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Evolutionary dynamics of HRSV following the implementation of nirsevimab immunoprophylaxis in Catalonia (2023-2024)

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SUMMARY

Objectives: To evaluate the effect of the selective pressure exerted by nirsevimab on human respiratory syncytial virus (HRSV) in Catalonia (2023–2024) by analysing viral mutations, diversity, and evolutionary dynamics, based on viruses characterised from non-immunised and previously immunised patients.
Methods: Respiratory samples were collected through the SIVIC sentinel network and three hospitals in Catalonia. HRSV-positive samples underwent whole-genome sequencing (WGS), or F gene sequencing when WGS was not feasible. Viral diversity, phylogenetics, and selection pressure were assessed.
Results: A total of 251 WGS (HRSV-A: 165; HRSV-B: 86) and 27 F gene sequences (HRSV-A: 13; HRSV-B: 14) were obtained for the non-immunised group. For immunised cases, 79 WGS (HRSV-A: 67; HRSV-B: 12) and 12 F sequences (HRSV-A: 10; HRSV-B: 2) were analysed. Lineage distribution remained similar between groups. Nucleotide diversity was similar for HRSV-A groups, though reduced in immunised HRSV-B.

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Genetic Drift
Genetic Fitness

Selection pressure analyses suggested a shift toward neutral evolution in immunised samples. Mutations N63S, K65R, I206T, and K209E (HRSV-A) and K68E, R209Q, and S211N (HRSV-B) were detected in nirsevimab epitope, with K209E and K68E absent in non-immunised samples.
Conclusions: Nirsevimab immunisation may influence HRSV evolution, particularly in HRSV-B. Continued genomic surveillance is crucial for early mAb-resistant mutants detection.

Introduction

Human respiratory syncytial virus (HRSV) imposes a substantial health burden in children younger than 5 years, with an estimated 33 million cases of HRSV-associated acute lower respiratory tract infections (LRTIs) worldwide. Among infants aged 0–6 months, HRSV-associated LRTIs led to approximately 1.4 million hospital admissions globally, with 45,700 deaths attributed to the virus in this age group. Notably, over 97% of HRSV-related deaths across all age groups occurred in low- and middle-income countries.[1] In the EU, Norway and the United Kingdom, HRSV also has a considerable impact, with 213,000 children under five and 158,000 adults hospitalised annually, some requiring intensive care.[2]

HRSV, as a member of the *Pneumoviridae* family, is an enveloped virus with a linear, negative-sensed, single-stranded RNA genome.[3] Antigenically, it is divided into two subgroups, HRSV-A and HRSV-B, which are further classified into multiple genetic lineages based on complete or near-complete genome sequences. Both subgroups co-circulate each season following a 4-year pattern, with HRSV-B typically predominating for three years, followed by a shift to HRSV-A in the subsequent year.[4] The acquisition of single mutations along its genome (genetic drift) is the primary source of genetic variation, while recombination is rarely detected.[5]

Given the significant health burden of HRSV and its genomic plasticity, substantial efforts have been focused on the development of several strategies for HRSV-disease prevention, including vaccination and passive immunisation with monoclonal antibodies (mAb).[6] Surface glycoproteins G and F, as primary targets of immune responses, are the most promising candidates for these preventive strategies. However, the high variability of the G protein limits its potential, whereas the highly conserved F protein emerges as the most suitable target for these interventions, as it has a critical role at early stages in host cell infection.[7] Up to six different antigenic sites (III, V, Ø, II, I, IV) have been identified in the pre-fusion and post-fusion conformations of the F protein, with different neutralisation potencies. Antigenic site Ø, exclusive to the pre-fusion conformation, is the epitope with the highest neutralising activity[8] and the target of the monoclonal antibody nirsevimab and other mAb candidates.[6] Nirsevimab was licensed by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) for extended use in preventing severe respiratory disease.[9,10]

During the first nirsevimab immunisation campaign (2023–2024) in certain regions of Spain, coverage with nirsevimab averaged 92% among <6 months infants born during the HRSV season and slightly decreased to 88% in the catch-up group (<1-year-old infants born before the season's onset).[11] The estimated effectiveness in reducing the risk of HRSV-associated hospitalisation in Spain was approximately 75%,[12] closely aligned with the 77% efficacy reported in randomised clinical trials.[13,14] In Catalonia, nirsevimab effectiveness was linked to reductions of 87.6% and 90.1% in hospital and ICU admissions, respectively.[15]

Although immunisation rates and efficacy levels are high, and the nirsevimab binding site exhibits a high degree of conservation,[4,16] new mutations may help the virus to evade its effectiveness. In fact, the emergence of mAb-resistant mutants has been reported in clinical trials and surveillance studies.[17,18] This highlights the importance of maintaining active virological surveillance, with particular attention to mutations in target epitopes of the current passive immunoprophylaxis with nirsevimab.

The objective of this study was to evaluate the effect of the selective pressure exerted by nirsevimab exposure through the identification of potential genetic markers associated with reduced nirsevimab neutralisation in the virus.

Materials and methods

Sample collection

Respiratory samples were collected between week 40 of 2023 (02/10/2023) and week 39 of 2024 (29/09/2024) using two approaches. Firstly, samples were obtained through the sentinel network for respiratory viruses' surveillance in Catalonia (SIVIC, https://sivic.salut.gencat.cat). Patients with suspected acute respiratory infections (ARI) attending sentinel hospitals or primary care centres on Mondays or Tuesdays during the period studied were eligible for inclusion in the SIVIC program. For selected patients spanning all age groups, clinical data were collected, and a naso-oropharyngeal swab was obtained and sent to designated reference laboratories for testing. Laboratory confirmation of respiratory viruses was performed at each hospital using a real-time multiplex RT-PCR assay (Allplex Respiratory Panels 1–3, Seegene, South Korea). Additionally, HRSV-positive samples with a CT value < 30 were forwarded to Hospital Universitari Vall d'Hebron for whole-genome sequencing and further genetic characterisation. Secondly, respiratory samples (nasopharyngeal aspirates or naso-oropharyngeal swabs) from children under one year of age with laboratory-confirmed HRSV after nirsevimab administration were collected from three different hospitals from Barcelona (Hospital Universitari Vall d'Hebron, Hospital Universitari Germans Tries i Pujol) and Lleida (Hospital Universitari Arnau de Vilanova). All samples received were divided into two groups based on immunisation status. The non-immunised group included samples from patients who had not received nirsevimab or whose samples were collected before its administration. The immunised group included samples collected from children after they had received nirsevimab.

The study protocol was approved by the Research Ethics Committee of Vall d'Hebron University Hospital under code PR(AG) 144–2025.

Genetic characterisation based on whole-genome sequencing

Prior to sequencing, HRSV genome amplification was performed with the SuperScript IV One-Step RT-PCR (Invitrogen, USA) in four overlapping 4-kb-amplicons based on the primers designed by Bose et al. and Schobel et al.[19,20] WHO Collaborating Centre for Reference and Research on Influenza in Australia kindly provided the PCR protocol. In cases where whole-genome sequencing was not possible, the complete F gene was sequenced through an in-house protocol.

Purification of PCR products was carried out using VAHTS DNA Clean Beads (Vazyme, China). Resulting amplicons were normalised and pooled. Libraries were prepared using the Illumina DNAPrep kit

(Illumina, CA, USA) and indexed with IDT for Illumina DNA/RNA UD Indexes Set A-D (384 IDX) (Illumina, USA). Quality assessment of the products was performed using the 4200 TapeStation System (Agilent, USA). The final pooled library was loaded onto a MiSeq Reagent Kit 600v3 or NextSeq 1000/2000 P2 (200 cycles) cartridges (Illumina, CA, USA) and sequenced on MiSeq or NextSeq 2000 platforms, respectively (Illumina, CA, USA).

FASTQ files obtained after sequencing were processed using Trimmomatic v0.39,[21] with the sliding window trimming feature enabled. A 10-base window was applied, and sequences were trimmed when the average quality within the window dropped below Q30. Leading and trailing bases with quality scores below Q30 were also removed. The resulting FASTQ files were further filtered with Bowtie2 v2.5.1[22] to remove host sequences (human genome assembly GRCh38). Primer sequences were trimmed using BBMap v38.91 (script: bbduk.sh; BBMap - Bushnell B. - sourceforge.net/projects/bbmap). After pre-processing, high-quality filtered reads were assembled de novo with SPAdes v3.15.2,[23] using the –rnaviral option. Resulting scaffolds were classified by subgroup using BLASTN v2.14.0+.[24] Scaffolds were aligned to subgroup-specific reference genomes with Minimap2 v2.26-r1175.[25] Sequences EPI_ISL_412866 (hRSV/A/England/397/2017) and EPI_ISL_1653999 (hRSV/B/Australia/VIC-RCH056/2019) were used as reference sequences for HRSV-A and B, respectively. Non-overlapping scaffolds were concatenated using N-strings based on their mapping positions. Overlapping scaffolds were merged based on sequence homology, CIGAR metrics, and kmer coverage defined by SPAdes. When overlapping sequences displayed less than 100% identity, the sequence with higher coverage was selected as the consensus.

Sequences with a genome coverage equal or greater than 90% were included in the analysis. For those samples with a genome coverage below that threshold, but with a coverage of the F gene equal or greater than 90%, the F coding sequences were also included in the analysis.

Phylogenetic analyses and lineage assignment

Whole-genome sequences from the study period were aligned using MAFFT v7.475.[26] Phylogenetic trees were constructed using IQ-TREE v2.3.6 (ModelFinder integrated).[27] Branch supports were obtained with the ultrafast bootstrap,[28] with a bootstrap value of 1000.

Lineage assignment was performed on whole-genome sequences by Nextclade v3.10.0[29] through the website https://clades.nextstrain.org, based on the classification proposed by Goya et al.[30] Fisher's exact test was performed to determine differences in lineage distribution between groups, considering statistically significant p-values < 0.05.

Nucleotide diversity

Nucleotide diversity (π) was calculated for each individual sample to assess within-host diversity. Viral diversity was estimated by computing the diversity statistic π, as defined by Illingworth J. R and Zhao L.[31]

In this study, nucleotide diversity was calculated keeping sites with a minimum allele frequency (MAF) of 0.01. Mutations with an allele frequency above 0.99 were considered fixed in the viral population and were therefore excluded from the analysis. Mutations within the 0.01–0.99 range underwent further filtering, with only positions covered at least 100 times (100x coverage) retained to minimise potential sequencing errors.

This statistic was computed for the whole genome, individual genomic features (coding and non-coding regions, 3' UTR and 5'UTR) and F regions for each sample. Group comparisons between the non-immunised and immunised cohorts were performed using the Wilcoxon rank-sum test, with statistical significance set at p-values < 0.05.

Mutation analysis of the F protein

Mutations were characterised for the fusion protein using the tool minMutFinder.[32] Nucleotide substitutions were kept when having an allele frequency (AF) equal or greater than 0.05. Mutations were analysed at consensus level (AF≥0.5) and at viral population level, assigning each mutation frequency to a range ($0.0 < AF \leq 0.2$, $0.2 < AF \leq 0.4$, $0.4 < AF \leq 0.6$, $0.6 < AF \leq 0.8$, $0.8 < AF \leq 1.0$).

Selection pressure analysis

Selection pressures on protein-coding sequences (CDS) and individual codons were assessed using Tajima's D[33] and the non-synonymous to synonymous substitution (dN/dS) ratio, respectively. Tajima's D was calculated with the tajima.test() function from the pegas (Population and Evolutionary Genetics Analysis System)[34] package v1.3 in R v4.4.2. The dN/dS ratio (ω) was determined via the Datamonkey Webserver using the Single Likelihood Ancestor Counting (SLAC) algorithm.[35] Codon sites were considered under significant selection if p-value < 0.05.

Results

Sample collection

During the study period, a total of 9839 hospital samples and 12,384 primary care samples were tested for HRSV, with positivity rates of 8% and 3%, respectively (data retrieved from https://sivic.salut.gencat.cat, accessed 2nd of January 2025). The distribution of the positive samples peaked between weeks 48 and 52 (Fig. 1).

A total of 414 samples, collected through both approaches (sentinel and the immunised cohort from three hospitals), met the sequencing criteria and were eligible for whole-genome sequencing. Sequencing was successfully completed for 92% (381/414) of these samples, of which 97% (369/381) met the inclusion criteria for this study.

Genetic characterisation based on whole-genome sequencing

In the non-immunised group, whole-genome sequences were successfully obtained for 251 HRSV-positive samples (165 HRSV-A and 86 HRSV-B), with 98% of these sequences achieving over 99% genome coverage (Suppl. File 1). Additionally, F gene sequences were recovered from 27 HRSV-positive samples (13 HRSV-A and 14 HRSV-B) in cases where whole-genome sequencing was not possible.

In the immunised group, whole-genome sequences were obtained for 79 HRSV-positive samples (67 HRSV-A and 12 HRSV-B), with 92% of them exceeding 99% genome coverage. For cases where whole-genome data could not be obtained, F gene sequences were retrieved for 12 additional HRSV-positive samples (10 HRSV-A and 2 HRSV-B). All whole-genome sequences are available at GISAID (EPI numbers provided in Suppl. File 1).

Phylogenetic analyses and lineage assignment

Phylogenetic trees constructed from whole-genome sequences were generated for both subgroups (Fig. 2). Distribution of Nextstrain-assigned lineages was remarkably similar between non-immunised and immunised samples for both HRSV subgroups, consistent with the phylogenetic analysis. For HRSV-A, 12 distinct lineages were identified in the non-immunised group, 10 of which were found in the immunised group. In both patient groups, the predominant lineages were A.D.1, A.D.1.5, A.D.3, and A.D.5.3, though

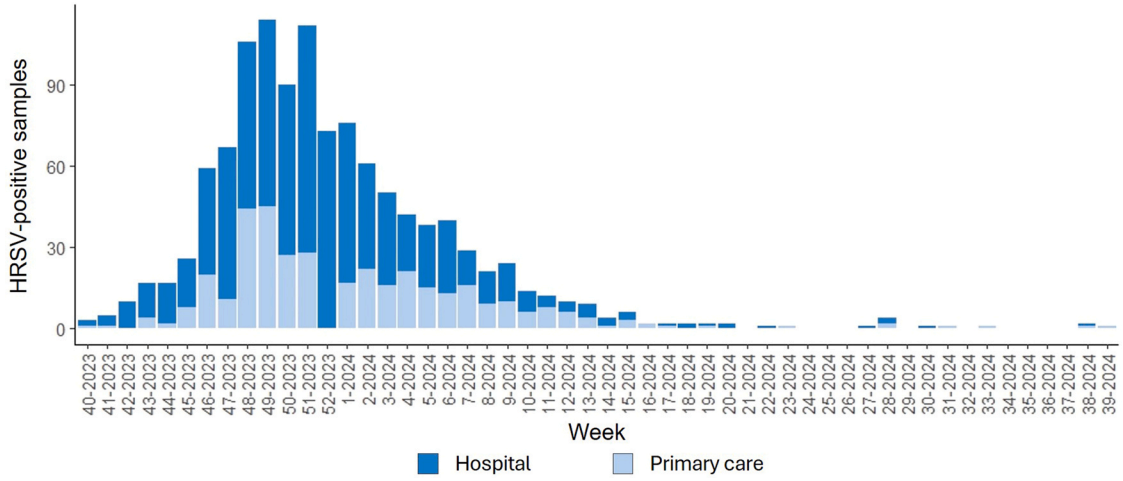

Fig. 1. Distribution of HRSV-positive samples from the SIVIC program (2023–2024). Weekly distribution of HRSV-positive samples collected during the 2023–2024 season (data retrieved from https://sivic.salut.gencat.cat, accessed 2nd of January 2025). Samples from primary care centres are represented in light blue, while hospital samples are shown in dark blue.

their prevalence varied slightly. In the non-immunised group, these lineages accounted for 14%, 15%, 28% and 15% of cases, whereas in the immunised group were 21%, 25%, 24% and 9%, respectively. For HRSV-B, less lineage heterogeneity was shown, with only two lineages detected in both patient groups: B.D.4.1.1 and B.D.E.1. These lineages represented 28% and 72% of cases in the non-immunised group, respectively, compared to 42% and 58% in the immunised group (Suppl. File 1). No statistically significant differences of lineage distribution between non-immunised and immunised groups were found in either HRSV subgroup.

In both HRSV-A and HRSV-B, viral sequences from non-immunised and immunised groups were phylogenetically mixed, with no monophyletic clustering observed among sequences from viruses exposed to nirsevimab.

Nucleotide diversity

Nucleotide diversity was explored at three levels: whole-genome sequences, specific genomic features, and F regions. At the whole-genome level, the median within-host viral diversity was comparable between non-immunised and immunised groups for HRSV-A, estimated at 0.0507 [IQR: 0.0396–0.0765] and 0.0515 [IQR: 0.0418–0.0726], respectively. For HRSV-B, the diversity in the immunised group showed a slight reduction (0.0464 [IQR: 0.0354–0.0765]) compared to the non-immunised group (0.0624 [IQR: 0.0416–0.0946]). Despite these patterns, no statistically significant differences emerged, with p-values of 0.9154 for HRSV-A and 0.1482 for HRSV-B. When comparing all samples by subgroup, without considering immunoprophylaxis status, HRSV-B showed a higher within-host diversity with a median of 0.0602 [IQR: 0.0413–0.0915], in front of 0.0509 [IQR: 0.0401–0.0764] from HRSV-A, with no significant differences (p-value=0.1804).

Zooming in on specific genomic regions (Fig. 3. A), no significant differences were found in HRSV-A groups. In contrast, for HRSV-B, the P gene exhibited significantly higher within-host diversity in the immunised group (p-value=0.0404). This increase was marked by a wider range of diversity values among samples, with some showing low diversity while others displayed substantially higher variability, suggesting greater heterogeneity in the viral populations under nirsevimab selective pressure.

In relation to the F CDS, neither the entire gene nor its subregions (Fig. 3. B) revealed statistically significant differences between non-immunised and immunised cohorts for HRSV-A. Median within-host diversity for the F gene was estimated at 0.0815 [IQR: 0.0315–0.1720] in the non-immunised group and 0.0855 [IQR: 0.0369–0.1720] in the immunised group (p-value=0.7796). Within epitope Ø, diversity decreased to 0.0484 [IQR: 0.0328–0.1446] for the non-immunised group and 0.0335 [IQR: 0.0218–0.0396] for the immunised group (p-value=0.0842). For HRSV-B, median diversity in the F gene was similar between non-immunised (0.0641; IQR: 0.0325–0.1771) and immunised (0.0564; IQR: 0.0356–0.1312) groups (p-value=0.5631). However, for epitope Ø, viral populations exposed to nirsevimab showed higher within-host variability, with median diversity rising to 0.0704 [IQR: 0.0310–0.1239] in the immunised group compared to 0.0333 [IQR: 0.0234–0.0436] in the non-immunised group, though these differences were not statistically significant (p-value=0.2149).

In relation to the other regions analysed in the F gene, HRSV-B groups showed significant differences for HRA region (p-value=0.0301), with higher median within-host diversity found for the immunised group. Additionally, for both subgroups HRSV-A and -B, the cytoplasmic tail showed a higher nucleotide diversity in the studied groups, in comparison to the other regions of the F gene.

Mutation analysis of the F protein

Several mutations were identified in the F protein, also within the nirsevimab target epitope, in both non-immunised and immunised groups. At consensus level (Fig. 4, Table 1, Suppl. Table 1), for epitope Ø (residue positions 62–69/196–212) in HRSV-A, mutations observed in the non-immunised group included N63S at 0.56% (1/178), K65R at 7.86% (14/178) and I206T at 0.56% (1/178), while in the immunised group, K65R and K209E were detected at 5.19% (4/77) and 1.30% (1/77), respectively. For HRSV-B, mutations in the non-immunised group comprised R209Q at 15% (15/100) of the samples and S211N at 82% (82/100). In the immunised group, the identified mutations were K68E at 7.14% (1/14), R209Q at 28.57% (4/14), and S211N at 71.43% (10/14). Mutations K209E and K68E, for HRSV-A and -B, respectively, were exclusive to the immunised cohort samples. Regarding epitope II (residue positions 254–277), targeted by palivizumab, only mutation S276N was identified, at 7.86% (14/178) and 5.19% (4/77) in non-immunised and immunised HRSV-A groups, respectively. For epitope IV (residue positions 426–447), targeted by the mAb clesrovimab (MK-1654), no mutations were found at consensus level.

Regarding minority mutations (AF < 0.5) in the F protein (Fig. 5, Table 1), several mutations were identified, most of them with allele

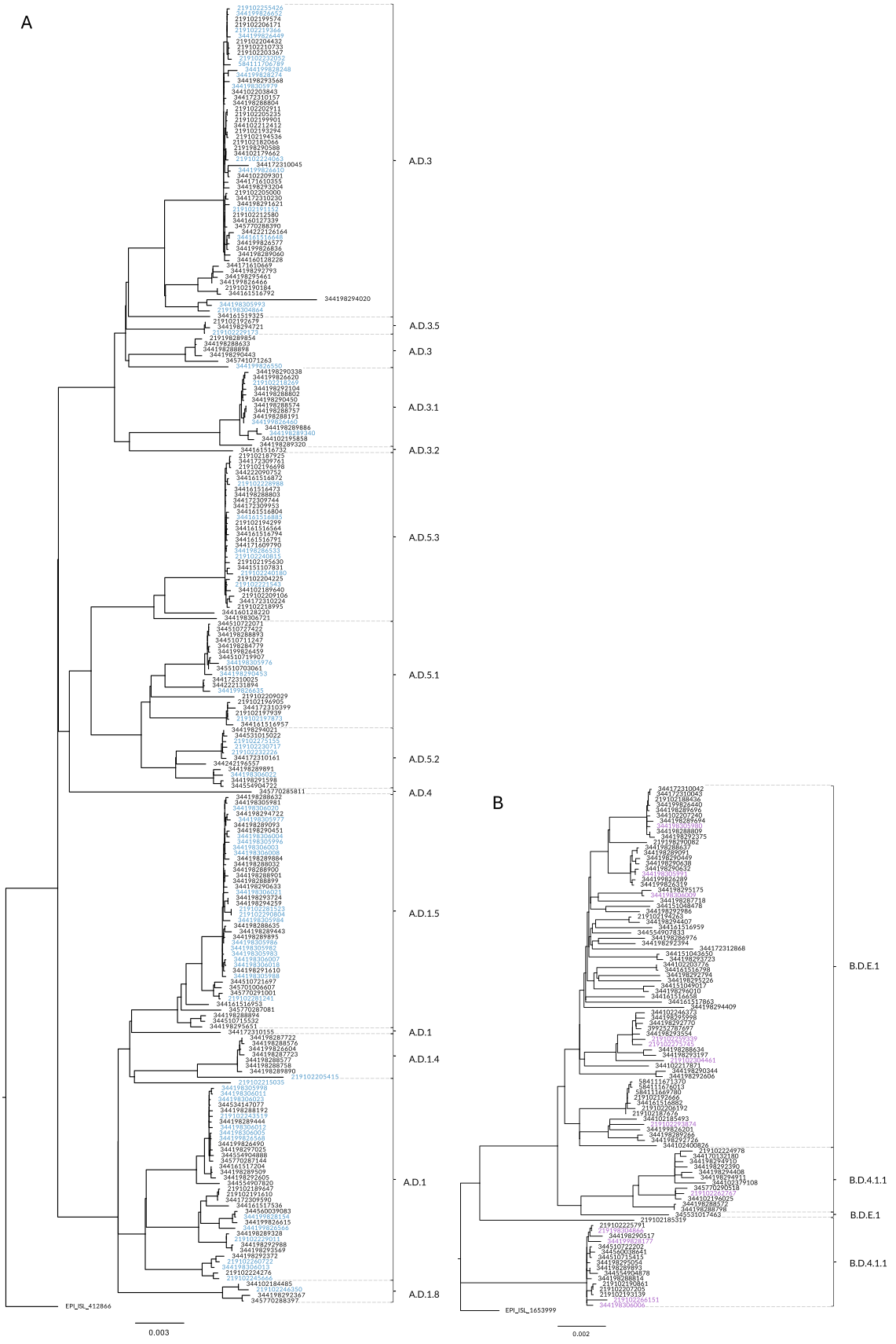

Fig. 2. Phylogenetic analysis of whole-genome HRSV sequences (2023–2024). Phylogenetic trees of whole-genome sequences for HRSV-A (A) and HRSV-B (B) from the 2023–2024 season. Sequences from the non-immunised group are shown in grey, while sequences from immunised patients are highlighted in blue (HRSV-A) and purple (HRSV-B).

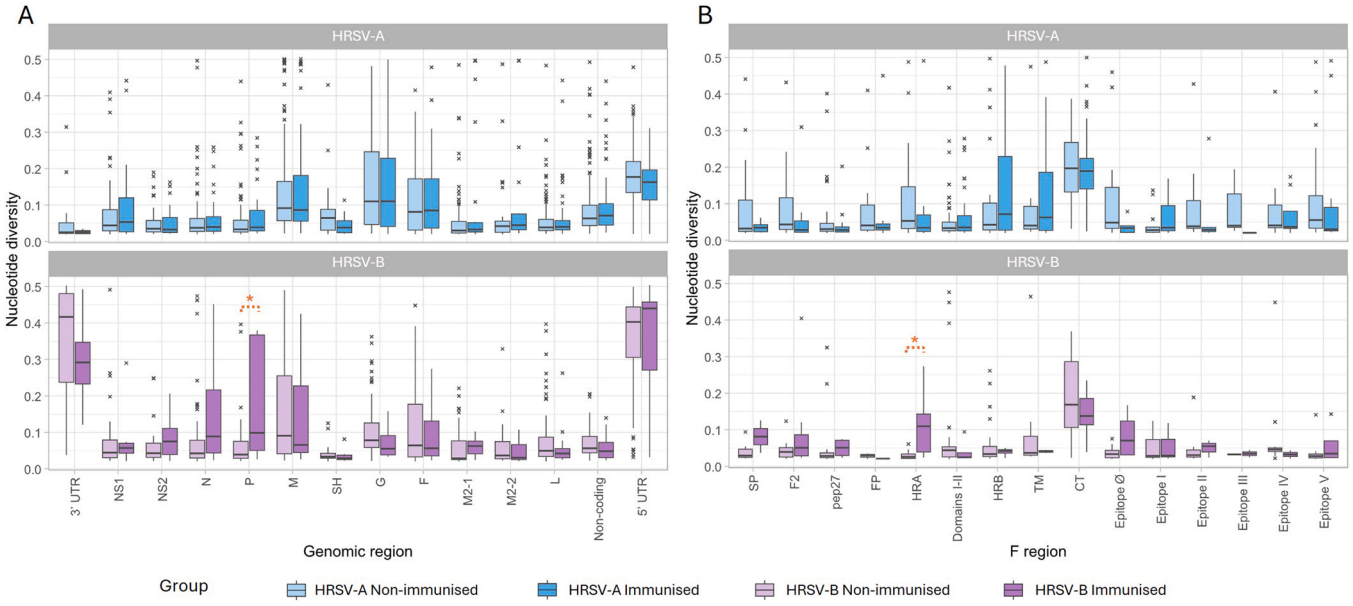

Fig. 3. Median nucleotide diversity across genomic and F gene regions. (A) Median nucleotide diversity across different genomic regions for HRSV-A and HRSV-B in the non-immunised and immunised groups. (B) Median nucleotide diversity across F gene regions for HRSV-A and HRSV-B in both groups. Abbreviations: SP – signal peptide, F2 – F2 subunit, FP – fusion peptide, HR – heptad repeat, TM – transmembrane domain, CT – cytoplasmic tail.

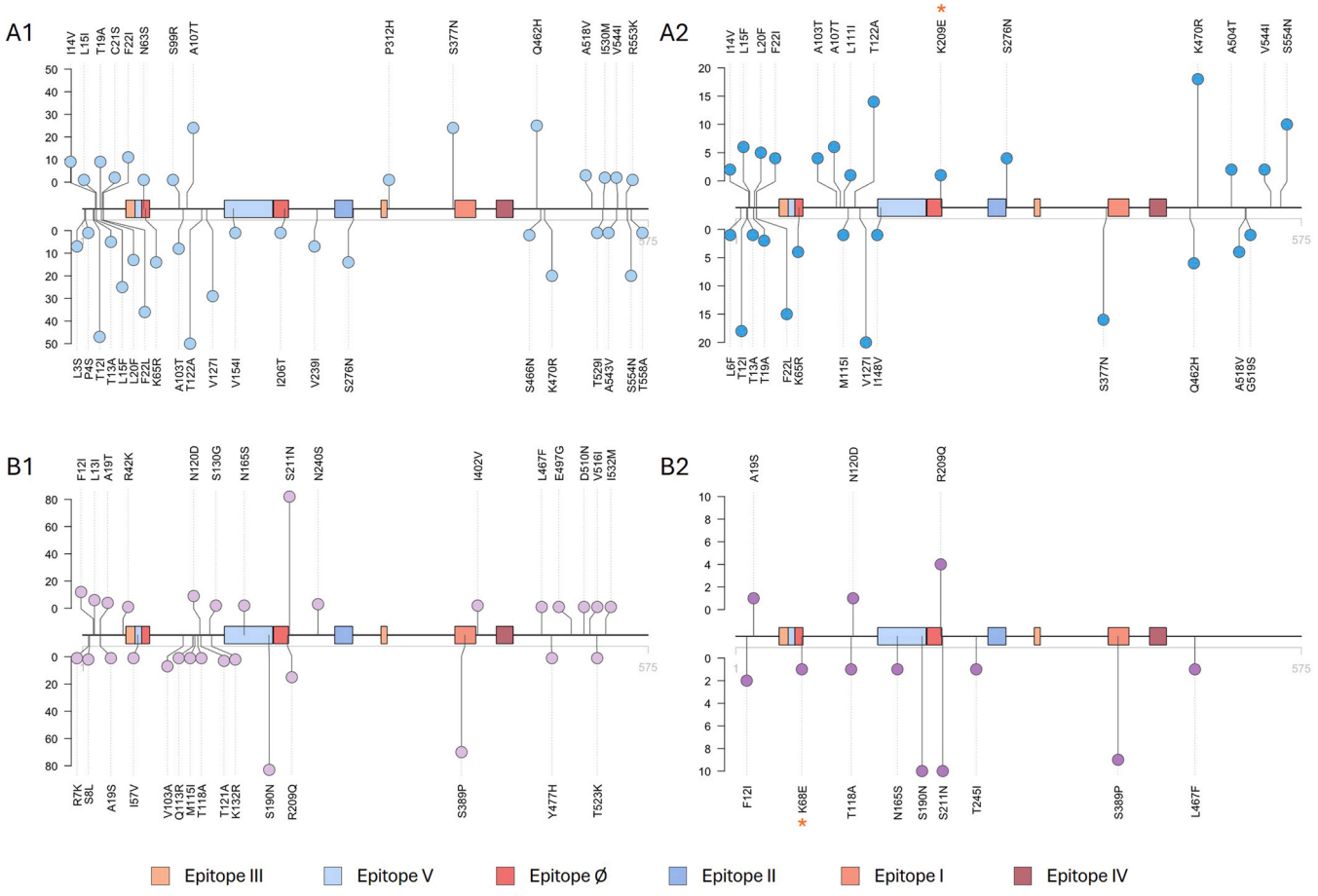

Fig. 4. Amino acid mutations at consensus level in the fusion protein for HRSV-A and HRSV-B. Amino acid mutations at consensus level (allele frequency ≥ 0.5) in the fusion protein for both the non-immunised (left side, labelled as 1) and immunised (right side, labelled as 2) groups. HRSV-A is shown at the top (labelled as A) and HRSV-B at the bottom (labelled as B). Mutations located in the nirsevimab epitope that were found exclusively in the immunised group are marked with an asterisk.

Table 1
Mutations identified in epitopes Ø, II and IV in the F protein.

HRSV subgroup	Epitope	Mutation	mAb neutralisation	Group	Frequency in dataset	Allele frequency
HRSV-A	Ø	N63S	Efficiently neutralised[18,36]	Non-immunised	0.56% (1/178)	≥0.5
		K65R*	Efficiently neutralised[17]	Non-immunised	7.86% (14/178)	≥0.5
				Non-immunised	0.56% (1/178)	0.09
				Immunised	5.19% (4/77)	≥0.5
		E66G	Efficiently neutralised[18]	Non-immunised	0.56% (1/178)	0.35
		L204S	Unknown	Non-immunised	0.56% (1/178)	0.08
		I206T	Unknown	Non-immunised	0.56% (1/178)	≥0.5
		K209E*	Unknown	Immunised	1.30% (1/77)	≥0.5
		Q210L	Unknown	Non-immunised	0.56% (1/178)	0.11
	II	N254H	Unknown	Immunised	1.30% (1/77)	0.09
		N262T	Unknown	Immunised	1.30% (1/77)	0.06
		M264I	Unknown	Non-immunised	0.56% (1/178)	0.06
		S276N	Efficiently neutralised[37]	Non-immunised	7.86% (14/178)	≥0.5
				Non-immunised	0.56% (1/178)	0.05
				Immunised	5.19% (4/77)	≥0.5
	IV	N428D	Unknown	Immunised	1.30% (1/77)	0.1
		S436P	Unknown	Immunised	1.30% (1/77)	0.08
		G446R	Unknown	Immunised	1.30% (1/77)	0.06
HRSV-B	Ø	K68E	Not efficiently neutralised[38]	Immunised	7.14% (1/14)	≥0.5
		L204S	Unknown	Immunised	7.14% (1/14)	0.16
		M206V	Not efficiently neutralised[38]	Immunised	7.14% (1/14)	0.06
		R209Q*	Efficiently neutralised[36]	Non-immunised	15% (15/100)	≥0.5
				Immunised	28.57% (4/14)	≥0.5
		S211N	Efficiently neutralised[17]	Non-immunised	82% (82/100)	≥0.5
				Immunised	71.43% (10/14)	≥0.5
	II	S255R	Unknown	Immunised	7.14% (1/14)	0.19
		E256G	Unknown	Immunised	7.14% (1/14)	0.09

Mutations identified in epitopes Ø (nirsevimab targeted), II (palivizumab targeted), and IV (clesrovimab targeted) of the F protein are listed. For mutations present in the consensus sequence, the frequency is ≥0.5. Minority mutations (AF < 0.5) show the allele frequency in the viral population.
Neutralisation effectiveness is classified as follows: "Not efficiently neutralised" if the fold change is > 5, "Resistant" if the fold change is > 100, and "Efficiently neutralised" if the fold change is < 5.
Mutations that form hydrogen bonds or salt bridges with nirsevimab are marked with an asterisk.

frequencies below 0.2. In the Ø epitope of the HRSV-A subgroup, the minority mutations K65R (1/178, AF=0.09), E66G (1/178, AF=0.35), L204S (1/178, AF=0.08) and Q210L (1/178, AF=0.11) were detected in the non-immunised group, while no minority mutations were found in the immunised group. For HRSV-B, the minority mutations L204S (1/14, AF=0.16) and M206V (1/14, AF=0.06) were identified in the immunised group, while no mutations were detected in the non-immunised group. Minority mutations in epitopes II and IV are shown in Table 1.

Selection pressure analysis

Tajima's D values were computed to assess deviations from neutrality for both HRSV-A and HRSV-B subgroups (Table 2) and identify evidence of selection. For HRSV-A, the Tajima's D values were consistently negative across all proteins in both groups, values closer to zero in the immunised group across most proteins. Notably, the SH protein in the immunised group showed a positive Tajima's D (0.3174, p-value=0.7509), in contrast to a negative value in the non-

Fig. 5. Within-host amino acid mutations in the fusion protein for HRSV-A and HRSV-B. Amino acid mutations in the fusion protein for HRSV-A (top, labelled as A) and HRSV-B (bottom, labelled as B) are shown for both the non-immunised (upper tile) and immunised (bottom tile) groups. Mutations are categorised into five frequency ranges based on their within-host allele frequency (AF): 0.0 < AF≤0.2; 0.2 < AF≤0.4; 0.4 < AF≤0.6; 0.6 < AF≤0.8; and 0.8 < AF≤1.0. The tiles are colour-coded to reflect the proportion of samples containing each mutation within a specific frequency range, with darker tiles indicating a higher number of samples exhibiting mutations at that range within the dataset.

Table 2
Tajima's D values for HRSV-A and HRSV-B proteins. Tajima's D values for all the proteins, separated by non-immunised and immunised, and HRSV-A and HRSV-B.

Protein	HRSV-A		HRSV-B	
	Non-immunised	Immunised	Non-immunised	Immunised
NS1	−1.4500 (0.1470)	−1.1602 (0.2460)	−1.3417 (0.1797)	−0.0415 (0.9669)
NS2	−1.0700 (0.2846)	−0.6553 (0.5123)	**−2.1640 (0.0305)**	−1.1384 (0.2550)
N	−1.2375 (0.2159)	−0.9605 (0.3368)	**−2.1162 (0.0343)**	−0.5437 (0.5867)
P	−1.6647 (0.0960)	−1.8064 (0.0709)	−1.7189 (0.0856)	−0.3933 (0.6941)
M	−1.1005 (0.2711)	−1.0379 (0.2993)	−1.3722 (0.1700)	−0.0553 (0.9559)
SH	−1.2380 (0.2157)	0.3174 (0.7509)	**−2.0810 (0.0374)**	−0.7515 (0.4524)
G	−0.9790 (0.3276)	−0.8150 (0.4151)	−1.6044 (0.1086)	−0.2885 (0.7729)
F	−1.2256 (0.2203)	−0.9329 (0.3509)	−1.9319 (0.0534)	−1.0079 (0.3135)
M2-1	−1.4792 (0.1391)	−1.0028 (0.3159)	**−2.3717 (0.0177)**	−0.4089 (0.6826)
M2-2	−1.5232 (0.1277)	−1.5324 (0.1254)	−1.8499 (0.0643)	−0.0669 (0.9467)
L	−1.3323 (0.1827)	−1.0814 (0.2795)	**−2.1255 (0.0335)**	−0.4002 (0.6890)

Normalised p-values are included in parentheses.
Statistically significant Tajima's D values in bold.

immunised group (−1.2380, p-value=0.2157), though these values were not statistically significant. For HRSV-B, a similar trend was observed, with strongly negative values in the non-immunised group, particularly in NS2, N, SH, M2–1 and L, all of which were statistically significant. In contrast, the immunised group exhibited mostly near-neutral values, with no significant deviations from neutrality.

Evolutionary pressures acting at codon level were assessed through the dN/dS ratio (Supplementary table 2). For HRSV-A, multiple proteins exhibited sites under negative selection in the non-immunised group, particularly in the G, F, and L proteins, having the highest number of constrained sites. The G protein, known for its role in immune evasion, had one positively selected site (314) in non-immunised cases, indicating diversifying selection at this residue position. For all proteins, fewer sites showed significant selective pressure in the immunised group. In the case of HRSV-B, selection was mainly negative, with constrained residues observed in multiple proteins, particularly G, F, and L, in the non-immunised group. Moreover, no positively selected sites were identified in the G protein for HRSV-B, unlike HRSV-A. The SH protein had one positively selected site at position 60, which was found only in the immunised group. No other sites under significant selective pressure were identified in any protein in the immunised group.

Discussion

Human respiratory syncytial virus (HRSV) poses a substantial burden in infants, contributing to significant morbidity and hospitalisations worldwide. In response, extensive efforts have focused on developing preventive strategies, including vaccines and mAbs. The passive immunisation with nirsevimab has represented a significant advancement, demonstrating high effectiveness in reducing HRSV-associated hospitalisations and intensive care admissions. Its widespread administration in most vulnerable population (children < 1 year old) was expected not only to reduce this burden but also to exert selective pressure on the virus, potentially driving its evolution and even leading to the acquisition of mutations associated with reduced mAb neutralisation. To our knowledge, this is the second study to evaluate the emergence of mutations in the nirsevimab-targeted epitope during the 2023–2024 season in immunised patients, and the first study analysing selective pressure signals associated with nirsevimab exposure using real-world data.

In this study, whole-genome and F gene sequences from viruses detected during the first nirsevimab immunisation campaign in Catalonia were analysed. Subgroup distribution of those samples (70% HRSV-A) was consistent with the predominance of this genetic group among circulating viruses according to the data gathered from sentinel network for respiratory virus surveillance in Catalonia (60% HRSV-A, data retrieved from https://sivic.salut.gencat.cat, accessed on 3rd of February 2025). A similar predominance of HRSV-A during the 2023–2024 season was also reported in other countries, such as France.[18] The consistency in subgroup predominance and proportions suggests that the viral genomes included in this study are representative of the circulating strains in the region, further supporting the reliability of the dataset for assessing viral diversity and evolutionary trends.

The distribution of HRSV lineages, based on whole-genome sequences, was comparable between non-immunised and immunised samples, with no statistically significant differences. When compared to European sequences (filtered by the same collection date range and genome coverage ≥ 90% from https://nextstrain.org/rsv/a/genome/all-time, accessed on 12th of February 2025), the identified lineages in the non-immunised were similar, though their prevalence varied. In Europe, the predominant lineage for HRSV-A was A.D.1, accounting for 34% (227/661) of cases, whereas it was found at 14% in the non-immunised group in our dataset. In contrast, lineage A.D.3 was the most prevalent in our study, while it represented only 15% (98/661) of European samples. For HRSV-B, the predominant lineage in Europe was B.D.E.1, comprising 70% (139/202) of cases, similar to our non-immunised dataset, followed by B.D.4.1.1 at 24% (49/202). While our dataset captures part of the lineage diversity seen in Europe, not all lineages were represented in our region, suggesting some geographical differences in patterns of circulating lineages.

Given the comparable lineage distribution between non-immunised and immunised samples in Catalonia, nucleotide diversity was examined to assess differences in genetic variation. Nucleotide diversity within a host can influence viral evolution and hence, transmission dynamics within populations, particularly in rapidly evolving RNA viruses like HRSV. Our data showed no statistically significant differences in overall nucleotide diversity across the whole genome between non-immunised and immunised cohorts. This maintenance of within-host nucleotide diversity in samples from immunised patients compared to non-immunised patients, has also been observed in studies of other RNA viruses, such as SARS-CoV-2 or influenza.[39–41] Despite this result at the whole-genome level, a statistically significant increase in nucleotide diversity was observed in some genomic regions (P gene and HRA region of F CDS) with critical roles in viral fitness in HRSV-B samples from immunised patients. This suggests that passive immunisation with nirsevimab may act as a selective pressure driving greater genetic variability in some regions, especially for HRSV-B. Additionally, increased nucleotide diversity was found for antigenic epitope Ø of HRSV-B immunised patients, although differences were not statistically significant. Further studies are needed to determine whether these genetic variations translate into functional changes in viral fitness, which could facilitate their spread and persistence in future seasons if they confer a biological advantage.

Analyses of selective pressure suggest that HRSV evolution is primarily driven by purifying selection, consistent with previous studies.[4] This evolutionary pattern is commonly observed in viruses that are well-adapted to their hosts and exhibit efficient transmission dynamics, which support genetic stability and functional conservation. However, external pressures such as immunoprophylaxis can challenge the ability of the virus to maintain optimal fitness by altering the within-host environment and disrupting this evolutionary equilibrium. In this study, immunised individuals exhibited a shift toward neutral selection, indicating a deviation from the overall trend. This may be attributed to a population bottleneck caused by mAb exposure, leading to a sharp reduction in viral population size from which the virus has not yet fully recovered. Following the strong selection imposed by the mAb, the viral population could have reached a more stable equilibrium under the new selective landscape, where functional constraints limit the incorporation of new mutations. Interestingly, in contrast to HRSV-A immunised samples, HRSV-B showed signs of increased diversity in certain regions, possibly due to the higher mutation rate reported for this subgroup,[4] which could have contributed to accelerate its recovery from the bottleneck. These findings suggest that HRSV-B may have a higher potential for developing immune escape mutations. Another possible explanation for those differences found between HRSV-A and HRSV-B could be subgroup-specific variation in how effectively nirsevimab neutralises the virus. If nirsevimab exerts stronger selective pressure on HRSV-A, it may lead to a greater reduction in viral population size and, consequently, lower genetic diversity. Further studies on nirsevimab neutralisation in relation to HRSV subgroups are needed to better understand potential differences in viral fitness.

Overall, our findings indicate that immunised individuals exhibit a distinct viral evolutionary pattern, characterised by a more stable population likely reflecting adaptation to the new within-host immunological context imposed by nirsevimab immunoprophylaxis. These results highlight the need for further studies on viral evolution under immune interventions to better understand the mechanisms driving adaptation and the emergence of resistant variants, which could, in the medium term, affect the efficacy of these treatments.

Several studies have reported the natural emergence of mAb neutralisation escape mutants in immunoprophylaxis-naïve patients, with frequencies ranging from 0.7% to 0.8% of sampled viruses.[42,43] In the presence of mAbs, these frequencies have been observed to increase to 5–9%.[37,44–46] The distinct viral evolutionary pattern observed in immunised patients from this study can lead to amino acid changes that may impact viral protein function, adaptation, and neutralisation profile by monoclonal antibodies. During season 2023–2024, several mutations have been identified within the nirsevimab epitope, with unclear or slightly reduced effects on nirsevimab neutralisation.

In HRSV-A, mutations with no effect in nirsevimab neutralisation were found, such as N63S and K65R. N63S was exclusively observed in non-immunised samples and has also been reported the 2023–2024 season in France, where it was detected in both untreated and immunised groups at similar proportions (1.3% and 1.7%, respectively).[18] In contrast, the K65R mutation was found in both cohorts, and was sporadically detected in previous seasons at very low frequency.[4] Additionally, two other mutations, I206T and K209E, were identified, though their effects on nirsevimab neutralisation remain unclear.

For HRSV-B, mutation K68E was found in strains from immunised individuals. When combined with three other mutations (I64T, I206M, Q209R), it has the potential to confer resistance to nirsevimab,[17] although this combination was not observed in our dataset. The substitutions F:206M and F:209R emerged in our region in season 2017–2018,[4] and are defining mutations for B.D.4.1.1 and B.D.E.1 lineages, which were the only lineages circulating during the study period. The presence of these mutations suggests a potential pathway for escape from nirsevimab-mediated neutralisation. Another notable mutation found in our dataset, R209Q, was detected at varying frequencies across different countries.[18,47] The F:209R residue has been identified as critical for antibody-antigen interactions, with the Q209R substitution previously shown to enhance neutralisation efficiency.[17] However, following widespread monoclonal antibody administration, the original F:209Q residue is re-emerging, suggesting a potential reversal of the earlier trend and a reduction in the enhanced antibody binding conferred by Q209R.

Special attention should be paid to changes at positions 65 and 209, as they form non-covalent bond with nirsevimab.[36] Although other mutations were detected at low frequencies within viral populations, they could potentially be selected by nirsevimab exposure and emerge as resistant variants. Continuous monitoring of these mutations is essential to understand their role in viral adaptation and resistance. Furthermore, the development of vaccines and other mAbs targeting the F protein, especially on nirsevimab-targeted Ø epitope, may drive the emergence of mutations in different regions, potentially leading to cross-resistance against other mAbs. Ultimately, this phenomenon could pose challenges by limiting therapeutic options for the prevention and treatment of HRSV infections. Given this scenario, further studies are necessary to refine our understanding of the evolutionary dynamics identified and the mutations found in this analysis. Moreover, in addition to genomic studies, phenotyping characterisation is crucial to gain deeper insight into the effect of single mutation, the viral escape mechanisms and the within-host dynamics of HRSV infections.

The genomic findings of this study provide valuable insights into evolutionary patterns and the impact of selective pressures associated with the widespread use of nirsevimab. Broader geographic and temporal sampling presents an opportunity to strengthen the conclusions of the study.

In conclusion, the findings of this study highlight the complexity of HRSV evolutionary dynamics and the impact of mAb immunisation on these processes. The faster recovery of HRSV-B compared to HRSV-A after the bottleneck effect derived from mAb exposure, suggests that special attention should be paid in seasons where this subgroup is predominant, in order to better understand the evolutionary strategies used to overcome constraints imposed by immunisation. The likely emergence of mutations that may reduce susceptibility to nirsevimab, as well as other mutations located within epitope Ø with uncertain significance, underscores the critical need for active genomic surveillance. Such surveillance will enable the early detection of potential mAb-resistant mutants, facilitating their containment, and later, monitoring their spread, thereby preserving the population-level effectiveness of available immunoprophylaxis.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve readability and clarity. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2025.106567.

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