances. *Neurodegenerative disease management* **6,** 147-160(2016).

101. Beers, D. R. & Appel, S. H. Immune dysregulation in amyotrophic lateral sclerosis: mechanisms and emerging therapies. *The Lancet Neurology* vol. **18** 211–220 (2019).
102. Rentzos, M. *et al.* Alterations of T cell subsets in ALS: A systemic immune activation? *Acta Neurol. Scand.* **125**, 260–264 (2012).
103. Liddelow, S. A. *et al.* Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* **541,** 481-487 (2017)
104. Tjalkens, R. B., Popichak, K. A. & Kirkley, K. A. Inflammatory Activation of Microglia and Astrocytes in Manganese Neurotoxicity. in *Advances in Neurobiology* vol. **18** 159–181 (2017).
105. Yuste, J. E., Tarragon, E., Campuzano, C. M. & Ros-Bernal, F. Implications of glial nitric oxide in neurodegenerative diseases. *Frontiers in Cellular Neuroscience* vol. **9,** 322 (2015).
106. Lewis, C. A., Manning, J., Rossi, F. & Krieger, C. The neuroinflammatory response in ALS: The roles of microglia and T cells. *Neurol. Res. Int.* **2012**, 803701 (2012).
107. Woodruff, T. M., Costantini, K. J., Taylor, S. M. & Noakes, P. G. Role of complement in motor neuron disease: animal models and therapeutic potential of complement inhibitors. *Advances in Experimental Medicine and Biology* vol. **632** 143–158 at
108. Arru, G. *et al.* Herv-k modulates the immune response in als patients. *Microorganisms* **9,** 1784 (2021)
109. Prasad, A., Bharathi, V., Sivalingam, V., Girdhar, A. & Patel, B. K. Molecular mechanisms of TDP-43 misfolding and pathology in amyotrophic lateral sclerosis. *Frontiers in Molecular Neuroscience* vol. **12,** 25 (2019).
110. Maekawa, S. *et al.* TDP-43 is consistently co-localized with ubiquitinated inclusions in sporadic and Guam amyotrophic lateral sclerosis but not in familial amyotrophic lateral sclerosis with and without SOD1 mutations. *Neuropathology* **29**, 672–683 (2009).
111. Kabashi, E. *et al.* TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat. Genet.* **40**, 572–574 (2008).
112. Wils, H. *et al.* TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 3858–3863 (2010).
113. Xu, Y. F. *et al.* Wild-type human TDP-43 expression causes TDP-43 phosphorylation, mitochondrial aggregation, motor deficits, and early mortality in transgenic mice. *J. Neurosci.* **30**, 10851–10859 (2010).
114. Martínez-González, L. *et al.* Motor neuron preservation and decrease of in vivo TDP-43 phosphorylation by protein CK-1δ kinase inhibitor treatment. *Sci. Rep.* **10**, (2020).

115. Francois-Moutal, L., Scott, D. D. & Khanna, M. Direct targeting of TDP-43, from small molecules to biologics: The therapeutic landscape. *RSC Chemical Biology* vol. **2** 1158–1166 (2021).
116. Li, W. *et al.* Human endogenous retrovirus-K contributes to motor neuron disease. *Sci. Transl. Med.* **7,** 307 (2015).
117. Bartel, D. P. MicroRNAs: Target Recognition and Regulatory Functions. *Cell* vol. **136** 215–233 (2009).
118. Huntzinger, E. & Izaurralde, E. Gene silencing by microRNAs: Contributions of translational repression and mRNA decay. *Nature Reviews Genetics* vol. **12** 99–110 (2011).
119. Ha, T.-Y. MicroRNAs in Human Diseases: From Cancer to Cardiovascular Disease. *Immune Netw.* **11**, 135 (2011).
120. Otmani, K. & Lewalle, P. Tumor Suppressor miRNA in Cancer Cells and the Tumor Microenvironment: Mechanism of Deregulation and Clinical Implications. *Frontiers in Oncology* vol. **11,** 708-765 (2021).
121. Hébert, S. S. & De Strooper, B. Alterations of the microRNA network cause neurodegenerative disease. *Trends in Neurosciences* vol. **32** 199–206 at
122. Ricci, C., Marzocchi, C. & Battistini, S. MicroRNAs as biomarkers in amyotrophic lateral sclerosis. *Cells* vol. **7,** 11-219 (2018).
123. Martinez, B. & Peplow, P. MicroRNA expression in animal models of amyotrophic lateral sclerosis and potential therapeutic approaches. *Neural Regeneration Research* vol. **17** 728–740 (2022).
124. Williams, A. H. *et al.* MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science* **326,** 1549-1554 (2009)
125. Zingale, V. D., Gugliandolo, A. & Mazzon, E. Mir-155: An important regulator of neuroinflammation. *International Journal of Molecular Sciences* vol. **23,** 90 (2022).
126. Arru, G. *et al.* Humoral immunity response to human endogenous retroviruses K/W differentiates between amyotrophic lateral sclerosis and other neurological diseases. *Eur. J. Neurol.* **25**, 1076-e84 (2018).
127. Conti, E. *et al.* Serum naturally occurring anti-TDP-43 auto-antibodies are increased in amyotrophic lateral sclerosis. *Sci. Rep.* **11**, 1978 (2021).
128. Verstraete, E. *et al.* TDP-43 plasma levels are higher in amyotrophic lateral sclerosis. *Amyotroph. Lateral Scler.* **13,** 446-451 (2012)
129. Foulds, P. *et al.* TDP-43 protein in plasma may index TDP-43 brain pathology in Alzheimer's disease and frontotemporal lobar degeneration. *Acta Neuropathol.* **116**, 141–

146 (2008).

130. Harrison, A. F. & Shorter, J. RNA-binding proteins with prion-like domains in health and disease. *Biochemical Journal* **474,** 1417-1438 (2017).
131. Neumann, M. *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science (80-.).* **314**, 130–133 (2006).
132. Afroz, T. *et al.* Immunotherapy targeting the C-terminal domain of TDP-43 decreases neuropathology and confers neuroprotection in mouse models of ALS/FTD. *Neurobiol. Dis.* **179**, 106050 (2023).
133. Douville, R., Liu, J., Rothstein, J. & Nath, A. Identification of active loci of a human endogenous retrovirus in neurons of patients with amyotrophic lateral sclerosis. *Ann. Neurol.* **69**, 141–151 (2011).
134. Arru, G. *et al.* Humoral immunity response to human endogenous retroviruses K/W differentiates between amyotrophic lateral sclerosis and other neurological diseases. *Eur. J. Neurol.* **25**, 1076-e84 (2018).
135. Simula, E. R., Arru, G., Zarbo, I. R., Solla, P. & Sechi, L. A. Tdp-43 and herv-k envelope-specific immunogenic epitopes are recognized in als patients. *Viruses* **13**, 2301 (2021).
136. Chang, Y.-H. & Dubnau, J. Endogenous retroviruses and TDP-43 proteinopathy form a sustaining feedback driving intercellular spread of Drosophila neurodegeneration. *Nat. Commun.* **14**, 966 (2023).
137. Vanderlugt, C. L. & Miller, S. D. Epitope spreading in immune-mediated diseases: Implications for immunotherapy. *Nature Reviews Immunology* **2,** 85-95(2002).
138. Phan, K. *et al.* Pathological manifestation of human endogenous retrovirus K in frontotemporal dementia. *Commun. Med.* **1,** 60(2021)
139. Stollar, B. D. Antibodies to DNA. *CRC critical reviews in biochemistry* vol. **20** 1–36 at
140. Shoenfeld, Y. & Toubi, E. Protective autoantibodies: Role in homeostasis, clinical importance, and therapeutic potential. *Arthritis and Rheumatism* **52,** 2599-2606 (2005).
141. Conrad, K., Bachmann, M. P., Matsuura, E. & Shoenfeld, Y. From animal models to human genetics: Research on the induction and pathogenicity of autoantibodies. in *Autoimmunity Reviews* **4,** 178-187 (2005).
142. Schwartz, M. & Baruch, K. Breaking peripheral immune tolerance to CNS antigens in neurodegenerative diseases: Boosting autoimmunity to fight-off chronic neuroinflammation. *Journal of Autoimmunity* **54,** 8-14(2014).
143. Stein, T. D., Alvarez, V. E. & McKee, A. C. Chronic traumatic encephalopathy: A spectrum of neuropathological changes following repetitive brain trauma in athletes and military

personnel. *Alzheimer's Research and Therapy* **6,** 4(2014).

144. Yoles, E. *et al.* Protective autoimmunity is a physiological response to CNS trauma. *J. Neurosci.* **21,** 11(2001).
145. Beard, J. D. & Kamel, F. Military service, deployments, and exposures in relation to amyotrophic lateral sclerosis etiology and survival. *Epidemiol. Rev.* **37,** 55-70 (2015)
146. Graham, N. S. N. & Sharp, D. J. Understanding neurodegeneration after traumatic brain injury: From mechanisms to clinical trials in dementia. *Journal of Neurology, Neurosurgery and Psychiatry* vol. **90** 1221–1233 (2019).
147. Curtin, F., Perron, H., Kromminga, A., Porchet, H. & Lang, A. B. Preclinical and early clinical development of GNbAC1, a humanized IgG4 monoclonal antibody targeting endogenous retroviral MSRV-Env protein. *MAbs* **7**, 265–275 (2015).
148. Steiner, J. P. *et al.* Human Endogenous Retrovirus K Envelope in Spinal Fluid of Amyotrophic Lateral Sclerosis Is Toxic. *Ann. Neurol.* **92**, 545–561 (2022).
149. Bhardwaj, N., Maldarelli, F., Mellors, J. & Coffin, J. M. HIV-1 Infection Leads to Increased Transcription of Human Endogenous Retrovirus HERV-K (HML-2) Proviruses In Vivo but Not to Increased Virion Production . *J. Virol.* **88**, 11108–11120 (2014).
150. Kouri, F. M. *et al.* MiR-182 integrates apoptosis, growth, and differentiation programs in glioblastoma. *Genes Dev.* **29**, 732–745 (2015).
151. Darbeheshti, F. *et al.* Investigation of BRCAness associated miRNA-gene axes in breast cancer: cell-free miR-182-5p as a potential expression signature of BRCAness. *BMC Cancer* **22**, 668 (2022).
152. Garofalo, M. *et al.* miR-221&222 Regulate TRAIL Resistance and Enhance Tumorigenicity through PTEN and TIMP3 Downregulation. *Cancer Cell* vol. **16** 498–509 (2009).
153. Liu, X. *et al.* A Necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circ. Res* **104,** 4(2009)
154. Lai, C.-C., Shih, T.-P., Ko, W.-C., Tang, H.-J. & Hsueh, P.-R. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges. *Int. J. Antimicrob. Agents* **55**, 105924 (2020).
155. Singhal, T. A Review of Coronavirus Disease-2019 (COVID-19). *Indian J. Pediatr.* **87**, 281–286 (2020).
156. Dotan, A. *et al.* The SARS-CoV-2 as an instrumental trigger of autoimmunity. *Autoimmun. Rev.* **20**, 102792(2021).
157. Garmendia, J. V., García, A. H., De Sanctis, C. V., Hajdúch, M. & De Sanctis, J. B. Autoimmunity and Immunodeficiency in Severe SARS-CoV-2 Infection and Prolonged

COVID-19. *Curr. Issues Mol. Biol.* **45**, 33–50 (2023).

158. Wang, E. Y. *et al.* Diverse functional autoantibodies in patients with COVID-19. *Nature* **595**, 283–288 (2021).
159. Temerozo, J. R. *et al.* Human endogenous retrovirus K in the respiratory tract is associated with COVID-19 physiopathology. *Microbiome* **10**, 65 (2022).
160. Balestrieri, E. *et al.* Evidence of the pathogenic HERV-W envelope expression in T lymphocytes in association with the respiratory outcome of COVID-19 patients. *EBioMedicine* **66**, 103341 (2021).
161. Kitsou, K. *et al.* Upregulation of Human Endogenous Retroviruses in Bronchoalveolar Lavage Fluid of COVID-19 Patients. *Microbiol. Spectr.* **9,** e0126021(2021)
162. Guan, W. *et al.* Clinical Characteristics of Coronavirus Disease 2019 in China. *N. Engl. J. Med.* **382**, 1708–1720 (2020).
163. Zhang, T., Wu, Q. & Zhang, Z. Probable Pangolin Origin of SARS-CoV-2 Associated with the COVID-19 Outbreak. *Curr. Biol.* **30,** 1346-1351(2020)
164. Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280.e8 (2020).
165. Xu, Z. *et al.* Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *Lancet Respir. Med.* **8**, 420–422 (2020).
166. Yang, X. *et al.* Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. *Lancet Respir. Med.* **8,** 475-481(2020)
167. Huang, C. *et al.* Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* **395**, 497–506 (2020).
168. Corman, V. M. *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance* **25,** 3 (2020).
169. Salehi, S., Abedi, A., Balakrishnan, S. & Gholamrezanezhad, A. Coronavirus disease 2019 (COVID-19): A systematic review of imaging findings in 919 patients. *American Journal of Roentgenology* **215,** 87-93(2020).
170. Zhao, J. *et al.* Antibody Responses to SARS-CoV-2 in Patients with Novel Coronavirus Disease 2019. *Clin. Infect. Dis.* **71,** 2027-2034 (2020).
171. Sanders, J. M., Monogue, M. L., Jodlowski, T. Z. & Cutrell, J. B. Pharmacologic Treatments for Coronavirus Disease 2019 (COVID-19): A Review. *JAMA - Journal of the American Medical Association* **323,** 1824-1836 (2020).
172. Beigel, J. H. *et al.* Remdesivir for the Treatment of Covid-19 — Final Report. *N. Engl. J.*

Med. **383,** 1813-1826 (2020)

173. Boulware, D. R. *et al.* A Randomized Trial of Hydroxychloroquine as Postexposure Prophylaxis for Covid-19. *N. Engl. J. Med.* **383,** 517-525 (2020)
174. Stone, J. H. *et al.* Efficacy of Tocilizumab in Patients Hospitalized with Covid-19. *N. Engl. J. Med.* **383**, 2333-2344 (2020)
175. Joyner, M. J. *et al.* Convalescent Plasma Antibody Levels and the Risk of Death from Covid-19. *N. Engl. J. Med.* **384,** 1015-1027(2021)
176. Kissler, S. M., Tedijanto, C., Goldstein, E., Grad, Y. H. & Lipsitch, M. Projecting the transmission dynamics of SARS-CoV-2 through the postpandemic period. *Science (80-.).* **368**, 860–868 (2020).
177. Callow, K. A., Parry, H. F., Sergeant, M. & Tyrrell, D. A. J. The time course of the immune response to experimental coronavirus infection of man. *Epidemiol. Infect.* **105**, 435–446 (1990).
178. Sagar, M. *et al.* Recent endemic coronavirus infection is associated with less-severe COVID-19. *J. Clin. Invest.* **131**, e143380 (2021).
179. Koma, T., Adachi, S., Doi, N., Adachi, A. & Nomaguchi, M. Toward Understanding Molecular Bases for Biological Diversification of Human Coronaviruses: Present Status and Future Perspectives. *Frontiers in Microbiology* vol. **11**, 2016 (2020).
180. Jaimes, J. A., André, N. M., Chappie, J. S., Millet, J. K. & Whittaker, G. R. Phylogenetic Analysis and Structural Modeling of SARS-CoV-2 Spike Protein Reveals an Evolutionary Distinct and Proteolytically Sensitive Activation Loop. *J. Mol. Biol.* **432**, 3309–3325 (2020).
181. Loos, C. *et al.* Evolution of Early SARS-CoV-2 and Cross-Coronavirus Immunity. *mSphere* **5**, e00622-20 (2020).
182. Grifoni, A. *et al.* Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. *Cell* **181,** 1489-1501.e15 (2020)
183. Braun, J. *et al.* SARS-CoV-2-reactive T cells in healthy donors and patients with COVID-19. *Nature* **587,** 270-274 (2020)
184. Mateus, J. *et al.* Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. *Science (80-.).* **370,** 89-94(2020)
185. Sotgia, F. & Lisanti, M. P. Using the common cold virus as a naturally occurring vaccine to prevent COVID-19: Lessons from Edward Jenner. *Aging (Albany. NY).* **12**, 18797–18803 (2020).
186. Premkumar, L. *et al.* The receptor-binding domain of the viral spike protein is an

immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Sci. Immunol.* **5**, eabc8413 (2020).

187. Premkumar, L. *et al.* The receptor-binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. *Sci. Immunol.* **5**, eabc8413 (2020)
188. Ng, K. W. *et al.* Preexisting and de novo humoral immunity to SARS-CoV-2 in humans. *Science (80-.).* **370**, 1339–1343 (2020).
189. Abdul-Rasool, S. & Fielding, B. C. Understanding Human Coronavirus HCoV-NL63~!2009-11-13~!2010-04-09~!2010-05-25~! *Open Virol. J.* **4**, 76–84 (2010).
190. Canducci, F. *et al.* Two-year prospective study of single infections and co-infections by respiratory syncytial virus and viruses identified recently in infants with acute respiratory disease. *J. Med. Virol.* **80,** 716-723(2008).
191. Batista Junior, J. Interaction Energies of the Human ACE2 Molecular Recognition by SARS-CoV-2. *Virol. Curr. Res.* **15***,* 42-48200 (2020)
192. Rawat, P., Jemimah, S., Ponnuswamy, P. K. & Gromiha, M. M. Why are ACE2 binding coronavirus strains SARS-CoV/SARS-CoV-2 wild and NL63 mild? *Proteins Struct. Funct. Bioinforma.* **89**, 389–398 (2021).
193. Bastard, P. *et al.* Autoantibodies against type I IFNs in patients with life-threatening COVID-19. *Science (80-.).* **370**, eabd4585 (2020).
194. Bastard, P. *et al.* Autoantibodies neutralizing type I IFNs are present in ~4% of uninfected individuals over 70 years old and account for ~20% of COVID-19 deaths. *Sci. Immunol.* **6**, eabl4340 (2021).
195. Chang, S. E. *et al.* New-onset IgG autoantibodies in hospitalized patients with COVID-19. *Nat. Commun.* **12**, 1–15 (2021).
196. Manry, J. *et al.* The risk of COVID-19 death is much greater and age dependent with type I IFN autoantibodies. *Proc. Natl. Acad. Sci. U. S. A.* **119**, e2200413119 (2022).
197. Zhou, Y. *et al.* Clinical and Autoimmune Characteristics of Severe and Critical Cases of COVID-19. *Clin. Transl. Sci.* **13**, 1077–1086 (2020).
198. Vlachoyiannopoulos, P. G. *et al.* Autoantibodies related to systemic autoimmune rheumatic diseases in severely ill patients with COVID-19. *Ann. Rheum. Dis.* **79**, 1661–1663 (2020).
199. Tovo, P. A. *et al.* Covid-19 in children: Expressions of type i/ii/iii interferons, trim28, setdb1, and endogenous retroviruses in mild and severe cases. *Int. J. Mol. Sci.* **22**, 7481 (2021).
200. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in

infectious disease. *Nature Reviews Immunology* **15,** 87-103 (2015).

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
Fondo Sociale Europeo, Azione I.1 “Dottorati Innovativi con caratterizzazione Industriale”

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