Review

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Is SARS-CoV-2 Spike Evolution Being Retargeted at the N-Terminal Domain?

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Since 2020, most of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) evolution has been focused on the receptor-binding domain (RBD) of the Spike protein. Nevertheless, the N-terminal domain (NTD) of Spike has been shown to represent the target for neutralizing antibodies, and accordingly, NTD mutations are relevant for immune escape. In 2024, after the introduction of the BA.2.86 saltation variant (heavily mutated at the NTD antigenic supersite), its descendant JN.1 has further explored NTD evolution in its progeny, largely focused on positions 22, 31, 59 and 60. In this review, we explore such convergent evolution in detail and hypothesize the underlying mechanisms.

Keywords: SARS-CoV-2; COVID-19; convergent evolution; receptor-binding domain; N-terminal domain; N-terminal domain evolution; immune evasion; glycosylation; Spike protein mutations

Introduction

In microbiology, convergent evolution results from the combination of mutation rates and evasion of the host immune response [1]. With anti-Spike (S) infection-neutralizing antibodies (nAbs) currently representing the best correlate of protection from Coronavirus disease 2019 (COVID-19) [2–4], convergent evolution in the S protein has been the main focus of investigations. S is a 1273-amino acid protein whose structure is simplified in Fig. 1.

Convergent evolution within the S protein during 2020–2023 has been mostly focused on the Angiotensin-Converting Enzyme 2 (ACE2) receptor-binding domain (RBD, positions 319–541), and especially at the receptor-binding motif (RBM, positions 437–508), with the minor exception of the swinging deletion (del) of HV69-70 [5]. The RBD transitions between a conformation inaccessible to the ACE2 receptor (termed either "closed" or "down") and another that allows binding (termed either "open" or "up") [6–9]. Variations in regions of the S protein far away from the RBD can have allosteric effects on such conformations of the RBD [10–14], with 2 subdomains (termed SD1 and SD2) playing essential roles in modulating S allostery [10]. This review focuses on analyzing the mutation trend of the N-terminal domain (NTD) of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Spike protein and its impact on immune escape and explores its possible mechanisms.

Emerging Mutations in the N-Terminal Domain (NTD)

The NTD of S (amino acid positions 27–293) is another dominant target for nAbs. The NTD is flanked by the signal sequence (positions 1–26) and by the NTD-to-RBD (N2R) domain (positions 293–330) [15]. Although along the pandemic all major lineages had some mutations within the NTD (Fig. 2, Ref. [16,17]), their contribution to immune evasion has been largely underestimated.

It was only in 2024 that we first observed the dominance of SARS-CoV-2 sublineages that have mutations or deletions within the NTD of Spike. The evolution of SARS-CoV-2 in 2024 largely overlaps with the JN.1* subtree (Fig. 3, Ref. [17,18]): we can see that T22, S31 and F59 have been subject to convergent evolution in the second half of 2024, but delS31 convergence is even more evident from the JN.1.11.1 subtree, which all of the dominant sublineages belong to (Fig. 4, Ref. [17,18]), such mutations were often acquired stepwise (with the notable exception of S60P, which lately joined NTD evolution by creating a separate cluster), so that convergent evolution charts can be obtained using software such as ConvMut [18] (Fig. 5, Ref. [17,18]).

Impact of NTD Mutations on Immune Evasion and Viral Fitness

Structural studies found an antigenic supersite located on the pinnacle of the NTD, consisting of the N-terminal region (residues 14–20), a β-hairpin formed by residues 140–

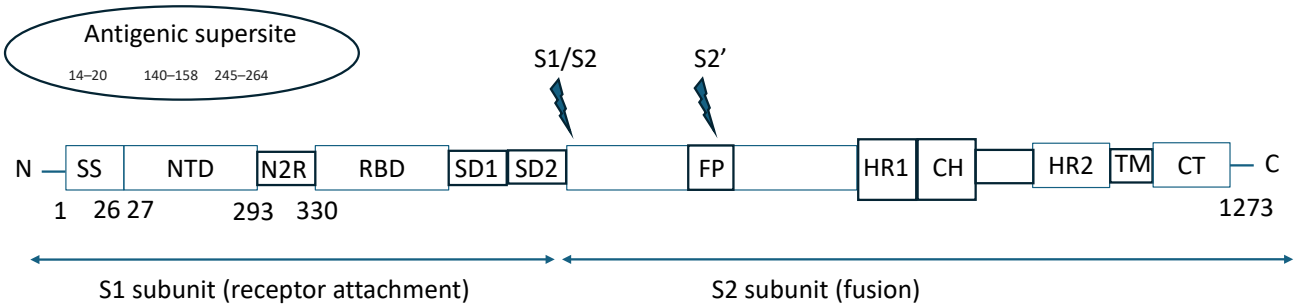

Fig. 1. Schematic representation of regions within the SARS-CoV-2 Spike protein. C, C-terminus; CH, central helix; CT, C-terminus domain; FP, fusion peptide; HR, heptad repeat; SS, signal sequence; N2R, NTD-to-RBD domain; N, N-terminus; NTD, N-terminal domain; RBD, receptor-binding domain; SD, subdomain; TM, trans-membrane; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2. The figure was generated using Microsoft PowerPoint 2021.

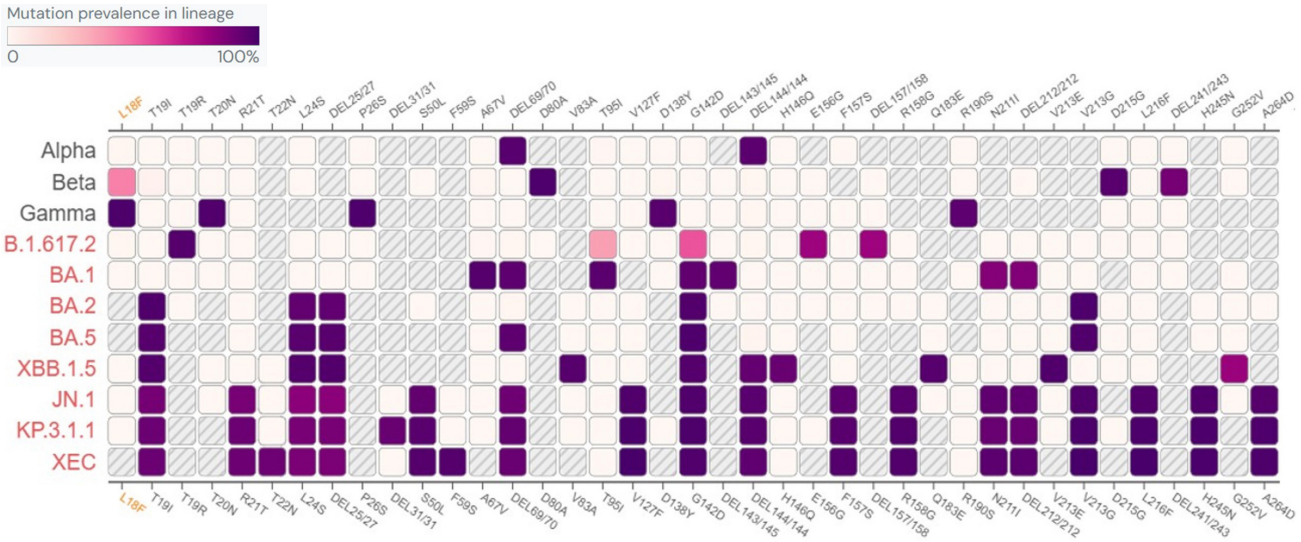

Fig. 2. Mutational spectrum within the Spike NTD in major SARS-CoV-2 sublineages. Generated using Outbreak.info (https://outbreak.info/) [16] based on GISAID data (https://www.gisaid.org/) [17]. The intensity of color represents the prevalence of the mutation.

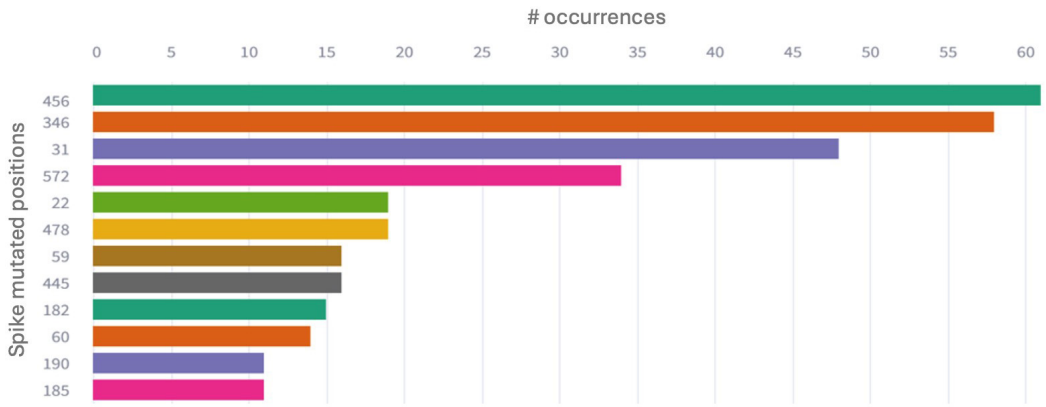

Fig. 3. Number of occurrences of Spike NTD mutations within the SARS-CoV-2 JN.1* subtree. Generated by ConvMut [18] based on GISAID data (https://www.gisaid.org/) [17].

158, and a loop spanning residues 245–264 (supersite loop) [19], with N-linked oligosaccharides at positions N17 and N149. Most neutralizing anti-NTD mAbs contact the NTD supersite near residue R246 by making use of hydrophobic

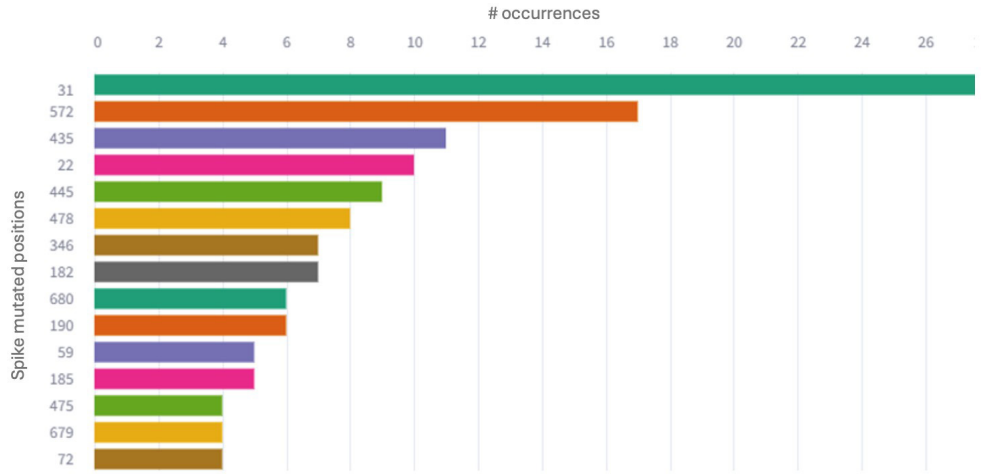

Fig. 4. Number of occurrences of Spike NTD mutations within the SARS-CoV-2 JN.1.11.1* subtree. Generated by ConvMut [18] based on GISAID.org data (https://www.gisaid.org/) [17].

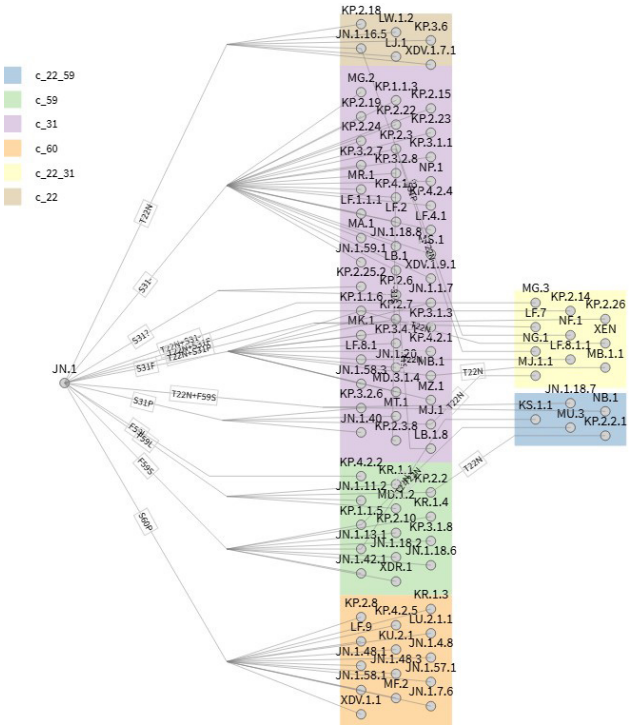

Fig. 5. Phylogenetic JN.1* subtree showing the convergent evolution at key amino acid residues within the NTD (22, 31, 59, and 60). Generated by ConvMut [18] based on GISAID data (https://www.gisaid.org/) [17]. Nodes (lineages) are connected by edges (mutations acquired along a phylogenetic kinship); nodes are grouped into colored clusters when they all acquire the same converging mutations.

residues at the tip of the Heavy Chain Complementarity-Determining Region 3 (HCDR3) loop (such as I94, W105, and W106) to [20]. B.1.1.7, B.1.351 (L18F, D80A, R246I), P.1 (L18F, T20N, P26S, D138Y, R190S), and XBB lineages all harbor frequent mutations within the NTD supersite. Recently, BA.2.86 showed ins16MPLF, R21T, S50L, del69-70 (a well-known site for yo-yo swings [5]), V127F, delY144, F157S, R158G, delN211, L212I, L216F, H245N, and A264D. Out of these, ins16MPLF and del15-23 (within the N1/N2 loop) drive antibody escape from XBB-elicited immunity [21].

The S NTD includes immunogenic epitopes [22,23] and can induce cross-neutralizing antibodies [24]. The NTD was used to evolve through insertions and deletions, as opposed to RBD mutations [15,25–27]. BA.1 harbored delHV69-70 and del143-145, BA.2.75 harbored K147E and W152R, BA.5 harbored delHV69-70, XBB harbored delY144, EG.5.1 harbored Q52H [28], BA.2.86/JN.1 harbored ins16MPLF [29,30], BA.2.87.1 harbored deletions 15–26 and 136–146 [31], and LB.1, KP.2.3 [32], and XDY harbored Q183H and delS31.

Some of these mutations have been linked to enhanced viral fitness in various variants [33], including delHV69-70 for viral infectivity [34], delY144 or K147E+W152R for NTD nAb evasion [35,36], and delS31 for increased infectivity and immune evasion [32]. Despite comparing SARS-CoV-2 sublineages invariably implies considering multiple additional mutations, the exact functional consequences of NTD mutation have been elucidated in several cases in the last months, thanks to bioinformatics and wet biology (Table 1, Ref. [37–41]). Structural analysis found a hydrogen bond between S31 and F59, and their mutations enable both escape from NTD-SD2-directed antibodies and hinder the upward movement of RBD [37]. The NTD has a similarly fundamental importance for anti-RBD mAb escape. In fact, mutations at residues 22 and 31 create novel glycosylation sites that impair RBD-targeting nAbs via allostery [42]. In addition, T22N has been shown to reduce cell-cell fusion, potentially reducing pathogenicity [38].

Pemivibart represents a case study on how NTD can modulate the RBD, and hence sensitivity to therapeutic

Table 1. Synopsis of Spike NTD mutations, their structural and functional consequences, and epidemiological effects.

Spike NTD mutation	Representative lineage(s)	Effect on ACE2 binding	Effect on infectivity	Effect on cell fusion	Mechanistic explanation	Consequences overpopulation immunity	Consequences over anti-Spike mAbs
T19I	Omicron other than BA.1	n.d.	n.d.	n.d.	removal of N17 glycosylation	n.d.	n.d.
T22N	XEC*	no change [37]	no change [40]	reduced cell-cell fusion [38]	novel *N*-linked glycosylation site → reduced S1 shedding [38]	1.5-fold resistance to KP.3.3 BTI sera [40]	n.d.
delS31	KP.3.1.1*, LP.8*	↓ 3.3-fold [37]	n.d.	n.d.	novel *N*-linked glycosylation site at N30 [38] and altered glycoforms at neighboring N61 [41] → impaired upward motion of the RBD [37] and reduced S1 shedding [38]	n.d.	impaired binding of class 4/1 antibodies [37]
S31P	LF.7*	n.d.	n.d.	n.d.	n.d.	n.d.	resistance to pemivibart [39]
F59S	XEC*	↓ 2.3-fold [37]	increase [40]	n.d.	the impaired upward motion of the RBD [37]	1.6-fold resistance to KP.3.3 BTI sera [40]	impaired binding of class 4/1 antibodies [37]

BTI, breakthrough infection; "n.d.", no data available; ACE2, Angiotensin-Converting Enzyme 2; →, consequence; ↓, reduction.

Table 2. Synopsis of the main Coronavirus disease 2019 (COVID-19) vaccines developed so far.

SARS-CoV-2	wild-type	wt+Beta	Delta	Beta+Delta	wt+BA.1	wt+BA.2	wt+BA.4/5	BA.4/5	? (RBD + NTD)	XBB.1.5	XBB.1.5+BA.5	JN.1	KP.2
Moderna	mRNA-1273/elasomeran (Spikevax®)	mRNA-1273.211	mRNA-1273.617	mRNA-1273.213	mRNA-1273.214 Spikevax® (wt/elasomeran +BA.1/imelasomeran)	-	mRNA-1273.222 (Spikevax® wt+BA.5)/ davesomeran	-	mRNA-1283 (refrigerator stable)	mRNA-1273.815/ andusomeran	mRNA-1273.231		X
Pfizer/BioNtech	BNT162b2/tozinameran (Comirnaty®)	-	-	-	BNT162b2 Comirnaty® Original/Omicron BA.1/ riltozinameran	BNT162b5	BNT162b2 Comirnaty® BNT162b6 (Original/Omicron BA.4-5)/famtozinameran	BNT162b7	-	Comirnaty® Omicron XBB.1.5/ raxtozinameran	-	x	X
Novavax	NVX-CoV2373 (Nuvaxovid®; Covovax™ by SII)	-	-	-	-	-	-	NVX-CoV2540	-	NVX-CoV2601 (Nuvaxovid® XBB.1.5)	-		

?, no public information about which SARS-CoV-2 lineage the RBD and NTD in this vaccine belong to.

anti-S mAbs, via allostery. 16 NTD mutations outside the pemivibart-binding epitope are each able to confer full resistance. Such 16 mutations are clustered at 3 sites: (1) T236A, N234H/S, G232C, N196S, and Y200C/D at the NTD-RBD interface; (2) K281E and K304E, located at the interface between the NTD and SD1 or SD2; and (3) mutations surrounding S31, including S31P, P39L, F55L, and R273K. Notably, S31P mutation can alter the NTD conformation in a way similar to the one delS31 creates in KP.3.1.1 and F59S creates in XEC [39].

End-of-2024 State of NTD Evolution

As of 31 December 2024, 3600 SARS-CoV-2 sublineages have been designated by the Phylogenetic Assignment of Named Global Outbreak (PANGO) group, and of them the JN.1* subtree includes 668, all generated in just 1 year.

- All of the 20 JN.1* sublineages that have acquired a mutation at T22 invariably show T22N, which is invariably caused by the single-nucleotide ACT to AAT transversion.
- Position 31 has been the most changing NTD residue in 2024: 25 JN.1* sublineages showed complete deletion of the codon, 2 showed the deletion of the third nucleotide of the codon, 14 showed S31F (driven by the single-nucleotide TTT to TCT transition), and 7 showed S31P (driven by the 2-nucleotide TTT to CCT transitions).
- At position 59, 11 JN.1* sublineages have shown mutations F59S (driven by the single nucleotide TTT to TCT transition), followed by 4 lineages showing F59L (driven by the single nucleotide TTT to CTT transition) and finally a single lineage showed F59I (driven by the single nucleotide TTT to ATT transversion).
- At position 60, 14 JN.1* sublineages have shown mutation S60P (driven by the single-nucleotide TCC to CCC transition).

Conclusions

In the second half of 2024, XEC and KP.3.1.1 have become globally dominant lineages largely thanks to their unique NTD mutations, including delS31 in KP.3.1.1, and T22N and F59S in XEC. At the end of 2024, LF.7.2.1 (Middle East and Europe), MC.10.1, NP.1 (Canada), and LP.8.1 (America) are gaining field. All of these variants harbor the convergent evolution hallmarks within their NTDs. When it comes to anti-Spike mAbs, there are no NTD-targeting mAbs under advanced clinical development so far, and the delusion stemming from the failure of anti-RBD mAbs will hamper their advancement. This manuscript has provided the first systematic summary of the Spike NTD mutation trends in 2024.

While NTD-specific vaccines have been researched and some of them are under clinical development, such recent massive NTD evolution imposes updates to the JN.1-based vaccines (Table 2). While Moderna and Pfizer/BioNTech have already focused on monovalent vaccines based on the more recent KP.2 lineage [43,44], substantial differences have already emerged between the NTD of KP.2 and the NTD of currently dominating sublineages. As always, predicting convergent evolution on the basis of genomic surveillance is providing opportunities to minimize the mismatch between the virus and the vaccine boosts: hopefully, in the coming years scientists and regulatory authorities will exploit these opportunities.

Availability of Data and Materials

Not applicable.

Author Contributions

DF: data curation, formal analysis. TA: visualization, methodology. AB: methodology, validation. DF wrote the first draft; TA and AB critically revised the manuscript. All authors have read and approved the final manuscript. All 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.

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