Article

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HERV-Derived Syncytin-1 and Syncytin-2 as Sources of Linear and Discontinuous Epitopes in Antiphospholipid Syndrome: A Pivotal Computational Study

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Background: To date, no studies have investigated the potential reactivation of human endogenous retroviruses (HERVs) in the pathogenesis of antiphospholipid syndrome (APS). HERV-derived syncytin-1 and syncytin-2 are localized in the plasma membrane of cells and physiologically expressed during pregnancy. The current study aimed to determine whether the epitopes of syncytins can trigger an immune response leading to APS in genetically predisposed individuals.
Methods: The TepiTool, ABCpred, and DiscoTope servers were utilized to predict T-cell and B-cell epitopes by inputting the FASTA sequences and 3D structures of syncytin-1, syncytin-2, and β2-glycoprotein I (β2GPI), which served as a reference antigen for APS. T-cell epitopes were selected based on their binding to a panel of human leukocyte antigen (HLA) class II alleles associated with APS according to the literature. Epitope predictions for the different proteins were statistically compared using GraphPad Prism.
Results: For syncytin-1, we identified a total of 721 T-cell epitopes, 51 linear B-cell epitopes, and up to 40 conformational epitopes. For syncytin-2, we predicted 705 T-cell epitopes and 28 linear B-cell epitopes, but a lower number of conformational epitopes, which also exhibited lower B-cell receptor (BCR)-binding scores. The predicted T-cell and B-cell conformational epitopes of both syncytin-1 and syncytin-2 demonstrated significantly higher binding affinity to selected HLA alleles and BCR compared with β2GPI. Furthermore, syncytin-1 exhibited significantly higher immunogenicity than syncytin-2.
Conclusions: Both syncytin-1 and syncytin-2 are computationally endowed with potential epitopes that may activate either T cells or B cells in individuals genetically predisposed to APS. While these findings may illuminate the possible role of HERVs in the development of APS, they warrant validation in further laboratory studies.

Keywords: antiphospholipid syndrome; β2-glycoprotein I; human endogenous retrovirus; syncytin-1; syncytin-2; T-cell epitopes; B-cell epitopes; epitope prediction

Introduction

Human endogenous retroviruses (HERVs) are DNA sequences of retroviral origin that have been incorporated into the human genome over the last 100 million years through multiple integrations of now-extinct exogenous retroviruses [1]. HERVs share with exogenous retroviruses the typical proviral structure consisting of two long terminal repeats (LTRs) that play a regulatory role and flank the internal part of the viral genes *gag*, *pro-pol*, and *env* [1]. These genes encode the structural components of the matrix, capsid and nucleocapsid; protease; reverse transcriptase; integrase; and the subunits of the envelope surface (SU) and transmembrane (TM) domain. HERVs constitute 8% of the human genome and are usually not reactivatable due to progressive somatic mutations that have altered their genetic sequence [2]. These primordial retroviruses are, therefore, stably anchored in the human genome and are passed on vertically to offspring [3]. The increasing tolerance to HERV elements in our genome is attributed to their role in many physiological processes. Over the years, scientific research has shown that HERVs can protect host cells from viral infections by antagonizing exogenous retroviruses that produce proteins or possess similar nucleic acids [3]. Additionally, the env particles of HERVs can act as "immune sentinels", protecting tissues from exaggerated immune responses and maintaining basal immune stimulation [1]. Another example of the contribution of HERVs to physiological processes is the role that syncytin-1 and syncytin-2, produced by HERV-W and HERV-FRD, respectively, play during placentation. Both are selectively expressed by the cells of the cytotrophoblast and determine the attachment of the embryo to the uterus, as well as being essential for the maintenance of maternal-fetal tolerance [1].

However, in other circumstances, unintentional reactivation of HERVs can underlie human diseases, including cancer, neurodegenerative disorders, and autoimmune diseases [2,4]. Indeed, autoimmune diseases are characterized by the breakdown of immune tolerance and the resulting chronic activation of the immune system against autoepitopes. The mechanism underlying this dysregulation is not yet fully understood, but the most recognized hypothesis is based on the interplay of the genetic predisposition of the affected individual in combination with various environmental factors such as exogenous infections and dysbiosis. Among the best-studied genetic factors are genes of the major histocompatibility complex (MHC), particularly the class I and II loci of the human leukocyte antigen (HLA), which play a fundamental role in antigen presentation and the regulation of the innate and adaptive immune response [5].

Although HERVs are an integral part of the genome and are tolerated as such, their upregulation can provide numerous pathogen-associated molecular patterns and damage-associated molecular patterns even in the absence of an external causative factor, thus providing continuous stimuli that trigger inflammation and contribute to an abnormal immune response [2,6]. HERVs could also act as superantigens and activate CD4+ T cells, leading to excessive production of cytokines and clinical consequences such as shock and multi-organ failure [2].

Multiple studies have shown that reactivation of HERVs can occur in rheumatoid arthritis (RA) [6], systemic lupus erythematosus (SLE) [7], and Sjögren's syndrome (SS) [8]. However, there is little evidence for the pathogenic role of HERVs in antiphospholipid syndrome (APS). APS is an autoimmune disease characterized by an increased risk of thrombosis and obstetric complications associated with specific anti-phospholipid antibodies (aPLs) such as anti-cardiolipin (aCL) antibodies, anti-β2-glycoprotein I (anti-β2GPI) antibodies, and lupus anticoagulant (LA) positivity [9]. Among these, autoantibodies recognizing cryptic epitopes embedded in β2GPI domain 1 (D1) have been shown to be significantly associated with an increased risk of APS clinical manifestations [10]. Indeed, anti-β2GPI antibodies may play a crucial role in the vascular, obstetric, and catastrophic variants of APS, as β2GPI is expressed on both endothelium and placental tissue. The binding of autoantibodies can trigger a prothrombotic phenotype, which is further enhanced by the intervention of circulating leukocytes, platelets, and complement activation [11]. Viruses are known trigger factors for APS in genetically predisposed individuals. aCL and, more rarely, anti-β2GPI antibodies can be detected transiently in individuals infected with exogenous retroviruses such as human immunodeficiency virus (HIV) or human T-lymphotropic virus (HTLV)-1, but without thrombotic consequences [12,13].

In carriers of certain HLA alleles that predispose to APS [14–36] and more generally to loss of immune tolerance, exogenous retrovirus infections could induce the production of aPLs that may cross-react with the antigens of HERVs. Molecular mimicry mechanisms associated with impaired autophagy, T-cell activation, type I interferon response, and pro-inflammatory cytokine secretion have already been postulated as the basis of SLE manifestations induced by exogenous retroviruses [7,37]. Importantly, APS and SLE share common pathogenic pathways and often overlap [38]. Given the affinity of aPLs for phospholipid-associated proteins, it is plausible that these antibodies could recognize HERV env proteins that are erroneously overproduced and expressed on the plasma membrane of cells. Pregnancy morbidity is a pathognomonic feature of APS, leading to the hypothesis that plasma membrane syncytins could be involved in the pathogenetic scenario.

To our knowledge, no study has attempted to detect autoantibodies against syncytin-1 and syncytin-2 in APS, although several reports suggest a pathogenic role of syncytin-1 in autoimmune diseases such as multiple sclerosis (MS) or type 1 diabetes [39,40]. A previous computational study conducted by our group showed alignments between syncytin-1 and syncytin-2 genes and human long non-coding (lnc)RNAs with potential impact on pregnancy outcomes in SLE [41]. Besides genomic crosstalk, it remains unclear whether the epitopes of syncytin-1 or syncytin-2 can be presented by specific HLA molecules that predispose to APS risk and trigger activation of autoreactive T and B lymphocytes.

Therefore, the primary objective of this computational study was to investigate the presence of potential T-cell and B-cell epitopes of syncytin-1 and syncytin-2 that could bind specific polymorphic HLA variants associated with APS or are predicted to interact with the B-cell receptor (BCR) of B lymphocytes. We also aimed to discuss the hypothesized pathogenic mechanism and clinical implications. Considering the central pathogenicity of β2GPI as an autoantigen, we secondarily aimed to compare the results obtained with syncytin-1 and syncytin-2 with β2GPI epitopes in terms of binding to the same HLA alleles or prediction of BCR ligation.

Materials and Methods

Immune Epitope Database (IEDB) TepiTool Analysis

To find T-cell epitopes, the protein FASTA sequence of the human syncytin-1 precursor was retrieved from the NCBI website https://www.ncbi.nlm.nih.gov/protein/NP_001124397.1 (reference sequence: NP_001124397.1, accessed on 18 January 2024). Similarly, the protein FASTA sequence of the human syncytin-2 preprotein was retrieved from https://www.ncbi.nlm.nih.gov/protein/NP_997465.1 (NCBI reference sequence: NP_997465.1, accessed on 18 January 2024). In both cases, the FASTA se-

quences were used separately as a key input for the TepiTool analysis of the Immune Epitope Database (IEDB) (https://www.iedb.org/, accessed on 18 January 2024) [42,43]. To find the T-cell epitopes, we performed a thorough search of the PubMed database and selected previously published articles that showed an association between specific HLA class II alleles and APS in different ethnic groups [14–36]. Table 1 (Ref. [14–29,31–36]) shows the alleles found to be significantly associated with APS and their highest frequencies in each population studied according to the Allele Frequencies Net database (https://www.allelefrequencies.net, accessed on 17 August 2024) [44]. Table 2 illustrates the panel of HLA class II alleles used as input for the TepiTool analysis, which were selected based on the literature search. For both syncytin-1 and syncytin-2, we searched for 15-mer epitopes with a predicted consensus rank <10. We applied the default settings for a moderate number of epitopes according to the prediction method recommended by the IEDB, removed duplicates, set overlapping residues to 10, and included a total of approximately 106 epitopes in the analysis. The standard IEDB analysis uses the Consensus method with an estimated area under the ROC curve (AUC) of 0.89 ± 0.05 and a sensitivity for predicting T-cell activation of 66.7% [45].

Using the same methods, we analyzed the affinity of selected HLA alleles for epitopes within the entire human β2GPI protein FASTA sequence (GenBank: AAP72014.1; https://www.ncbi.nlm.nih.gov/protein/AAP72014.1, accessed on 20 May 2024) and performed separate searches for each of the five domains of β2GPI, with particular attention to D1, which has been shown to be the most immunogenic in APS pathogenesis [10]. The FASTA amino acid sequences of the β2GPI domains were identified according to the work of Steinkasserer and Ioannou [46,47].

ABCpred Analysis

For the prediction of linear B-cell epitopes, we used the bioinformatics tool ABCpred, https://webs.iitd.edu.in/raghava/abcpred/ [48,49], a machine learning-based method consisting of artificial neural networks with an estimated accuracy of 65.93% with equal sensitivity and specificity [50]. Briefly, we inserted the protein FASTA sequence of the human syncytin-1 precursor (reference sequence: NP_001124397.1, accessed on 18 January 2024) and syncytin-2 preprotein (NCBI reference sequence: NP_997465.1, accessed on 18 January 2024) separately into the query box and initiated a search with the suggested default settings (threshold 0.51; no overlapping filters; window length for prediction: 16).

We repeated the same analysis by replacing the FASTA amino acid sequence of syncytin-1 and syncytin-2 with that of human β2GPI (GenBank: AAP72014.1; https://www.ncbi.nlm.nih.gov/protein/AAP72014.1, accessed on 20 May 2024) and its five domains, which were analyzed separately [46,47].

DiscoTope Analysis

We also consulted version 2.0 of the bioinformatics server DiscoTope [51] to predict the discontinuous epitopes of syncytin-1 and syncytin-2 that may bind to B cells. DiscoTope is an IEDB tool that enables the prediction of conformational or discontinuous epitopes in the 3D structures of proteins. The method consists of calculating the contact number of amino acid residues, which correlates with the localization of the epitope, and the propensity score for the probability that a particular residue is part of an epitope [52]. The DiscoTope score is then calculated by combining the propensity score with the contact numbers. For this analysis, the two protein structures were searched in the RCSB Protein Data Bank (PDB) (https://www.rcsb.org/, accessed on 31 January 2024) [53]. For syncytin-1, we used both the crystal structures in the fusion and post-fusion conformation (5HA6 and 6RX1, respectively) [Aydin, H., Thavalingam, A., Bikopoulos, G., Sultana, A., Lee, J.E. Structural and functional characterization of the human Syncytin 1 fusion protein, to be published]. For syncytin-2, the 1Y4M [54] and the 6RX3 crystal structure of human syncytin-2 in post-fusion conformation [55] were used. We performed a separate search for each chain of the fusion and post-fusion conformation of the proteins. The threshold value of –3.7, corresponding to a specificity of 75% and a sensitivity of 47%, was applied.

Using DiscoTope analysis with the same settings, we also analyzed the conformational epitopes of chain a of the 6XST crystal structure of β2GPI (https://www.rcsb.org/, accessed on 7 June 2024).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, MA, USA (https://www.graphpad.com/, accessed on 18 August 2024). The predicted T-cell and B-cell epitopes of syncytin-1 or syncytin-2 were statistically compared with each other and with those of β2GPI using the two-tailed t-test. Significance was assumed at p-values $<$ 0.05 and a confidence interval of 95%.

Epitope Mapping

To assess the protein localization of the identified epitopes of syncytin-1 and syncytin-2, the UniProt database (https://www.uniprot.org/, accessed on 17 June 2024) was consulted [56]. In particular, we performed two different searches for Q9UQF0 human syncytin-1 and P60508 human syncytin-2 with respect to domains (regions and motifs) that have been characterized for their biological function. The T-cell and B-cell epitopes of the syncytins were mapped based on the amino acid positions within the two proteins and the spanning regions or domains.

Table 1. List of HLA class II alleles that are correlated with the risk of antiphospholipid syndrome (APS) according to the literature review.

HLA allele	Ethnic group	Highest allele frequency (in decimals)	Reference
HLA-DR4	-Anglosaxon	0.20	[22]
	-European	0.23	[19–22]
	-South of Spain	0.16	[18]
	-Caucasian	0.23	[24]
	-British Caucasian	0.20	[14]
	-British	0.20	[16]
	-Hungarian	unknown	[27]
	-Belgian	0.06	[25]
HLA-DR5	-Mexican	0.13	[31]
HLA-DR7	-European	0.24	[20,22]
	-French	0.18	[29]
	-British	0.20	[16,33]
	-British Caucasian	0.20	[17]
	-Caucasian	0.24	[24]
	-Latin	0.10	[22]
	-Belgian	0.11	[25]
HLA-DRw53	-Italian	0.20	[19–22]
	-South of Spain	0.16	[18]
	-Caucasian	0.28	[24]
	-French	unknown	[29]
	-Japanese	unknown	[28,36]
	-Mexican-American	unknown	[23]
	-British	unknown	[33]
*HLA-DRB1*04:03*	-Japanese	0.06	[36]
*HLA-DRB1*04:05*	-Japanese	0.15	[36]
*HLA-DRB1*08:02*	-Japanese	0.10	[36]
*HLA-DRB1*09:01*	-Japanese	0.16	[36]
*HLA-DRB1*11:01*	-Japanese	0.05	[36]
*HLA-DRB1*12:01*	-Mexican	0.01	[31]
*HLA-DRB1*12:02*	-Japanese	0.08	[36]
*HLA-DRB1*13:02*	-Greek	0.04	[26]
	-White population	0.07	[26]
	-African-American	0.08	[26]
	-Mexican-American	0.02	[26]
	-British Caucasian	0.03	[14,17]
	-Black American	0.07	[23]
*HLA-DRB1*14*	-British	0.02	[16]
*HLA-DRB1*15:02*	-Japanese	0.13	[36]
*HLA-DMA*01:02*	-White population	0.15	[15]
*HLA-DQA1*01:02*	-British Caucasian	0.20	[17]
*HLA-DQA1*02:01*	-British Caucasian	0.16	[17]
*HLA-DQA1*03:01*	-Greek	0.07	[26]
	-White population	0.23	[26]
	-African-American	0.17	[26]
	-Mexican-American	0.69	[26]
	-British Caucasian	0.23	[14]
*HLA-DQA1*03:02*	-British Caucasian	0.009	[14]
HLA-DQB1	-American	0.24	[34,35]

Table 1. Continued.

HLA allele	Ethnic group	Highest allele frequency (in decimals)	Reference
*HLA-DQB1*03:01*	-British Caucasian	0.16	[14]
	-Mexican-American	0.55	[23]
	-White American	0.94	[23]
	-Black American	0.17	[26]
*HLA-DQB1*03:02*	-European	0.22	[23]
	-Hungarian	unknown	[32]
	-Mexican-American	0.77	[23]
	-White American	0.11	[23]
*HLA-DQB1*03:03*	-British Caucasian	0.03	[17]
	-Mexican-American	0.06	[23]
	-White American	0.04	[23]
	-Black American	0.01	[23]
	-Japanese	0.18	[36]
*HLA-DQB1*03:04*	-British Caucasian	0.003	[14]
*HLA-DQB1*06:02*	-Hungarian	unknown	[32]
*HLA-DQB1*06:04*	-Greek	0.03	[26]
	-White population	0.07	[26]
	-African-American	0.02	[26]
	-Mexican-American	0.02	[26]
	-British Caucasian	0.03	[14,17]
	-Black American	0.02	[23]
*HLA-DQB1*06:05*	-Black American	0.0004	[23]
	-British Caucasian	0.01	[14,17]
*HLA-DQB1*06:06*	-British Caucasian	unknown	[14,17]
*HLA-DQB1*06:07*	-British Caucasian	unknown	[14,17]
*HLA-DQB1*06:09*	-British Caucasian	0.01	[14,17]
HLA-DQ3	-South of Spain	0.29	[18]
HLA-DQ7	-South of Spain	0.29	[18]
	-Caucasian	0.39	[24]
HLA-DQ8	-Belgian	0.11	[25]

Abbreviation: HLA, human leukocyte antigen.

Results

Predicted Epitopes of Syncytin-1

T-Cell Epitopes

Using TepiTool, we identified a total of 721 epitopes of syncytin-1 that were predicted to bind HLA class II alleles (**Supplementary Material 1** and Table 3). In particular, the epitope FRPYVSIPVPEQWNN was predicted to have the highest binding affinity (percentile rank 0.01) to HLA-DRB1*04:01, HLA-DRB1*04:07, HLA-DRB1*04:16, HLA-DRB1*04:21, HLA-DRB1*04:26, and HLA-DRB1*04:64, all of which are associated with APS in Caucasian individuals. In contrast, the epitope CLPLNFRPYVSIPVP was predicted to bind with the lowest percentile rank value (0.02) to the allele HLA-DRB1*15:02, which is associated with APS in the Japanese population.

Importantly, according to the UniProt database, 140 of these epitopes were located in the InterPro Representative Domain 345-422, which includes the immunosuppressive (ISU) domain at position 380–396 (https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=cd09851) [57]. The InterPro Representative Domain is shared by HERVs and other exogenous retroviruses such as human HTLV-1 and HTLV-2 and includes an N-terminal heptad repeat containing a fusion peptide, a CKS17-like ISU region, a CX6CC motif that forms a disulfide bond, and a C-terminal heptad repeat. We also found 79 T-cell epitopes in the region essential for fusogenic function, 38 in the fusion peptide region, 35 in the CXXC motif having fusogenic properties [58], and seven in the ISU region. These data are undoubtedly important, as neutralization of such domains by autoantibodies could impair the fusogenic and immunosuppressive function of syncytin-1.

Table 2. Panel of HLA class II alleles used for the TepiTool analysis.

Host species	Human
Allele class	Class II
Selected alleles	DQA1*01:02/DQB1*06:02
	DQA1*03:01/DQB1*03:02
	DQA1*04:01/DQB1*04:02
	DQA1*05:01/DQB1*02:01
	DRB1*04:01
	DRB1*04:02
	DRB1*04:03
	DRB1*04:04
	DRB1*04:05
	DRB1*04:06
	DRB1*04:07
	DRB1*04:08
	DRB1*04:09
	DRB1*04:10
	DRB1*04:11
	DRB1*04:13
	DRB1*04:14
	DRB1*04:15
	DRB1*04:16
	DRB1*04:17
	DRB1*04:19
	DRB1*04:21
	DRB1*04:22
	DRB1*04:23
	DRB1*04:24
	DRB1*04:26
	DRB1*04:28
	DRB1*04:29
	DRB1*04:31
	DRB1*04:42
	DRB1*04:64
	DRB1*07:01
	DRB1*07:03
	DRB1*07:04
	DRB1*07:09
	DRB1*08:02
	DRB1*09:01
	DRB1*11:01
	DRB1*11:02
	DRB1*11:03
	DRB1*11:04
	DRB1*11:05
	DRB1*11:06
	DRB1*11:07
	DRB1*11:08
	DRB1*11:09
	DRB1*11:10
	DRB1*11:11
	DRB1*11:13
	DRB1*11:14
	DRB1*11:15
	DRB1*11:16
	DRB1*11:19
	DRB1*11:20
	DRB1*11:21
	DRB1*11:25
	DRB1*11:27
	DRB1*11:29
	DRB1*11:37
	DRB1*11:52
	DRB1*12:01
	DRB1*12:02
	DRB1*12:03
	DRB1*12:05
	DRB1*12:06
	DRB1*13:02
	DRB1*14:01
	DRB1*14:02
	DRB1*14:03
	DRB1*14:04
	DRB1*14:05
	DRB1*14:06
	DRB1*14:07
	DRB1*14:08
	DRB1*14:09
	DRB1*14:10
	DRB1*14:11
	DRB1*14:12
	DRB1*14:13
	DRB1*14:14
	DRB1*15:02
	DRB4*01:03

B-Cell Epitopes

Linear Epitopes. Linear B-cell epitopes are a minority of B-cell epitopes consisting of peptides that can be extrapolated from the amino acid FASTA sequence of a protein of interest [50]. According to the ABCpred server, syncytin-1 was predicted to contain 51 linear B-cell epitopes, of which the epitopes CMHANTHYWTGKMINP, IGGITTSTQFYYKLSQ, and TGMSDGGGGVQDQAREK had a predictive score $\geq$0.90, as shown in Table 4.

When querying the UniProt.org database, we found two linear B-cell epitopes belonging to the region of the fusion peptide and one to the region essential for fusogenic function, while one epitope corresponded to the ISU domain and three to the InterPro Representative Domain 345–422.

Table 3. Epitopes of syncytin-1 predicted to bind to HLA class II alleles with a percentile rank $\leq$ 0.5 according to TepiTool analysis.

Epitope sequence	Percentile rank	HLA allele
FRPYVSIPVPEQWNN	0.01	HLA-DRB1*04:01
		HLA-DRB1*04:07
		HLA-DRB1*04:16
		HLA-DRB1*04:21
		HLA-DRB1*04:26
		HLA-DRB1*04:64
	0.02	HLA-DRB1*04:31
CLPLNFRPYVSIPVP	0.02	HLA-DRB1*15:02
FRPYVSIPVPEQWNN	0.03	HLA-DRB1*04:08
		HLA-DRB1*04:19
	0.1	HLA-DRB1*04:24
	0.11	HLA-DRB1*04:14
	0.12	HLA-DRB1*04:09
TEKVKEIRDRIQRRA	0.14	HLA-DRB1*04:02
DAPSYRSLSKGTPTF	0.14	HLA-DRB1*11:05
FRPYVSIPVPEQWNN	0.16	HLA-DRB1*04:05
		HLA-DRB1*04:28
		HLA-DRB1*04:29
	0.19	HLA-DRB1*08:02
	0.2	HLA-DRB1*04:17
GTPTFTAHTHMPRNC	0.29	HLA-DRB1*11:14
		HLA-DRB1*11:20
		HLA-DRB1*13:02
CLPLNFRPYVSIPVP	0.3	HLA-DRB1*07:09
FRPYVSIPVPEQWNN	0.31	HLA-DRB1*04:03
		HLA-DRB1*04:06
EVISQLTRVHGTSSP	0.32	HLA-DRB1*11:04
DAPSYRSLSKGTPTF	0.34	HLA-DRB1*11:01
		HLA-DRB1*11:09
		HLA-DRB1*11:10
		HLA-DRB1*11:15
		HLA-DRB1*11:29
FRPYVSIPVPEQWNN	0.34	HLA-DRB1*14:09
DAPSYRSLSKGTPTF	0.35	HLA-DRB1*08:02
SYVISKPRNKRVPIL	0.36	HLA-DRB1*11:21
VSSRIEAVKLQMEPK	0.39	HLA-DQA1*03:01/DQB1*03:02
SYVISKPRNKRVPIL	0.39	HLA-DRB1*11:02
EVISQLTRVHGTSSP	0.39	HLA-DRB1*11:06
SYVISKPRNKRVPIL	0.39	HLA-DRB1*11:16
EAVKLQMEPKMQSKT	0.4	HLA-DRB1*14:06
EVISQLTRVHGTSSP	0.42	HLA-DRB1*11:25
VNDIKGTPPEEISAA	0.45	HLA-DRB1*07:01
		HLA-DRB1*07:03
FRPYVSIPVPEQWNN	0.46	HLA-DRB1*14:02
EAVKLQMEPKMQSKT	0.48	HLA-DRB1*14:09
VNDIKGTPPEEISAA	0.5	HLA-DRB1*07:09

Table 4. Predicted linear B-cell epitopes of syncytin-1 according to ABCpred analysis.

Rank	Sequence	Start position	Score
1	CMHANTHYWTGKMINP	79	0.94
2	IGGITTSTQFYYKLSQ	337	0.93
3	TGMSDGGGVQDQAREK	112	0.90
4	DIKGTPPEEISAAQPL	514	0.88
4	RRPLDRPASPRSDVND	499	0.88
4	VKEIRDRIQRRAEELR	418	0.88
4	FVCGTSAYRCLNGSSE	270	0.88
4	CIRWVTPPTQIVCLPS	251	0.88
4	YVSIPVPEQWNNFSTE	197	0.88
4	NCWICLPLNFRPYVSI	185	0.88
5	GKMINPSCPGGLGVTV	89	0.87
5	RSLSKGTPTFTAHTHM	53	0.87
5	PGNIDAPSYRSLSKGT	44	0.87
5	VKEVISQLTRVHGTSS	129	0.87
6	AVKLQMEPKMQSKTKI	482	0.86
6	CWTYFTQTGMSDGGGV	105	0.86
7	SFTLTAPPPCRCMTSS	16	0.85
8	PFVIGAGVLGALGTGI	322	0.84
8	PMTIYTEQDLYSYVIS	296	0.84
9	SKTKIYRRPLDRPASP	493	0.83
9	GVQDQAREKHVKEVIS	119	0.83
10	YHSATLCMHANTHYWT	73	0.82
10	HTHMPRNCYHSATLCM	65	0.82
11	LGEECCYYVNQSGIVT	400	0.81
11	FNTTLTGLHEVSAQNP	168	0.81
11	RVHGTSSPYKGLDLSK	138	0.81
12	TGPWGLLSQWMPWILP	435	0.79
12	RRAEELRNTGPWGLLS	427	0.79
12	AERGGTCLFLGEECCY	391	0.79
12	SYVISKPRNKRVPILP	307	0.79
12	EITHTSNLTCVKFSNT	228	0.79
13	EFLWRMQRPGNIDAPS	36	0.78
13	ERVADSLVTLQDQLNS	359	0.78
13	HETLRTHTRLVSLFNT	155	0.78
14	NTTYTTNSQCIRWVTP	242	0.77
15	TEINTTSVLVGPLVSN	211	0.76
15	HEVSAQNPTNCWICLP	176	0.76
16	PASPRSDVNDIKGTPP	505	0.74
16	YKLSQELNGDMERVAD	348	0.74
16	PCRCMTSSSPYQEFLW	24	0.74
17	TQIVCLPSGIFFVCGT	259	0.73
18	SCPGGLGVTVCWTYFT	95	0.71
18	PEEISAAQPLLRPNSA	520	0.71
19	NGSSESMCFLSFLVPP	281	0.69
20	KRVPILPFVIGAGVLG	316	0.66
21	LLLLFGPCIFNLLVNF	460	0.65
21	MPWILPFLGPLAAIIL	445	0.65
21	MALPYHIFLFTVLLPS	1	0.65
22	PLVSNLEITHTSNLTC	222	0.62
23	NRRALDLLTAERGGTC	382	0.59
24	LVTLQDQLNSLAAVVL	365	0.56

Epitopes are ordered by their score as determined by a trained recurrent neural network. A higher score of the peptide indicates a higher probability that it is an epitope. All epitopes shown are above the selected threshold.

Conformational Epitopes. The results of DiscoTope analysis showed that either chain a or chain b of syncytin-1 contains amino acid residues that can bind to BCRs in both the fusion and post-fusion configurations, with no statistically significant difference between the two 3D structures (**Supplementary Material 2** and Table 5).

Considering the 5HA6 3D structure, chain a was predicted to have 40 B-cell conformational epitopes with a DiscoTope score between 3.421 and –3.69, while chain b was predicted to have 39 epitopes with a DiscoTope score between 3.303 and –3.443. The post-fusion conformation of syncytin-1 (PDB 6RX1) was predicted to contain 35 discontinuous B-cell epitopes in chain a with a DiscoTope score between 2.527 and –3.494. Positive predictions involved amino acids in positions zero and from 343 to 434, which are located in the extracellular domain of syncytin-1 according to the UniProt.org database [56]. No positive predictions were found for amino acids of the transmembrane and cytoplasmic domains, **Supplementary Material 2**. As with the linear T-cell and B-cell epitopes, some of the amino acid residues were part of the InterPro Representative Domain 345-422, the CXXC 186-189 motif, and the CX6CC motif, both of which are critical for the cell-cell fusion process [58].

Predicted Epitopes of Syncytin-2

T-Cell Epitopes

We found a total of 705 epitopes of syncytin-2 predicted to bind HLA class II alleles associated with APS (**Supplementary Material 3** and Table 6). The epitope GTGTGIAGITKASLT was predicted to bind to the HLA-DRB1*14:01, HLA-DRB1*14:04, and HLA-DRB1*14:11 alleles with the highest avidity (percentile rank 0.09–0.16). The highest probability of interaction was calculated for the alleles HLA-DRB1*14:01 and HLA-DRB1*14:04 (percentile rank 0.09), both of which may predispose Caucasian individuals to APS.

The UniProt database for P60508 human syncytin-2 revealed that the T-cell epitopes were localized in the InterPro Representative Domain 345–422 in 182 cases, in the fusion peptide region in 80 cases, and in the CKS-17 motif with immunosuppressive and fusion function [59] in 36 cases. These data are consistent with the results of the syncytin-1 analysis and indicate a possible disruption of essential syncytin-2 functions by anti-syncytin-2 antibodies. However, statistical analysis showed that the syncytin-2 T-

Table 5. Top five conformational epitopes of each syncytin-1 chain in fusion and post-fusion conformation according to DiscoTope analysis.

Protein conformation	Chain ID	Residue ID	Propensity Score	DiscoTope Score
Syncytin-1 5HA6	a	434	3.866	3.421
		430	4.569	3.353
		433	4.116	2.838
		427	3.414	2.332
		426	3.066	1.909
	b	430	4.512	3.303
		433	4.314	3.1
		434	3.389	2.309
		427	3.327	2.254
		431	4.07	1.762
Syncytin-1 6RX1	a	430	3.635	2.527
		433	3.143	1.861
		427	2.743	1.737
		426	2.588	1. 6
		349	1.699	1.274

Table 6. Epitopes of syncytin-2 predicted to bind to HLA class II alleles with a percentile rank $\leq$ 0.5 according to TepiTool analysis.

Epitope sequence	Percentile rank	HLA allele
GTGTGIAGITKASLT	0.09	HLA-DRB1*14:01
		HLA-DRB1*14:04
GTGTGIAGITKASLT	0.16	HLA-DRB1*14:11
THNQFRHQPRFPKPP	0.22	HLA-DRB1*11:14
		HLA-DRB1*11:20
		HLA-DRB1*13:02
PIPIYGNSPLPRVRR	0.27	HLA-DRB1*14:02
	0.28	HLA-DRB1*11:07
GTGTGIAGITKASLT	0.3	HLA-DRB1*11:52
		HLA-DRB1*14:05
		HLA-DRB1*14:08
THNQFRHQPRFPKPP	0.33	HLA-DRB1*11:11
KGLMRPANSLLSTVK	0.44	HLA-DRB1*04:02
KVQDNIRQLLNQASS	0.48	HLA-DRB1*04:42
PIPIYGNSPLPRVRR	0.48	HLA-DRB1*14:06
QIDSLAAVVLQNRRG	0.49	HLA-DQA1*01:02/DQB1*06:02
KVQDNIRQLLNQASS	0.5	HLA-DRB1*04:04
		HLA-DRB1*04:23

cell epitopes had a significantly lower binding affinity to selected HLA alleles than those of syncytin-1 ($p < 0.0001$).

B-Cell Epitopes

Linear Epitopes. The search in the ABCpred server yielded a total of 28 linear epitopes of syncytin-2 (Table 7). As with syncytin-1, three epitopes achieved a score $\geq$0.90; these were QQTYQTYTHNQFRHQP, EWTSIEAELHISYRWD, and YSTNCWLCTSSSTETP.

Three epitopes were located in the InterPro Representative Domain 345–422, two in the fusion peptide region, while one B-linear epitope was predicted for each of the CKS-17, CX6CC, and CXXC motifs. No significant difference was found between the ABCpred scores of syncytin-1 and syncytin-2 ($p > 0.05$).

Conformational Epitopes. Similar to syncytin-1, chains a, b, and c of syncytin-2 were predicted to harbor discontinuous B-cell epitopes (**Supplementary Material 4** and Table 8). However, compared to syncytin-1, the DiscoTope scores resulting from analyzing the fusion and post-fusion configurations of syncytin-2 were significantly lower than

Table 7. Predicted linear B-cell epitopes of syncytin-2 according to ABCpred analysis.

Rank	Sequence	Start position	Score
1	QQTYQTYTHNQFRHQP	155	0.93
2	EWTSIEAELHISYRWD	64	0.91
3	YSTNCWLCTSSSTETP	39	0.90
4	TETPGTAYPASPREWT	51	0.88
4	TIGYVTPDIFIAPGNL	318	0.88
5	DTMAKALTTMQEQIDS	393	0.87
5	PICVMAKRKNGTNVGT	124	0.87
6	CLPSNWTGTCTIGYVT	308	0.86
6	YTHNQFRHQPRFPKPP	161	0.85
7	LVLILTPSLAAYRHPD	6	0.85
7	MQEQIDSLAAVVLQNR	402	0.85
7	TGTGIAGITKASLTYS	367	0.85
7	LQSTGSPYSTNCWLCT	32	0.85
8	GQSIHQCLPSNWTGTC	302	0.85
8	EKCCFWVNQSGKVQDN	436	0.84
8	FGTSLTPLFHFHISTC	276	0.84
8	RNFWFRPADYNQCLQI	203	0.84
8	NLMGIAPICVMAKRKN	118	0.84
9	AGLGILAGTGTGIAGI	359	0.83
10	GTLLDKSSRFCQGRPS	183	0.82
11	SRFCQGRPSSCSTRNF	190	0.81
11	PDIRQKPPIFGPIFTN	101	0.81
12	ELHISYRWDPNLKGLM	71	0.80
12	RATQGWLNWEGTWKWF	465	0.80
12	SKEIANNIDTMAKALT	385	0.80
13	LCTSSSTETPGTAYPA	45	0.79
13	GMTIATSYLGISAVSE	259	0.79
13	YRHPDFPLLEKAQQLL	17	0.79
14	IPIYGNSPLPRVRRAI	337	0.78
14	SLFWENKTKGANQSQT	236	0.78
15	QGGICLALDEKCCFWV	427	0.77
16	LQTNLSAGRHPRNIQE	520	0.76
16	NQSQTPCVQVLAGMTI	247	0.76
16	TNVGTLPSTVCNVTFT	135	0.76
17	TQFVSSRLQAIKLQTN	508	0.75
18	LGISAVSEFFGTSLTP	267	0.74
19	AEWVLLDQTRNSLFWE	225	0.73
20	LLSTVKQDFPDIRQKP	92	0.72
20	VRRAIHFIPLLAGLGI	348	0.72
20	QCLQISNLSSTAEWVL	214	0.72
21	EGTWKWFSWVLPLTGP	474	0.71
21	STVCNVTFTVDSNQQT	142	0.71
22	RWDPNLKGLMRPANSL	77	0.70
22	RHQPRFPKPPNITFPQ	167	0.70
22	FGPIFTNINLMGIAPI	110	0.70
23	QGAFYICGQSIHQCLP	295	0.69
24	NQSGKVQDNIRQLLNQ	443	0.64
25	KGLMRPANSLLSTVKQ	83	0.61
26	VVLQNRRGLDMLTAAQ	412	0.56
27	RQLLNQASSLRERATQ	453	0.54

Table 7. Continued.

Rank	Sequence	Start position	Score
27	PKPPNITFPQGTLLDK	173	0.54
28	RGLDMLTAAQGGICLA	418	0.53

Epitopes are ordered by their score as determined by a trained recurrent neural network. A higher score of the peptide indicates a higher probability that it is an epitope. All epitopes shown are above the selected threshold.

those of syncytin-1 ($p = 0.0001$) and significantly higher for the 1Y4M configuration compared to the 6RX3 post-fusion configuration ($p = 0.01$).

In particular, PDB structure 1Y4M was predicted to harbor seven epitopes in chain a (DiscoTope scores between –0.157 and –2.778), eight epitopes in chain b (DiscoTope scores between –0.629 and –3.429), and eight epitopes in chain c (DiscoTope scores between –0.233 and –3.437). On the other hand, the PDB 6RX3 crystal conformation of syncytin-2 harbored 36 epitopes in chain a (DiscoTope scores between –0.372 and –3.681), 37 in chain b (DiscoTope scores between 0.185 and –3.471), and 29 in chain c (DiscoTope scores between –0.374 and –3.641). In syncytin-2, the amino acid residues predicted to be immunogenic by DiscoTope analysis were also located in the extracellular part of the protein (**Supplementary Material 4**), particularly at positions 46–52 in the prefusion configuration and between amino acid residues 369 and 468 in the post-fusion configuration. Involved residues were identified in the InterPro Representative Domain 345–422 as well as in ISU and fusogenic motifs CXXC, CKS-17, and CX6CC. The analysis also revealed epitopes within the fusogenic peptide. As with syncytin-1, the neutralization of such domains by autoantibodies may therefore promote inflammation and additionally impair the formation of cytotrophoblast.

Predicted Epitopes of β2GPI and Comparison with Syncytins

T-Cell Epitopes

Next, using the same prediction tools, we analyzed the T-cell epitopes potentially harbored by the entire β2GPI and by each of its five domains to compare the results obtained with those of the analyses of syncytin-1 and syncytin-2. The TepiTool search revealed a total of 335 T-cell epitopes in the whole β2GPI, 41 T-cell epitopes in β2GPI D1, 10 T-cell epitopes in β2GPI D2, 24 T-cell epitopes in β2GPI D3, 41 T-cell epitopes in β2GPI D4 and 190 T-cell epitopes in β2GPI D5 (**Supplementary Material 5**). In 28 cases, the epitopes were located in two contiguous domains, namely in 19 cases between D1 and D2 and in nine cases between D4 and D5. Interestingly, the highest probability of interaction was predicted for epitopes within β2GPI D5, followed by β2GPI D1 and D4. Specifically, the epitope HSSLAFWKTDASDVK in D5 was predicted to bind to HLA-

Table 8. Top five conformational epitopes of each syncytin-2 chain in the 1Y4M and post-fusion conformation according to DiscoTope analysis.

Protein conformation	Chain ID	Residue ID	Propensity Score	Discotope Score
Syncytin-2 1Y4M	a	46	–0.177	–0.157
		47	–0.548	–0.485
		48	–0.885	–1.243
		49	–1.267	–1.236
		50	–1.606	–1.651
	b	48	–0.711	–0.629
		47	–0.954	–0.844
		46	–0.599	–0.99
		49	–1.197	–1.174
		51	–1.812	–1.834
	c	46	–0.263	–0.233
		49	–0.912	–0.807
		47	–0.637	–0.909
		50	–1.601	–1.417
		48	–0.709	–1.547
Syncytin-2 6RX3	a	453	0.359	–0.372
		454	0.372	–0.475
		450	–0.033	–0.604
		468	–0.277	–0.821
		464	–0.267	–0.926
	b	454	0.989	0.185
		453	0.83	0.045
		380	–0.199	–0.176
		464	0.386	–0.348
		450	–0.008	–0.582
	c	453	0.357	–0.374
		454	0.172	–0.538
		450	–0.014	–0.587
		369	–1.528	–1.352
		457	–0.778	–1.378

DRB1*04:02 and HLA-DQA1*04:01/DQB1*04:02 with a percentile rank of 0.14 and 0.19, respectively. Conversely, epitopes within D1 had higher percentile rank scores (2.6–9.9) and thus a lower predicted binding affinity to the HLA selected in our analysis.

None of the epitopes in β2GPI, considered as a whole protein or split into domains, showed homologies to epitopes of syncytin-1 or syncytin-2. Although in many cases the same HLA alleles or haplotypes were recognized, the degree of molecular affinity was highly variable and significantly greater for epitopes of syncytins compared to β2GPI, Table 9. In detail, the mean $\pm$ standard deviation (SD) of the percentile rank of T-cell epitopes predicted to bind to the selected HLA alleles was 0.89 $\pm$ 1.0 for syncytin-1, 1.74 $\pm$ 1.5 for syncytin-2, and 2.96 $\pm$ 2.0 for β2GPI. When compared to β2GPI, the *t*-test for paired samples showed a higher binding prediction for both syncytin-1 and syncytin-2 ($p < 0.0001$).

Linear and Conformational B-Cell Epitopes

The ABCpred server analysis predicted 27 linear B-cell epitopes of β2GPI, most of which were contained in β2GPI D1, Table 10. No significant differences were found between syncytin-1 and β2GPI ABCpred scores ($p > 0.05$). On the contrary, the linear B-cell epitopes of β2GPI showed significantly higher BCR-binding affinity compared to those of syncytin-2 ($p = 0.04$).

DiscoTope analysis positively predicted eight conformational epitopes for the β2GPI crystal structure 6XST, whose amino acid residues were mainly located in D4, followed by D1 and D5, **Supplementary Material 6**. No positive predictions were found in D2 and D3 of the protein. The DiscoTope scores were between –1.638 and –3.569 and thus overall lower than those found in the same analysis with the crystal structures of the syncytins. Indeed, in statistical comparison with 6XST, significantly higher DiscoTope scores were found for syncytin-1 in both the 5HA6

Table 9. Comparison between the lowest percentile rank of the T-cell epitopes of syncytin-1, syncytin-2, and β2GPI binding to the same HLA alleles.

HLA allele	syncytin-1 (percentile rank)	syncytin-2 (percentile rank)	β2GPI (percentile rank)
HLA-DQA1*01:02/DQB1*06:02	0.51	0.49	0.88
HLA-DQA1*03:01/DQB1*03:02	0.39	3.3	8.8
HLA-DQA1*04:01/DQB1*04:02	0.84	0.99	0.19
HLA-DQA1*05:01/DQB1*02:01	1.4	4.4	0.67
HLA-DRB1*04:01	0.01	1.4	2.9
HLA-DRB1*04:02	0.14	0.44	0.14
HLA-DRB1*04:03	0.31	0.9	0.91
HLA-DRB1*04:04	1.1	0.5	1.0
HLA-DRB1*04:05	0.16	5.9	6.5
HLA-DRB1*04:06	0.31	0.9	0.91
HLA-DRB1*04:07	0.01	1.2	1.8
HLA-DRB1*04:08	0.03	1.8	2.5
HLA-DRB1*04:09	0.12	2.2	6.6
HLA-DRB1*04:10	1.7	2.3	3.0
HLA-DRB1*04:11	0.9	3.6	3.3
HLA-DRB1*04:13	0.82	0.82	1.1
HLA-DRB1*04:14	0.11	0.65	0.65
HLA-DRB1*04:15	0.81	0.65	0.42
HLA-DRB1*04:16	0.01	1.4	2.9
HLA-DRB1*04:17	0.2	5.2	6.7
HLA-DRB1*04:19	0.03	1.8	2.5
HLA-DRB1*04:21	0.01	1.4	2.9
HLA-DRB1*04:22	1.1	0.53	2.3
HLA-DRB1*04:23	1.1	0.5	1.0
HLA-DRB1*04:24	0.1	3.8	6.0
HLA-DRB1*04:26	0.01	1.4	2.9
HLA-DRB1*04:28	0.16	6.0	5.8
HLA-DRB1*04:29	0.16	5.9	6.5
HLA-DRB1*04:31	0.02	1.5	2.3
HLA-DRB1*04:42	1.3	0.48	0.95
HLA-DRB1*04:64	0.01	1.5	2.0
HLA-DRB1*07:01	0.45	3.9	0.48
HLA-DRB1*07:03	0.45	3.9	0.48
HLA-DRB1*07:04	0.53	3.3	0.95
HLA-DRB1*07:09	0.3	4.3	0.78
HLA-DRB1*08:02	0.19	1.1	4.8
HLA-DRB1*09:01	0.82	1.8	3.1
HLA-DRB1*11:01	0.34	0.65	3.3
HLA-DRB1*11:02	0.39	2.3	1.4
HLA-DRB1*11:03	1.3	3.2	1.2
HLA-DRB1*11:04	0.32	2.1	1.3
HLA-DRB1*11:05	0.14	0.61	4.2
HLA-DRB1*11:06	0.39	2.0	1.3
HLA-DRB1*11:07	0.8	0.28	2.6
HLA-DRB1*11:08	0.63	0.72	4.0
HLA-DRB1*11:09	0.34	0.65	3.3
HLA-DRB1*11:10	0.34	0.65	3.3
HLA-DRB1*11:11	1.6	0.33	3.1
HLA-DRB1*11:13	1.1	0.67	1.3
HLA-DRB1*11:14	0.29	0.22	3.2
HLA-DRB1*11:15	0.34	0.65	3.3
HLA-DRB1*11:16	0.39	2.3	1.4

Table 9. Continued.

HLA allele	syncytin-1 (percentile rank)	syncytin-2 (percentile rank)	β2GPI (percentile rank)
HLA-DRB1*11:19	0.64	0.64	4.4
HLA-DRB1*11:20	0.29	0.22	3.2
HLA-DRB1*11:21	0.36	1.8	1.5
HLA-DRB1*11:25	0.42	2.4	1.5
HLA-DRB1*11:27	0.64	1.1	3.2
HLA-DRB1*11:29	0.34	0.65	3.3
HLA-DRB1*11:37	0.51	0.81	3.5
HLA-DRB1*11:52	2.2	0.3	3.3
HLA-DRB1*12:01	3.5	4.1	6.1
HLA-DRB1*12:02	2.4	3.3	6.4
HLA-DRB1*12:03	3.6	4.4	7.6
HLA-DRB1*12:05	3.5	4.1	6.1
HLA-DRB1*12:06	3.5	4.1	6.1
HLA-DRB1*13:02	0.29	0.22	3.2
HLA-DRB1*14:01	1.6	0.09	2.6
HLA-DRB1*14:02	0.46	0.27	3.3
HLA-DRB1*14:03	4.9	1.2	4.8
HLA-DRB1*14:04	0.98	0.09	3.8
HLA-DRB1*14:05	2.2	0.3	3.3
HLA-DRB1*14:06	0.4	0.48	0.58
HLA-DRB1*14:07	2.6	0.61	4.5
HLA-DRB1*14:08	2.2	0.3	3.3
HLA-DRB1*14:09	0.34	1.4	4.1
HLA-DRB1*14:10	1.3	1.7	1.3
HLA-DRB1*14:11	1.9	0.16	3.2
HLA-DRB1*14:12	1.1	2.1	1.1
HLA-DRB1*14:13	0.56	1.8	3.4
HLA-DRB1*14:14	2.2	2.0	7.7
HLA-DRB1*15:02	0.02	1.2	0.34
HLA-DRB4*01:03	3.0	1.8	0.68

Abbreviation: β2GPI, β2-glycoprotein I.

and 6RX1 conformations ($p < 0.0001$) and for syncytin-2 ($p = 0.004$ for the 1Y4M and $p = 0.02$ for the 6RX3 conformation).

Discussion

The results of this computational analysis suggest that either syncytin-1 or syncytin-2 may contain T-cell and B-cell epitopes that could potentially trigger autoimmunity in individuals genetically predisposed to APS. T and B cells can indeed recognize different epitopes (e.g., linear vs. conformational), provided they belong to the same antigen, and trigger a synergistic immune response [60]. According to these data, it could be hypothesized that abnormally expressed syncytins in the placenta or other organs are taken up by dendritic cells, processed and passed on to naïve T lymphocytes as epitopes in combination with specific polymorphic HLA class II molecules. This process may be enhanced by the simultaneous recognition of conformational epitopes of syncytin-1 and syncytin-2 by B lymphocytes via the BCRs, with subsequent digestion and presentation of the epitopes to preactivated T cells in the docking site of MHC class II molecules [60]. This event would lead to the production of autoantibodies via a cognate T-B-cell interaction, Fig. 1. Indeed, it is clear that B lymphocytes not only produce antibodies but can also act as antigen-presenting cells and regulators of the immune response. B cells could therefore play an important role in this scenario, as they may be able to capture overexpressed syncytin-1 and syncytin-2, process them into epitopes, and present them to T effector lymphocytes via specific HLA class II alleles that could favor the survival and activation of autoreactive clones.

As previously mentioned, autoimmune diseases share a common feature: the breakdown of immune tolerance, which results in chronic activation of the immune system against autoepitopes that are typically tolerated by the organism. The underlying reasons for this phenomenon are not yet fully understood; however, it is clear that genetic factors play a fundamental role, as evidenced by the familial clustering of these diseases. Polymorphic HLA variants are crucially involved in the predisposition to autoimmunity, as they can bind self and foreign epitopes, enabling recognition by the T-cell receptor (TCR). Under physio-

Table 10. Predicted linear B-cell epitopes of β2GPI according to ABCpred analysis.

Rank	Sequence	Start position	Domain	Score
1	HGNWTKLPECREVKCP	191	3–4	0.96
2	YTTFEYPNTISFSCNT	97	2	0.95
3	DGTIEVPKCFKEHSSL	317	5	0.94
4	PEEIECTKLGNWSAMP	243	4	0.93
5	NDTITCTTHGNWTKLP	183	3	0.91
6	HVAIAGRTCPKPDDLP	15	1	0.90
7	EVKCPFPSRPDNGFVN	202	4	0.89
8	KPGYVSRGGMRKFICP	52	1	0.88
9	NYPAKPTLYYKDKATF	217	4	0.86
10	GEEITYSCKPGYVSRG	44	1	0.85
10	CAPIICPPPSIPTFAT	137	2–3	0.85
10	KWSPELPVCAPIICPP	129	2–3	0.85
11	PPSIPTFATLRVYKPS	144	3	0.84
11	NGADSAKCTEEGKWSP	117	2	0.84
12	MRKFICPLTGLWPINT	61	1	0.83
12	VVPLKTFYEPGEEITY	34	1	0.83
12	LGNWSAMPSCKASCKV	251	4–5	0.83
12	AVFECLPQHAMFGNDT	170	3	0.83
12	KCTEEGKWSPELPVCA	123	2	0.83
12	ISFSCNTGFYLNGADS	106	2	0.83
13	NKEKKCSYTEDAQCID	302	5	0.82
14	CHDGYSLDGPEEIECT	234	4	0.81
15	PSCKASCKVPVKKATV	258	4–5	0.80
16	RVKIQEKFKNGMLHGD	279	5	0.79
16	KATVVYQGERVKIQEK	270	5	0.79
17	MLHGDKVSFFCKNKEK	290	5	0.78
18	TLRVYKPSAGNNSLYR	152	3	0.77
19	ENGAVRYTTFEYPNTI	91	2	0.76
20	RTCPKPDDLPFSTVVP	21	1	0.74
21	CFKEHSSLAFWKTDAS	325	5	0.73
22	TLKCTPRVCPFAGILE	76	1–2	0.72
23	GLWPINTLKCTPRVCP	70	1–2	0.68
24	PSAGNNSLYRDTAVFE	158	3	0.66
25	LFSSFLCHVAIAGRTC	8	1	0.65
26	DDLPFSTVVPLKTFYE	27	1	0.58

Epitopes are ordered by their score as determined by a trained recurrent neural network. A higher score of the peptide indicates a higher probability that it is an epitope. All epitopes shown are above the selected threshold.

logical conditions, T cells that recognize self-epitopes presented by HLA class I and II molecules with high affinity are eliminated or suppressed, preventing the induction of autoimmunity and maintaining surveillance against foreign non-self-antigens [61]. However, individuals with polymorphic HLA variants may exhibit increased avidity for self-antigens, leading to the activation of autoreactive T cells and the development of autoimmune diseases. For instance, the allele HLA-B27 predisposes to ankylosing spondylitis, and the HLA-DRB1*01 and HLA-DRB1*04 alleles are associated with RA. Additionally, the HLA-DR3 and HLA-DR8 alleles are found more frequently in patients with SLE, and the HLA-DQ2 and HLA-DQ8 alleles predispose to celiac disease [61,62].

Regarding APS, various studies have demonstrated an association between several HLA class II alleles and the risk of developing the disease [14–36]. Importantly, scientific data indicate that HLA polymorphisms appear to predispose only to the risk of aPL formation, suggesting that other gene variants may be required to determine the clinical manifestations of APS [20]. Although the presence of aPLs without clinical manifestations is relatively common in the general population [38], APS remains a relatively rare autoimmune disease [63].

The pathophysiological mechanism underlying APS consists of a complex immunothrombotic scenario characterized by several pathogenic events [9,11,64]. aPLs, particularly anti-β2GPI antibodies, can disrupt the anticoagu-

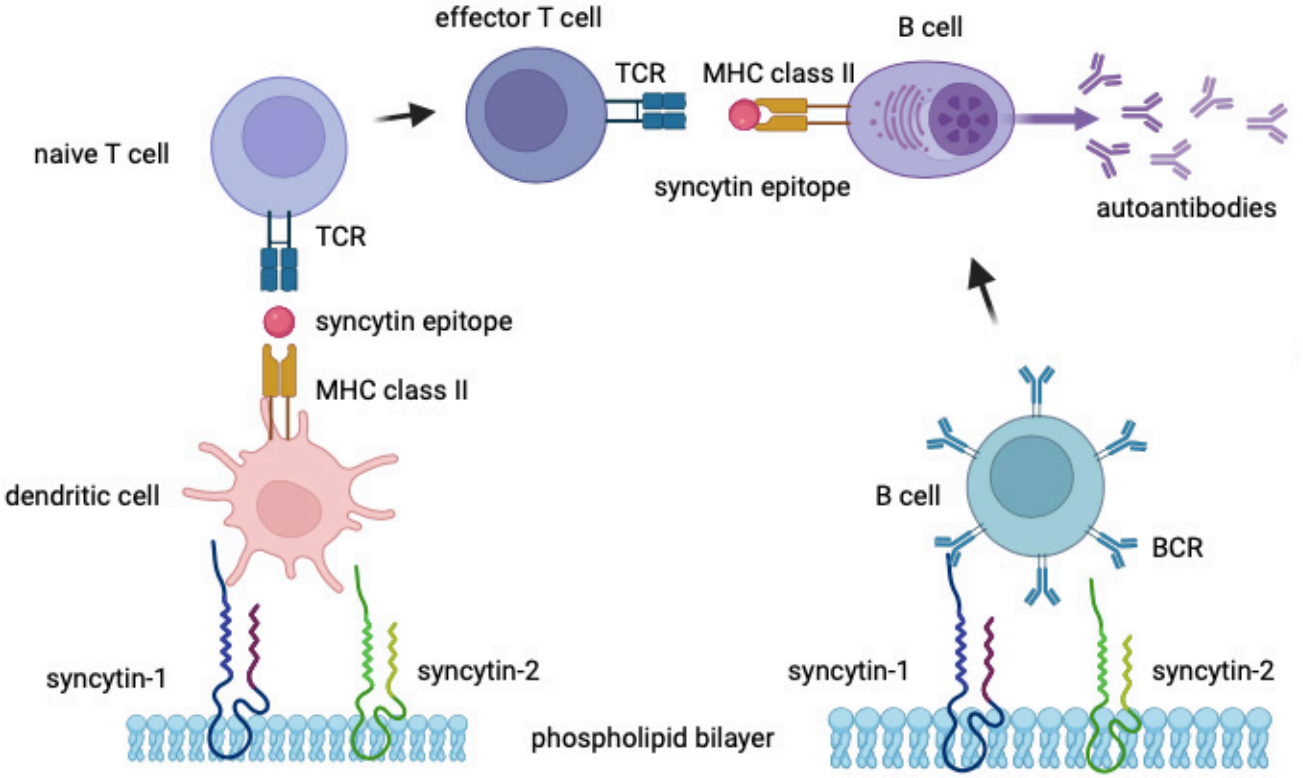

Fig. 1. Hypothetical scenario illustrating syncytin-1 and syncytin-2 as potential autoantigens in antiphospholipid syndrome (APS). Syncytin-1 and syncytin-2 harbor several T-cell and B-cell epitopes that can trigger the activation of T-cell and B-cell responses, ultimately leading to the formation of autoantibodies. When these proteins are overexpressed under certain circumstances such as pregnancy or infection, they can be taken up by dendritic cells and processed into epitopes that are presented to naïve T cells through polymorphic MHC class II molecules. This process would activate CD4+ T lymphocytes. At the same time, B lymphocytes may encounter linear or conformational epitopes of syncytin-1 and syncytin-2. After binding to the BCR, syncytins can be internalized and processed into epitopes that can be presented by B lymphocytes to effector CD4+ T cells within the docking site of MHC class II molecules. This mechanism can enable the B cell to enter a plasma cell stage and eventually produce anti-syncytin autoantibodies. Abbreviations: BCR, B-cell receptor; MHC, major histocompatibility complex; TCR, T-cell receptor. The figure was created with https://www.biorender.com/.

lant cascade by inhibiting the functions of protein C, protein S, and protein Z, leading to decreased production of factor X and increased levels of thrombin and plasminogen inhibitor type 1 [64]. Direct binding to endothelial cells and monocytes can trigger the release of tissue factor and endothelin-1, as well as the expression of phosphatilylserine and annexin A2. Once bound to the endothelium, anti-β2GPI antibodies can further interact with Toll-like receptor 4 (TLR4), initiating a pro-coagulant and pro-inflammatory signaling pathway. Additionally, anti-β2GPI antibodies can directly activate platelets, promoting thromboxane production, adhesion and aggregation, and thrombocytopenia, which occurs in one-third of patients [9,38]. Furthermore, evidence suggests that the complement system plays a crucial role in the pathogenesis of APS thrombosis, as no aPL-mediated effects are observed in experiments on animal models where the complement cascade is blocked [11].

Pregnancy complications can be explained on the basis of direct aPL-mediated functional damage to placental tissue and activation of a pro-inflammatory cascade with the help of complement. The overexpression of anionic phospholipids and β2GPI on the trophoblast that occurs during placentation can trigger the binding of aPLs, particularly in a permissive vascular and hormonal environment [64]. aPLs can block annexin A5, an anticoagulant protein that indirectly inhibits the thromboplastin-specific complex, thereby making the phospholipid bilayer more susceptible to clot formation [38]. Additionally, aPLs can decrease trophoblast cell differentiation and invasiveness. The activation of the complement cascade and the recruitment of neutrophils to the placenta, which release neutrophil extracellular traps (NETs), can lead to inflammatory damage, ultimately resulting in fetal growth restriction and abortion [11,64].

Since aPLs can also be present in asymptomatic carriers [10,38,65], the occurrence of thrombotic events in only some cases has been explained by the “double hit” hypothesis [66,67]: the presence of aPLs represents the “first hit” that determines a pro-coagulant state, while the “second

hit"—such as infection, trauma, or surgery—would determine the activation of coagulation and trigger inflammatory pathways that eventually lead to clinical manifestations. This hypothesis has been recently discussed, as obstetric APS, APS associated with other diseases such as SLE, or catastrophic APS (CAPS) may rely on additional pathogenic mechanisms [11,64,68]. Indeed, viral diseases, including the recent coronavirus disease-19 (COVID-19) and acquired immunodeficiency syndrome (AIDS), may trigger the production of aPLs and induce a pro-coagulant state, although the pathogenicity of aPLs in these contexts may not be entirely equivalent to that of aPLs found in primary APS [12,65,67].

Reactivation of HERVs is a common tract of autoimmunity and has been observed in SLE [69,70], pregnancy [1,71], and infections [72], all of which can trigger or promote APS.

The env products of HERV-W and HERV-FRD, known as syncytins, are overexpressed during placentation; however, evidence suggests that constant expression can occur in various human tissues, including kidney, spleen, and bone marrow [73]. Syncytin-1 is a 73-kDa glycoprotein of 538 amino acids, produced by a defective HERV-W env that was acquired from primates approximately 25 million years ago [1]. By binding to specific receptors, syncytin-1 induces the formation of pseudopodia on the plasma membrane and alters the phospholipid composition, promoting fusion between two trophoblast cells [74]. This mechanism favors the formation of the multinucleated syncytiotrophoblast. Due to its location, the abundant production of syncytin-1 has been shown to play an important role in immunosuppression and tolerance. Syncytin-2, on the other hand, is an env protein produced by HERV-FRD, which was integrated into the human genome more than 40 million years ago [1]. Like syncytin-1, syncytin-2 possesses fusogenic properties that facilitate the transition from cytotrophoblast to syncytiotrophoblast. However, it appears to have greater immunomodulatory potential than syncytin-1, as it suppresses the production of pro-inflammatory T helper (Th)1 cytokines (e.g., tumor necrosis factor-α, interferon-γ, and interleukin-2) to prevent cytotoxic processes that could be harmful to the fetus [75]. Both normal expression and function of syncytin-1 and syncytin-2 are essential for successful human pregnancy. Studies indicate that syncytin expression may be deregulated in the placenta or trophoblastic tissue of women with preeclampsia, hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome, or spontaneous abortions due to abnormal karyotypes [71,76,77]. Similarly, aberrant distribution of syncytin between basal and apical syncytiotrophoblasts may affect placental villus differentiation and syncytiotrophoblast formation [71]. Changes in syncytin expression may be related to defective DNA methylation, impaired placental perfusion, and hypoxia [71,77].

Given that obstetric complications account for 6% of APS morbidity [38], it could be hypothesized that antibodies directed against syncytin-1 and syncytin-2 epitopes could impair their physiological function and contribute to pregnancy morbidity. Notably, a recent study found high serum levels of anti-syncytin-1 antibodies in two of 27 SLE patients, while none were detected in healthy participants [78]. Furthermore, several studies have reported that abnormal expression of syncytin-1 may be associated with certain autoimmune diseases, particularly MS. One study demonstrated that syncytin-1 levels increase in monocytes during both MS relapses and acute infections, and that *in vitro* ligation of syncytin-1 can promote monocyte activation by up-regulating CD80 [40]. Additionally, there is evidence that patients with MS can produce aPLs, such as aCL antibodies or antibodies against other phospholipids of myelin [79]. It could therefore be hypothesized that some of the aPLs found in MS patients are actually formed in response to an overproduction of syncytin-1. Conversely, the pathogenic role of syncytin-2 in autoimmune diseases remains unclear.

Our computational results indicate that syncytin-1 has a higher immunogenic potential compared to syncytin-2 and β2GPI in terms of the overall number of epitopes with the best TepiTool percentile rank and DiscoTope scores. In contrast, the linear B-cell epitope predictions between syncytin-1 and syncytin-2, as well as between syncytin-1 and β2GPI were characterized by statistically non-significant differences. It is important to emphasize that linear B-cell epitopes constitute approximately 10% of the total B-cell epitopes and are primarily found in denatured antigens rather than in natural antigens [80].

Regarding T-cell epitopes, our analysis revealed that the alleles HLA-DRB1*04:01 and HLA-DRB1*15:02 had the highest predictive scores for binding to syncytin-1 epitopes. The allele HLA-DRB1*04:01 has been widely reported in the literature as one of the best candidate genes for predisposition to autoimmunity in general and APS specifically [14,16,18–22,24,25,27]. Its frequency in the Caucasian population is estimated at 7.82%, compared to 2.28% in Japan [81]. Conversely, the allele HLA-DRB1*15:02, which was predicted to recognize a different epitope of syncytin-1 with a lower percentile rank, is highly prevalent in the Japanese population compared to Caucasians (allele frequency of 22.89% vs. 1.86%) [81]. In contrast, the allele HLA-DRB1*14:01 was identified as the best predicted allele for binding to syncytin-2 epitopes. The frequency of this allele is similar in Caucasian and Oriental populations (4.83% vs. 5.10%) [81], suggesting that the same epitope may be equally immunogenic across various ethnic groups. These findings are significant for the potential use of such epitopes in the development of targeted therapies for APS in different populations.

Our analysis also demonstrated that the linear and conformational T-cell and B-cell epitopes of syncytin-1 and syncytin-2 are primarily located within the InterPro Representative Domain, as well as in ISU motifs and other regions responsible for syncytium formation, as illustrated in Figs. 2,3. The InterPro Representative Domain is a con-

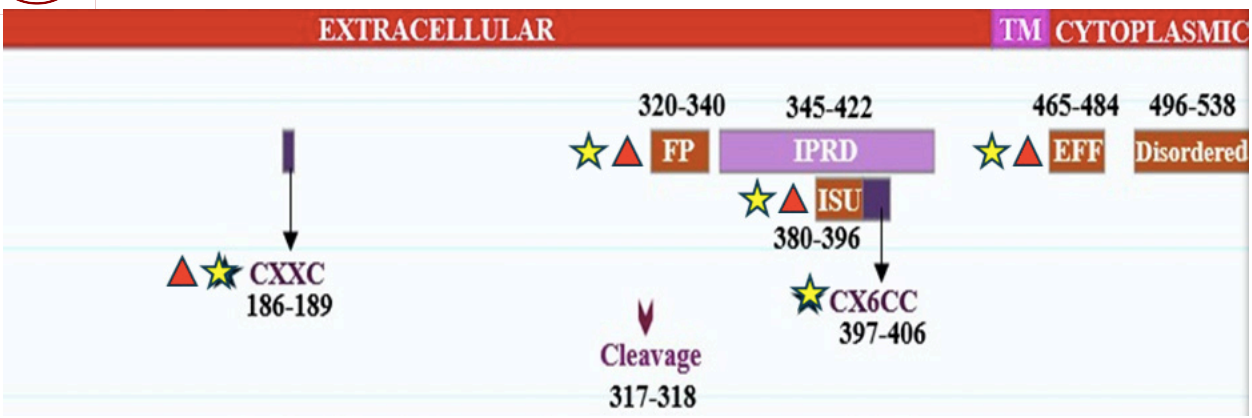

Fig. 2. T-cell (red triangle) and B-cell (yellow star) epitope mapping to regions and motifs of syncytin-1. Protein features were obtained from UniProt.org. Abbreviations: TM, transmembrane; FP, fusion peptide; IPRD, InterPro Representative Domain; ISU, immunosuppressive; EFF, essential for fusogenic function.

Fig. 3. T-cell (red triangle) and B-cell (yellow star) epitope mapping to regions and motifs of syncytin-2. Protein features were obtained from UniProt.org. Abbreviations: TM, transmembrane; CYT, cytoplasmic; FP, fusion peptide; IPRD, InterPro Representative Domain.

served sequence shared by HERVs and exogenous retroviruses. Therefore, it is likely that exogenous retroviral infections may lead to molecular mimicry and cross-reactivity phenomena, potentially resulting in an anti-syncytin immune response. The subsequent formation and binding of antibodies against the amino acid residues of such HERVs could impair the immunosuppressive and fusogenic functions of syncytin-1 and syncytin-2, thereby contributing to the amplification of the immunologic cascade and placental abnormalities.

This study revealed no molecular similarity between the epitopes predicted in syncytins and those of β2GPI, which is the major antigenic target in APS. However, the epitopes of the syncytins or of β2GPI may be presented by the same HLA alleles or haplotypes, albeit with varying degrees of avidity. Interestingly, TepiTool analysis indicated higher avidity scores for syncytins, particularly for syncytin-1, while β2GPI was predicted to have a lower number of T-cell epitopes, which also exhibited lower avidity scores for HLA class II binding. Importantly, most of the predicted T-cell epitopes were localized in D5. Similarly, DiscoTope analysis predicted a lower number of conformational B-cell epitopes from β2GPI, which were primarily located in D4. Conversely, ABCpred searches revealed that β2GPI B-linear epitopes were mainly found in D1 and had comparable predictive scores to syncytin-1, but higher avidity scores than syncytin-2. These data suggest that T-dependent aPLs may recognize more cryptic epitopes in domains of β2GPI other than D1. This perspective aligns with the findings of Hattori *et al.* [36], who observed that the reduced form of β2GPI, but not the native form, triggered a proliferative response of T cells to a major cryptic antigenic determinant in D5. The authors concluded that events altering the normal configuration of β2GPI, including its combination with endogenous or exogenous proteins on the cell membrane, may generate autoepitopes that escape immune surveillance. In this context, the aggregation of β2GPI with syncytins may generate neoepitopes that could induce an autoreactive T-cell response. Indeed, several studies indicate that antibodies against D4 or D5 of β2GPI can occur in healthy carriers or in the context of other infectious or non-APS-mediated diseases, and are not associated with thrombosis or obstetric complications [10,11]. Conversely, linear B-cell domains on β2GPI appear to have a similar or higher

avidity for BCRs than syncytins. Therefore, β2GPI could contain various T and B-cell epitopes, with the B-cell epitopes being more pathogenic concerning the clinical complications of APS.

However, this research has several limitations, the most significant of which is its computational nature. Indeed, computational predictions do not account for changes in protein conformation, charge, pH, or binding to proteins other than antibodies [82]. Regarding the prediction of T-cell epitopes, the TepiTool calculation method relies on the IEDB database, which is continuously updated with new experimental data from humans and other animal species [42]. This means that new epitopes binding to the same HLA alleles could be predicted in the future. Our analysis intentionally focused on potential T-cell epitopes that bind to HLA class II alleles already described in the literature for known associations with APS or aPL positivity in different ethnic groups. However, we acknowledge that the selected alleles may not represent the entire polymorphic HLA landscape across different populations. Moreover, APS is a rare disease, and its epidemiology in various ethnic groups is challenging to clarify due to inconsistent methods or population sampling [63]. Furthermore, our search for T-cell epitopes was limited to the panel of human HLA class II alleles currently available in the TepiTool database. Associated with this methodological problem is the impossibility to study the epitope binding affinity of other HLA alleles considered predisposing or protective for APS, such as HLA-G [83]. Our analysis indicates that the allele HLA-DRB1*11:01, regarded as protective against anti-β2GPI antibody formation in Caucasian, African-American, and Mexican-American subjects [26], is instead pathogenically associated with the anti-β2GPI immune response in studies involving Japanese individuals [36]. This allele may interact with several epitopes of syncytin-1 (lowest percentile rank 0.34), syncytin-2 (lowest percentile rank 1.8), and β2GPI (lowest percentile rank 3.3). These data seem to confirm the relatively low binding affinity of β2GPI to an example of an APS-protective HLA allele, while syncytin-1 and syncytin-2 exhibit different behaviors.

In silico prediction of B-cell epitopes is further complicated by the folding mechanisms and conformational structures of the proteins [82]. The methods employed for predicting B-cell epitopes are based on propensity scales and deep neural network analyses, which are characterized by overall modest accuracy and sensitivity. These methods may also be affected by challenges in mapping interactions between paratopes and epitopes, as well as the structural flexibility of the epitopes [50]. Consequently, future experimental studies utilizing peptide microarrays, phage display libraries, ELISA, flow cytometry, or functional T-cell assays are required to confirm whether syncytin-1 and syncytin-2 epitopes are indeed immunogenic in human samples [50].

The absence of *in vitro* experiments to characterize the immunogenic properties of the identified epitopes and the immunological pathways potentially involved, including mechanisms of peripheral tolerance, cross-reactivity, and epitope spreading, represents another significant limitation. Regarding the first point, it is important to emphasize that part of the linear and conformational epitopes of syncytin-1 and syncytin-2 identified in our analysis involve the immunosuppressive domain, which can physiologically shift the immune response from Th1 to Th2 lymphocytes and from pro-inflammatory M1 to anti-inflammatory M2 macrophages [1,84]. The notion that autoantibodies may disrupt the function of the ISU domains of syncytins is noteworthy in the context of APS, as it could explain phenomena such as the loss of maternal-fetal tolerance. However, it is also known that HLA class II molecules on B cells can induce peripheral T-cell tolerance to self-epitopes, potentially silencing the immune response to syncytins when their epitopes are presented by B lymphocytes [85].

Moreover, it has been observed that syncytin-1 can exhibit molecular mimicry with other human and viral proteins [78,86]. However, it remains unknown whether the epitopes identified in our analysis can cross-react with those of other self or foreign proteins. Importantly, our study did not detect any molecular similarity between the epitopes of syncytins and β2GPI.

Finally, although there is some evidence for the expression of HERV env in human endothelium, particularly under inflammatory conditions [87], the presence and biological role of syncytins in vascular tissue require further investigation, as does their potential involvement in the immunopathogenic scenarios of primary APS, secondary APS and CAPS.

All these points should be considered in the future research agenda, which should include *in vitro* or *ex vivo* experiments aimed at detecting anti-syncytin antibodies in APS, assessing their correlation with disease severity, and evaluating their biological role in living cells or systems.

Conclusions

This computational study suggests that both syncytin-1 and syncytin-2 appear to contain linear and discontinuous epitopes that could be recognized by HLA class II molecules known to predispose individuals to APS, as well as by BCRs. Our silico analyses indicated that syncytins possess greater immunogenic potential compared to β2GPI in terms of the number of T-cell and B-cell conformational epitopes and the overall degree of binding affinity. Many of the identified epitopes were mapped to the InterPro Representative Domain, the ISU, and other fusogenic motifs. Therefore, the hypothetical formation of anti-syncytin antibodies in APS could adversely affect placentation or alter the immunological balance in favor of autoreactive cells. Although these results may illuminate the possible role of HERVs in the development of APS, they warrant validation

in further laboratory studies. Indeed, demonstrating the immunogenicity of these epitopes could pave the way for future research aimed at developing targeted immunotherapies for APS.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

Conceptualization, RT; methodology, RT; software, RT; validation, RT; formal analysis, RT; investigation, LDP and RT; data curation, RT and LDP; writing—original draft preparation, LDP; writing—review and editing, RT; supervision and critical revision of the article, RT. Both authors have read and agreed to the published version of the manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202436189.195.

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