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Neutral evolution test of the spike protein of SARS-CoV-2 and its implications in the binding to ACE2

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Abstract

As the SARS-CoV-2 has spread and the pandemic has dragged on, the virus continued to evolve rapidly resulting in the emergence of new highly transmissible variants that can be of public health concern. The evolutionary mechanisms that drove this rapid diversity are not well understood but neutral evolution should open the first insight. The neutral theory of evolution states that most mutations in the nucleic acid sequences are random and they can be fixed or disappear by purifying selection. Herein, we performed a neutrality test to better understand the selective pressures exerted over SARS-CoV-2 Spike protein, as well as in four of the identified health concern variants. Lys and Thr have higher occurrence rate on the Receptor Binding Domain (RBD) than in the overall sequence whereas Cys, His, and importantly Arg have low occurrence rate both in the whole protein and the RBD. Amino acids that have lower occurrence than the expected neutral control influence in the stability and or functionality of the protein. Our results show that most unique mutations either for SARS-CoV-2 or the variants of health concern are under selective pressures, which could be related either to the evasion of the immune system, increasing the virus' fitness or altering protein – protein interactions with host proteins. Altogether all these forces have shaped the Spike protein. Understanding the evolutionary forces that act upon Spike protein may help designing better treatments and vaccines that target variants of health concern.

Keywords: Spike evolution, Neutrality test, Phylogenetic analyses, Binding of Spike-ACE2, SARS-CoV-2 variants, Selective pressure

Introduction

The ongoing COVID-19 pandemic caused by the rapid global transmission of SARS-CoV-2[1] illustrates the planetary consequences of recurrent episodes of zoonotic transmission from animals to human populations. At least seven coronaviruses have been identified to infect humans causing principally respiratory difficulties but only three of them pose potential pandemic threats[2–4]. Among the 4 genera of coronavirus, only the *Alphacoronavirus* and the *Betacoronavirus* can infect humans[5]. These two genera have a common ancestor that infects bats while the *Gammacoronavirus* and *Deltacoronavirus* have a bird coronavirus origin[5]. This means that human coronavirus may be directly related to bat coronavirus or to other mammals as intermediate hosts. Phylogenetic analyses have revealed that SARS-CoV-2 is a *Betacoronavirus* related to the bat *Rhinolophus affinis* coronavirus Bat-SL-RaTG13 and the Malayan pangolin (*Manis javanica*) coronavirus, and that SARS-CoV-2 and SARS-CoV belong to the same B lineage, whereas MERS-CoV belongs to the C lineage [1,6].

Structural and genomic analysis of viral components are key for understanding the evolution of the virus and being able to propose therapeutic strategies both to combat the pandemic and to prevent further spread. As all the coronavirus, SARS-CoV-2 recognizes and fuses into the host cells membranes through the Spike glycoprotein[7]. The SARS-CoV-2 Spike glycoprotein (SARS2-S) attaches to the human Angiotensin Converting Enzyme 2 (ACE2) expressed on the cell membrane and is then processed by host's proteases[8] which are necessary for fusion. SARS2-S is made up of the Subunit 1(S1) that contains the Receptor Binding Domain (RBD) and Subunit 2 (S2), responsible for fusion with the cell membrane[9]. Given the essential role of this protein in the virus life cycle, it is assumed that it has undergone strong evolutionary pressures to ensure the propagation of the virus. The human membrane protease ACE2 has been identified as the viral receptor for several coronavirus that infect humans, including other bat SARS-like coronavirus, the SARS-CoV and SARS-CoV-2, as well as the *Alphacoronavirus* hCoV- NL63[6,7]. Different analyses revealed that the RBD of Spike proteins of SARS-CoV-2, SARS-CoV and MERS-CoV allow binding to the receptor from various species while staying within a range of possible mutations[10,11], although neither the binding affinities nor the effect of such mutations on the affinity have been quantified. What has been certainly demonstrated through structural analysis is that the binding affinity of SARS2-S protein to its receptor ACE2 is greater than the one of SARS-CoV to the same receptor[12,13]. The amino acids most probably responsible for the increase in affinity that could have resulted in enhancing the spread of the virus SARS-CoV-2 have already been proposed[4,9,14–16].

Herein, we analyze the sequence of the Spike protein of the SARS-CoV-2 and compare it to the sequences of Spike proteins from other coronavirus, to best fit an evolution model that explains the amino acids preferences that have been selected for a higher affinity binding to the host's receptor. To understand the evolution of the spike protein, we applied an amino

acid substitution test (neutrality test) to identify the amino acids that deviate from neutral mutations. This test is directly related to the degeneracy of the Standard Genetic Code[17] and is applied to both the whole sequence of the Spike protein and to the sequence of the RBD.

Materials and methods

Data sources

The nucleotidic and amino acidic sequences of the Spike protein of 54 coronavirus were obtained from the GenBank (https://www.ncbi.nlm.nih.gov/). There were 6 *Alphacoronavirus* and 48 sequences belonging to *Betacoronavirus* genus. The structures of SARS2-S (6X6P and 6XR8), the RBDs of SARS2-S in complex with ACE2 (6M0J), SARS-S RBD bound to ACE2 (2AJF), were downloaded from the Protein Data Bank (https://www.rcsb.org/). Also, the reference structure of SARS2-S was downloaded from the SARS-CoV-2-dedicated ZhangLab webpage (https://zhanglab.ccmb.med.umich.edu/COVID-19/). SARS-CoV-2 variants' Spike sequences were retrieved from Situation Reports deposited in the site outbreak.info.

Neutral Evolution Model

Forty-eight Spike sequences of *Betacoronavirus* were pairwise aligned. Each pair of protein sequences was aligned using MUSCLE[18] with default parameters. The protein alignment was used as template to derive a nucleotide alignment that would not have gaps that could split codons. From the nucleotide alignment a table of mutations was computed that account for the total of changes in codons. The table of codon mutations was transformed into an amino acid mutation matrix by adding up the values of the codons for a given amino acid. Hence, this matrix considers synonymous and non-synonymous mutations. The amino acid mutation matrix was computed for every pair of sequences and added up. Then, the matrix was normalized by rows, so that each row adds up to 1, and yields a probability transition matrix. The stationary distribution of the probability transition matrix was derived and compared to the control of neutral evolution as described in[17]. To assess the statistical robustness of the sample of sequences, a jackknife procedure was applied. The procedure of deriving the stationary distribution from the probability transition matrix of a sample of sequences was repeated to all possible subsets of 50 sequences. A confidence interval of 95% was computed around the stationary distribution derived from the set of 48 sequences. The whole process was also applied to a set of 9 RBD ACE2 sequences and a confidence interval of 95% for the stationary distribution was computed and compared to the neutral control of evolution.

Phylogenetic analysis

All the evolutionary analyses were conducted with MEGA X software[19]. The multiple alignments of the spike sequences were performed with MUSCLE algorithm. The test for

the best evolutionary method for the *Betacoronavirus* Spike sequences resulted to be WAG+G+I+F, where the Invariable (I) value was 0.081 and the Gamma (G) value was 1.143. Then a phylogenetic tree was constructed by Maximum Likelihood analysis. Another evolutionary test model for the Spike sequences that bind to ACE2 was also WAG+G+I+F, where I is equal to 0.064 and the G value was 6.54. SARS2-S sequence was compared to both groups i) the most proximal amino acid (a.a) sequences and ii) the ACE2 binding CoVs. Consequently, unique mutations for SARS2-S and conserved residues were identified manually using both groups.

Structural analysis

The structures were cleaned to have the most accurate protein and complexes of the RBDs to its receptor. The structural analysis was visualized and analyzed with Chimera[20], and I-TASSER[21–24]. The complex of SARS2-S RBD with the receptor was used to point out unique mutations and conserved residues. Distances between the amino acids involved in the protein – protein interaction were computed. Other parameters like the hydrophobicity and electrostatic potential were calculated for the a.a. in the interface. The same was calculated for the complex SARS-S RBD with the same receptor. The number of contacts, number of hydrogen bonds, hydrophobicity and mean distances were compared. Glycosylation sites were identified in both the linear and structural model. Besides, Spike mutations of the most prominent health concern variants of SARS-CoV-2 were identified in the three-dimensional model of SARS2-S and the structural models were predicted for each of them with I-TASSER platform. The reference structure was downloaded from the SARSCoV2-dedicated ZhangLab webpage (https://zhanglab.ccmb.med.umich.edu/COVID-19/) inhere the accurate predicted and curated structures of all the SARS-CoV-2 proteins are deposited. For each mutation physicochemical characteristics were discussed.

Results

The neutral theory of molecular evolution assumes that evolution is driven by random stochastic point mutations that eventually may be fixed by genetic drift or natural selection. From this point of view, we applied a neutrality evolution model to better understand the type of selective pressure acting upon a.a. present in the Spike protein[17]. This analysis revealed that in the *Betacoronavirus* genus Trp, Cys, His, Gly, Pro, Leu, Ser, and Arg underwent negative selective pressures, as the number of changes in these a.a. are lower than the expected by neutral evolution. In contrast, Tyr, Lys, Gln, Phe, Asn, Asp, Thr and Val, displayed positive selection (**Figure 1**). Mutations giving Met, Glu, Ile and Ala exhibited neutral or nearly neutral forces. To accurately analyze the great adaptation of SARS-CoV-2 to its receptor, we tested the neutrality of mutations in the RBD of the ACE2 binding sequences, which are crucial for specific receptor recognition, and thus for infection. Importantly, Cys, His, Gly, Pro, Val, Ala, Leu, and Arg showed negative selection, whereas Lys, Gln, Phe, Asn, Asp, Ile, Thr, and Ser manifested positive selection

(**Figure 1**). This means that it is less likely to find an Arg that appeared by random mutation than a Lys or a Thr, because the hexa-codonic Arg is under high negative selective pressure.

For the phylogenetic analysis, we carried out a multiple alignment of the a.a. sequences of Spike proteins of coronavirus including *Alphacoronavirus* and *Betacoronavirus.* Consequently, we identified the most related Spike sequences to SARS-CoV-2 (**Figure 2**). In agreement with previous analysis, the phylogenetic tree computed shows that the bat coronavirus RaTG13 Spike protein exhibits the highest similarity with the SARS2-S followed by the pangolin coronavirus (PnCov) Spike protein[6]. As expected, the S2 subdomains had a high degree of similarity, so, we focused in the RBD sequence of the S1 subdomain to identify mutations that could be advantageous for SARS2-S binding to ACE2. Therefore, a multiple alignment was performed including the Spikes of SARS-CoV-2, RaTG13, PnCoV, and several Spikes known to bind to human ACE2[8]. We discarded the *Alphacoronavirus* HCoV NL63 because even though it binds to the human ACE2, the orientation of the RBD is completely different. We identified point mutations in the RBD of SARS2-S that could be responsible for binding. Most a.a. were conserved among all the sequences, but there are few mutations that are unique for SARS2-S (**Table S1**). Some of these mutations are present in CoV RaTG13 and PnCoV Spikes, what would suggest that these spike proteins could bind to the human ACE2. Interestingly, most mutations are located at the interface with ACE2 **(Figure S1)**. **Figure 3a** shows the interaction between SARS2-S RBD and ACE2 and **Figure 3b** show a close- up where the side chains of the amino acids involved in the protein-protein interaction are shown with sticks. The conserved residues are shown in pale pink as the rest of the structure (*i.e.*, Tyr 449, Tyr 453, Asn 487, Tyr 489, Thr 500, Gly 502, Tyr 505) (**Table 1**) while the a.a. that are unique for SARS2-S, are shown in red **(Figure 3b**). Among the 17 a.a. involved in the interaction, 10 are unique for SARS2-S, including Lys 417, Gly 446, Leu 455, Phe 456, Ala 475, Phe 486, Gln 493, Gly 495, Gln 498 and Asn 501 (**Table 2**). Compared to SARS-S, SARS2-S forms more hydrogen bonds with the receptor, 8 and 11, respectively. This is because all a.a. involved in forming hydrogen bonds are shared, except for 2 mutations that lead to the formation of new hydrogen bonds with the receptor's surface (*i.e.*, Gly 446 and Lys 417). Apart from the identification of a.a. that are unique for SARS-CoV-2, our analysis revealed that a.a. important for maintaining the structure of both the domain and the complete protein, such as Cys residues as well as the glycosylated a.a. (*i.e.* Asn) and most Gly and Pro, are highly conserved.

Moreover, we measured the distances between the amino acids at the interface of SARS2-S with ACE2 and compared them with the distances of SARS-S with ACE2 (Supplementary Information). Seventeen a.a. of SARS2-S contact 17 a.a of the receptor, with a mean distance of 3.563 Å. In contrast, 15 a.a. of SARS-S contact 18 a.a. of the receptor, at a mean distance of 3.605 Å. We also compared the hydrophobicity and electrostatic potential of the

interface of the spike proteins, and we observed that both have similar values (SARS2-S interface: minimum -27.2, mean -4.743 and maximum 22.83 of hydrophobicity potential while SARS-S's values are -26.41, mean -3.47 and maximum 23.19) (**Figure 4**). Both Phe 456 and Phe 486 in SARS2-S generate more hydrophobic contacts than the Leu at the same positions present in SARS-S. Also, there is slightly bigger hydrophilic surface at the other edge of the interface in SARS2-S. All these factors may contribute to the higher binding affinity of SARS2-S to ACE2 that has been reported[12,13].

Then we concentrated in the mutations of spikes proteins of SARS-CoV-2 variants to unravel the evolutionary behavior of the virus. The variants that have received most attention due to their importance for public health are those identified in the United Kingdom (B.1.1.7/ UK), in South Africa (B.1.135/ SA), in Brazil (B.1.1.248 / P.1/ BR), and in California USA (B.1.429/ CAL.20C/ CL). Interestingly, three variants share two mutations N501Y and D614G (UK, SA, BR variants). Tyr is a positive selected a.a. for the complete spike protein of *Betacoronavirus*, but neutral for the RBDs of ACE2 binding sequences. This means that in this domain, Tyr is not subjected to any selective pressure and therefore this change occurred randomly. In contrast, these same variants have a Gly which is clearly negatively selected at position 614 (**Figure 1**). It is important to note that the first reported variant was the one isolated from UK which has the highest percentage of neutral mutations (*circa* 30%), probably because selective pressures had not shaped the variant yet. The structure of the spike protein of SARS-CoV-2 and the variants of concern are illustrated in **Figure 5**. The structure of SARS2-S is shown with a zoom of the RBD painted in green (**Figure 5A**). Cys of the RBD are shadowed in yellow and the two glycosylated Asn are magenta. All sites of point mutations in the variants are shadowed in cyan and deletions in grey. Predicted structures of four SARS-CoV-2 variants (UK, BR, SA, and CL) with mutations shown in cyan are shown in **Figure 5B**, as well as the comparison with the reference structure. At the center, the reference structure overlapped with the predicted structure of variants. To note, the predicted structure of BR and CL variants protrude the RBD at a different position than that in the reference structure and the UK and SA variants. In total, the UK's variant had 1 positively selected mutation, 3 negatively selected, and 3 neutral mutations, besides three deletions (**Table 3**). SA variant has 2 a.a. positively selected, 1 a.a. negatively selected and 2 neutral mutations. Additionally, the BR variant shares a third mutation with the one first isolated from South Africa, E484K; Lys is under positive selective pressure. In total, the BR variant has the higher number of mutations: 6 positively selected, 3 negatively selected and 2 neutral. Lastly, the CL variant has only 2 negatively selected and 1 neutral mutation.

Finally, we looked at residues that are potential glycosylation sites. For SARS2-S the reported glycosylation sites of two of the structures 6X6P and 6XR8 revealed that there are 14 Asn forming glycoside bonds (**Table 4**). Compared to the other ACE2 binding sequences, three Asn (17, 149 and 657) are unique to SARS2-S, while the rest (11) are

shared. To note, PgCoV and bat CoV RaTG13 express all potential glycosylation sites identified in the causal agent of COVID-19. These three unique Asn could have been important for the spread of the viruses previously mentioned but not directly affecting binding to the receptor ACE2. However, it seems that most Asn with the capability to form a glycosidic bond are unlikely to mutate.

Discussion

The neutral theory of evolution states that most mutations in nucleic acid sequences are random, and these can be fixed by different evolutionary mechanisms. We exploited a neutrality test to interrogate the molecular evolution of the spike protein. Herein, we constructed the neutral evolution model for spike proteins of *Betacoronavirus*, focusing on the evolution of the RBD and the possible implications for binding to its receptor. Positive selective pressures cause a.a. to be fixed in higher frequencies than neutral mutations, while negative selective pressures cause a.a. to appear in lower frequencies than a neutral variant. Therefore, most fixed mutations under negative selective pressures remain because they are advantageous for the protein either increasing stability, affinity to a ligand, or others. These pressures are similar among homologous proteins of similar organisms and, importantly, they are the major driving forces for adaptation.

Applying the neutrality test, we identified the amino acids that had suffered negative selection through evolution of spike proteins of *Betacoronavirus*. One of these is Trp, that is encoded by only one codon and thus its frequency of occurrence is expected to be the lowest. However, Arg, Pro, Gly, His and Cys that can be coded by 6, 4, 4, 2 and 2 codons respectively, appeared at frequencies significantly lower than the expected by neutral mutations. Of note the polybasic motif (RRAR) in SARS2-S which is a tremendously important site for infection, resulted from the insertion of the motif PRRA (4 a.a. under negative selection). This insertion has been a crucial virulence factor that enables the cleavage by furin protease which generates a neuropilin-1 binding motif that enhances internalization[25,26]. Within the context of the neutral theory of evolution, the probability for inserting these four a.a. was very low. However the insertion of a furin cleavage site (RXXR) is not new in CoVs[27]. This motif implies the insertion of 12 nucleotides, but since Arg is hexa-codonic and Ala is tetra-codonic, the probability of appearance of this sequence was not that low. Once presented, it may have remained because this motif has provided a high increase in virulence to the etiological agent of COVID-19. Other *Betacoronavirus* present similar polybasic motifs but they were achieved by point mutations rather than by an insertion of four amino acids, such as, the Spikes of the Murine Hepatitis Virus (YP009824982.1), Murine CoV RA59/R13 (ACN89689.1), Murine CoV RA59/SJHM (ACN89705.1), Rat CoV (YP003029848.1), HCoV HKU1 (YP173238.1), HCoV OC43 (YP009555241.1), Rabbit CoV (YP005454245.1), Canine CoV (AQT26498.1), Human enteric CoV (ACJ35486.1), Bovine CoV (NP150077.1) and the Sambar deer CoV US/OH-WD388TC/1994 (ACJ67012.1).

The neutral evolution model applied to the RBD sequences that bind to ACE2, shows that there are amino acids that have similar selective pressure as within the whole protein. The negatively selected a.a. such as Cys, His, Gly, Pro and Arg are more likely to affect the thermodynamic stability of the protein, which could impact the structure, the function, or protein- protein interactions. Thus, they are conserved in certain positions and it is unlikely that they appear by mutation in other positions. For example, eight Cys establish disulphide bonds that preserve the structure of the RBD (C336-C361, C379-C432, C391-C525, and C480-C488). In contrast, Asn has been positively selected such that the probability of finding an Asn that appeared by mutation is higher. This does not mean that Asn in certain positions are not conserved; in the RBD, the two glycosylated Asn (N331, N343) are conserved and mutations in these positions are not favored. The alignment with sequences of other RBD suggests that those Asn residues are probably glycosylated as well. Like Asn, the amino acids Glu, Lys, Gln, Phe, Asp and Thr have higher occurrence than predicted by neutral mutations; this means that there must be a selective pressure that favors them.

Interestingly, in the RBD, Lys and Thr are the two a.a. which show the highest deviation from the neutral mutation model; their chemical characteristics may favor interaction with the receptor or may be important for maintaining the domain's structure. This is not related to the predicted probability of occurrence, which is relatively high for Thr but relatively low for Lys (**Figure 1**). Lys is a basic a.a. coded by 2 distinct triplets (AAA and AAG) and the side chain consists of four carbons ending with an amino group which gives it a positive charge. Thr is a small and polar amino acid which has a hydroxyl group; and in contrast to Lys it is coded by four different codons (ACU, ACC, ACA and ACG). By neutral mutation, the probability of occurrence of Thr is higher because any mutation in the third position maintains Thr, therefore a mutation in any of the first two positions, or an insertion or deletion that moves the reading frame are required for a non- synonymous mutation.

The non-polar Phe has been positively selected in the RBD of SARS2-S where there are two Phe involved in the interaction with ACE2, Phe 456 and Phe 486. Both substitute for Leu expressed in other ACE2 binding sequences which contribute to generate more hydrophobic interactions with atoms from ACE2: SARS2-S Phe456 with Asp 30, Thr 27, Lys 31 from ACE2 and SARS2-S Phe486 with Leu 79 and Met 82 and Tyr 83 from ACE2. These hydrophobic interactions were probably the driving force for maintaining the mutations. In comparison to the neutral model of the whole spike protein, Tyr has neutral evolution in the RBD, meaning that there is no selective pressure that favors or disfavors the mutations towards Tyr. However, there are four Tyr in the interface with the receptor, and three variants of SARS-CoV-2 also express a fifth one. This may suggest a great importance of Tyr's properties in protein – protein interactions.

The variants of SARS-CoV-2 were analyzed with the same neutrality test. The UK variant shows several mutations that could contribute to its higher transmissibility. The P681H substitution may cause slight differences in the secondary structure immediately before the

304 furin cleavage site. Pro introduces slight bends to protein structures because the amino
305 group is binding both the αC and the lateral R group in a cyclic form. The resulting
306 structure could change the susceptibility for furin cleavage, but further analyses are needed
307 to confirm this. Interestingly, three deletions of amino acids in this variant do not change
308 the structure and function of the protein, but probably contribute to the decrease of
309 recognition by patients' serum.

310 The mutation D1118H of UK variant introduced another His in S2 that may alter the
311 structure and function because the opposite charge. However, other mutations may not
312 influence the structure and function of the protein. In this same variant, UK, the substitution
313 S982A transduces into an a.a. under neutrality but in the same group according to its polar
314 requirement[28,29]. In the SA variant, the mutation A701V turns to an amino acid with
315 positive selection through a mutation in the second position of the triplet. The Brazil variant
316 is the variant with more mutations all along the Spike protein so far described, and
317 furthermore most of these mutations imply changes towards a.a. with positive selection,
318 such as L18F, T20N, D138Y and H655Y. Also, P26S and R190S changed to Ser (slightly
319 negative selected) and T1027I is a neutral mutation. The California variant has only one
320 mutation drove by neutrality S13I, and two mutations negatively selected W152C and
321 L452R, the first resulting in the change of a.a. with similar polar requirements, and the
322 second mutation involving a.a with different polar requirements.

323 The reference sequence of SARS2-S has Lys 417 which enables the formation of a
324 hydrogen bond with the receptor in comparison to other ACE2 binding sequences, however
325 SA and BR variants substitutes this into an Asp (K417D). It remains to confirm whether if
326 these Spikes could be able to maintain the hydrogen bond as the N atom of the R chain
327 which was donating the electron, is absent. Similarly, the mutation E484K, shared with BR
328 variant too, occurs towards an a.a. with almost the same polar requirement even though the
329 charge changes. This mutation leads to the a.a. with the highest positive selection. Other
330 shared mutation is D614G substitution which is present in UK, SA and BR. The mutation
331 translates to a small a.a. without charge which is negatively selected. It has been proven
332 that this substitution increases binding to ACE2 in comparison with the ancestral virus,
333 therefore infectivity and transmission of the variant also has raised[30–32]. D614G alters the
334 affinity to the receptor due to a conformational change that causes the RBDs to turn into the
335 up position, which is necessary for receptor recognition [33]. Besides, N501Y substitution
336 shared by the same variants probably alters more the binding to the receptor rather than
337 favoring evading the immune response. Actually this substitution enables infection to mice
338 cells through interaction with mouse ACE2[34]. It is probable that the strong selective
339 pressure exerted on both mutations, has already drove them to fixation in human
340 populations by genetic drift. Shared mutations among different health concern variants may
341 arose by convergent evolution coming from a strong selection.

Mutations in the S2 subdomain would probably be less frequent to reach fixation because the sequence is crucial for the function, as consequence, there is a high degree of conservation. This subdomain is involved in fussing with the membrane, so non- polar amino acids are required. On the contrary, S1 is very important for receptor recognition, specifically the Carboxyl Terminal Domain (CTD) where the RBD is contained, so the sequence of this domain is crucial for adapting to the actual host or for the ability to infect other species. Interestingly, the first reported variants had more mutations in S2, and this number seems to progressively decrease in the latter variants. The exact biological significance of this observation is not clear. It is probable that the last variant reported could either i) had been mutating before and by the time it was reported it may have had enough time to fix or disappear neutral mutations at S2, or ii) it may have appeared recently and have mutated only in S1. Either way, the fact that Cal.20 variant had few mutations draws attention. Now, we cannot sustain any hypothesis because we discussed only four variants and we lack the accurate mutation rate of each variant and the transmission rate at different places.

Also, the accumulation of mutations is linked to the capability to correct errors. Coronavirus have RNA-dependent RNA polymerases which are prone to mistake, unlike other RNA virus, they have also a 3' to 5' exoribonuclease (nsp14-ExoN) that proofreads the new sequence [35]. Nsp14-ExoN is one of the major factors enabling long and stable RNA genomes. Therefore, the accumulation of errors slows down, and synonymous mutations become the most frequent. However, the neutrality test constructed here considers both synonymous and non-synonymous mutations, and this allowed us to obtain information of the types of selective pressures that influenced deviation from neutral mutations.

In other Coronavirus the highly glycosylated Amino Terminal Domain (NTD), play an important role in attachment to the host cell and immune response evasion. For influenza C virus and some coronavirus (HCoV HKU1 and HCoV OC43), attachment through 9-O-acetylated sialic acid receptors is crucial and constitute another species barrier [36,37]. The interaction with specific hosts' proteins facilitates the approaching of the fusion machinery to the cell membrane. Furthermore, saccharides mask potential epitopes recognized by antibodies, making it difficult to the immune system to eliminate the virus. It has been observed that the Spike glycoprotein must be shielded by the protective glycans from the immune system[38]. It remains to be determined whether the mutations near glycosylation sites interfere with the formation of glycosidic bonds in the variants of concern. Although L18F, T20N, P26S and D138Y substitutions from the Brazil variant and del144 from the UK variant are not precisely replacing glycosylation sites, they have been reported reduce recognition by antibodies[39]. Besides probably affecting glycosylation, these mutations could create new epitopes exposed on the protein surface making previous antibodies unable to recognize them. Hence, it is important to undercover whether if the glycosylation

of SARS-CoV-2 spike protein influence receptor recognition, membrane fusion, or immune evasion. Knowing the role of saccharides bound to the spike protein could help us understand more of the physiopathology of the virus and to develop better prophylactic or therapeutic strategies, effective against all variants.

Conclusion

The long-lasting pandemic, the wide geographic distribution, and the rapid contagiousness mainly during epidemic waves, have influenced the generation of variants of SARS-CoV-2. At global or local scale, the evolution of this virus can be appreciated. Vaccines and drugs have been developed and tested aiming to stop transmission which would also result in preventing the virus from mutating and developing new variants that cannot be recognized by newly developed treatments. Therefore, evolutionary studies play an important role in the prevention of epidemiological catastrophes and in the development of better treatments that covers most viral variants. The first evolutionary mechanism to consider is the fact that most mutations occur stochastically, meaning that neutral mutations are not the result of a selective pressure and do not respond to conferring any advantage or disadvantage to the virus. Some of them will be maintained until another substitution occurs; however, there are selective pressures that influence the fixation or disappearance of mutations. Selective pressures are similar among the viral particles in different hosts, and in the long term they shape proteins. Important selective pressures for SARS-CoV-2 evolution are related to the pathogenesis as mutations that improve fitness, alter interactions with host's proteins or evade the immune response. Consequently, they select mutations and conduct to adaptation. Shared mutations with different geographical origins may have been subjected to common selective pressures over specific residues, meaning that each mutation must have a given a significantly advantage.

Comparison of homologue proteins enables to construct an evolutionary model for stochastic mutations. The neutrality test computed shows the type of selective pressure for each amino acid in the spike protein of *Betacoronavirus*. This evolutionary study enables to understand and describe changes in SARS2-S sequence that affects its stability, structure, or function. One of the most relevant mutations is the insertion of a four- a.a. motif that allows the cleavage of a protease, that despite not being favored, a clear advantage in terms of the virus transmissibility won over neutral evolution mechanisms. Furthermore, selective pressures in the RBD favored the ability of SARS-CoV-2 to infect humans and chemical features were gained (*i.e.* increasing the number of hydrogen bonds and forming more hydrophobic contacts with the receptor). To note, SARS-CoV-2 continues to evolve rapidly throughout the globe, generating lineages with accumulated mutations. Here it has been shown that most of these mutations have been selected by selective pressures. Yet, most mutations in the interface and the ones in the variants of interest were favored implying other evolutionary mechanisms such as selection as important driving forces in the spike

419 glycoprotein that have enhanced the viral transmission. In the end mutations have allowed
420 SARS-CoV-2 to become a threat to mankind, on the scale of a pandemic.

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Figures and tables legends

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Figure S1 Protein- protein interaction between RBD of SARS2-S and ACE2. **A)** Tridimensional structure of the RBD (red) of the Spike of SARS-CoV-2 interacting with ACE2 (blue) and **B)** the linear representation of Spike protein showing the location of the RBD. Each loop in contact with the receptor is colored as in Table S1.

Table 1. Conserved residues involved in protein- protein interaction with ACE2 among the ACE2 binding Spikes. The selection type according to the neutrality test are indicated.

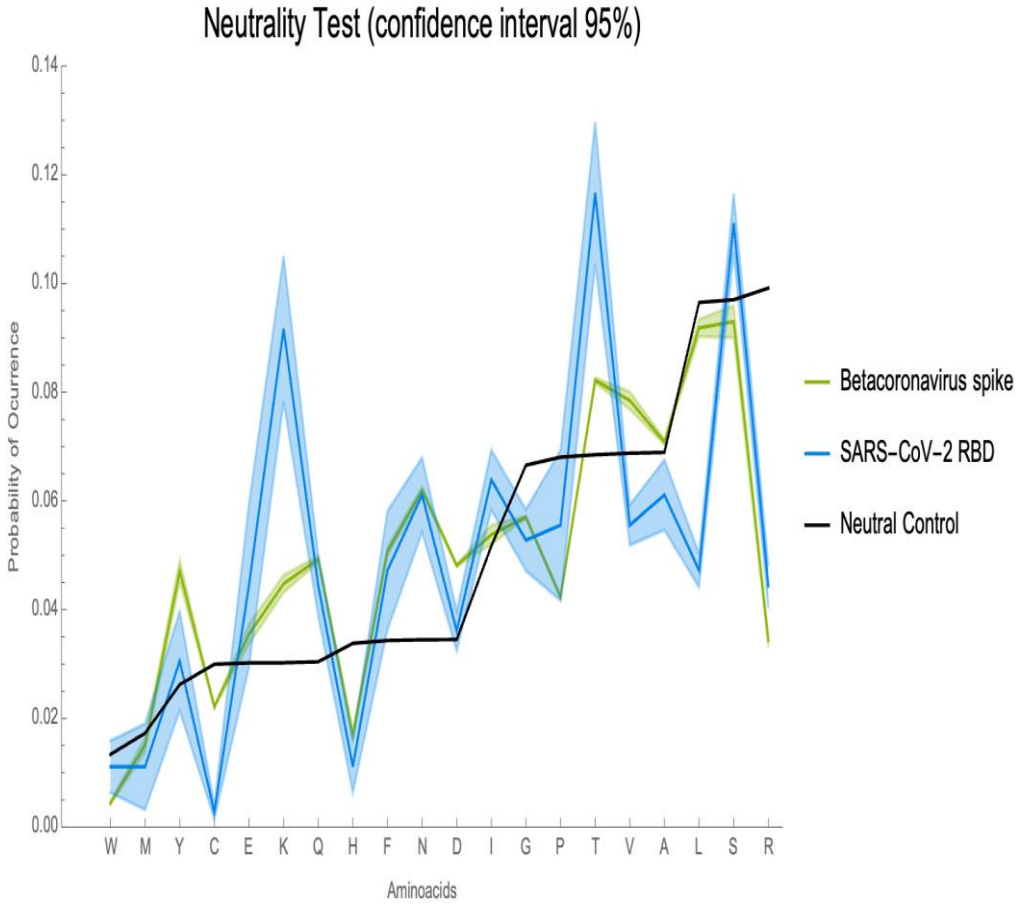
Table 2. Unique residues for SARS2-S involved in protein-protein interaction with ACE2. The selection type according to the neutrality test are mentioned specifically for SARS2-S. Other ACE2 binding Spike proteins expressed different a.a. Here SARS-S is shown as an example. The empty spaces in SARS-S are a.a. that do not interact with ACE2.

Table 3. Type of selective pressure for mutations in health concern variants. The mutations colored by physicochemical properties are enlisted for each variant and the type of selective pressure applied for each a.a. is shown. The type of pressure is specified either for the whole protein or for amino acids positioned in the RBD.

Table 4. Potential glycosylation sites. Comparison of the glycosylation sites reported from the structures of SARS2-S with all the ACE2 binding sequences.

Table S1. Unique mutations in the RBD for SARS-CoV-2. Compared to the other ACE2 binding Spikes, SARS-CoV-2 Spike protein has 49 mutations in the RBD, the majority selected by a positive pressure. The point mutations of the SARS-CoV-2 Spike RBD are listed with the corresponding amino acid expressed in the rest of the Spike proteins that bind to ACE2; the amino acids are highlighted depending on the chemical nature: in yellow the non-polar, in green the polar and neutral amino acids, in blue the positively and in red the negatively charged amino acids. Amino acids in contact with ACE2 are in bold type letters.

589 **Figure 1**



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595 **Figure 2**

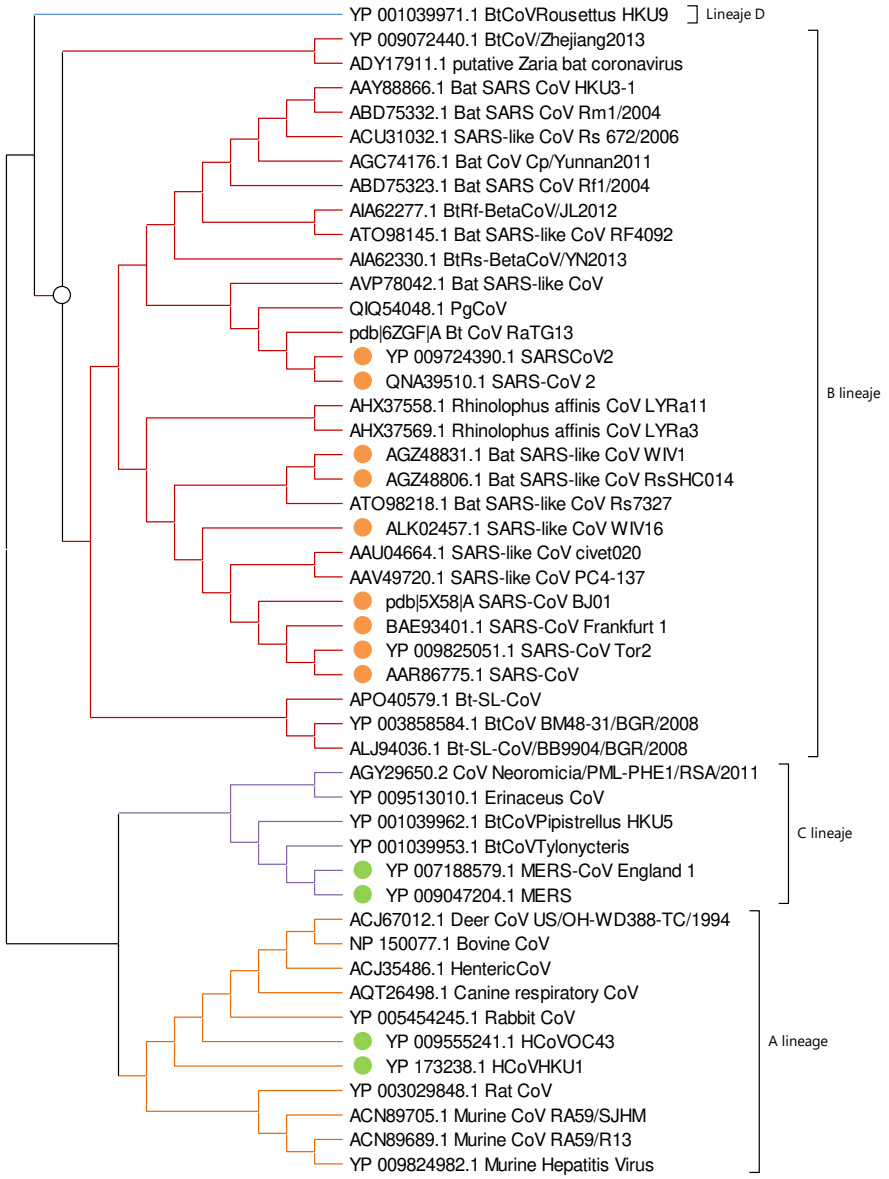

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Figure 3

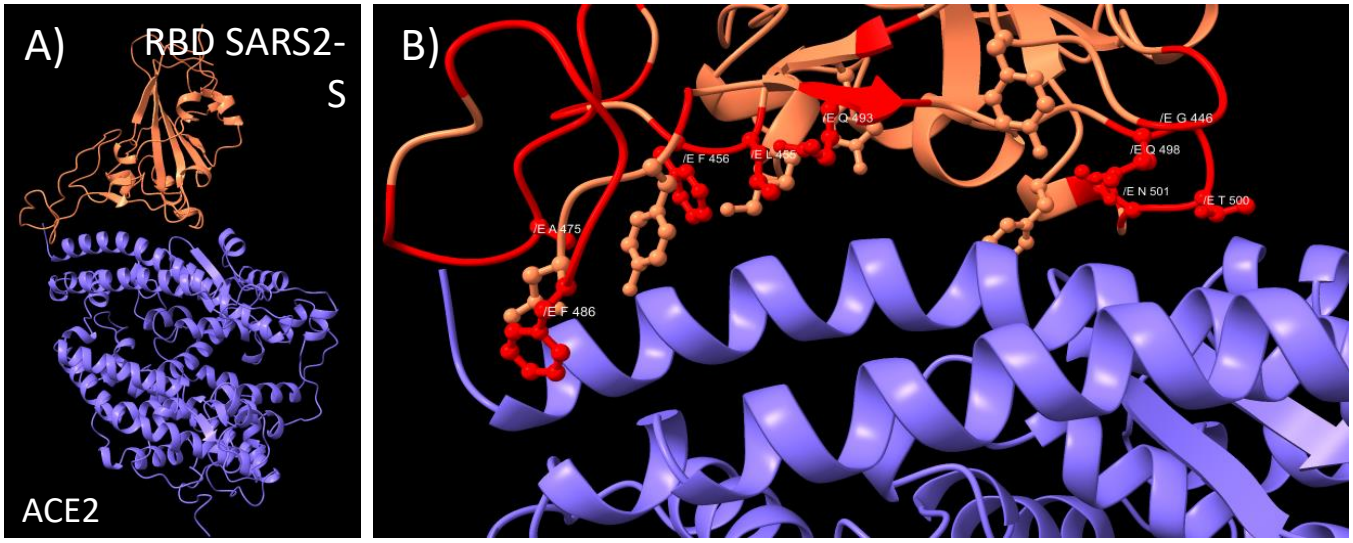

Figure 4

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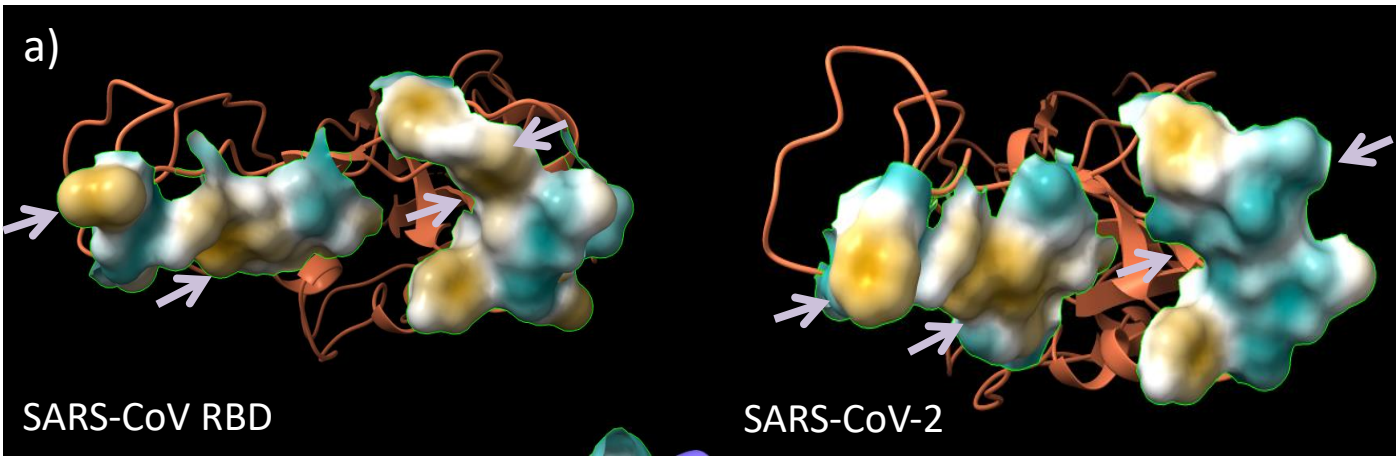

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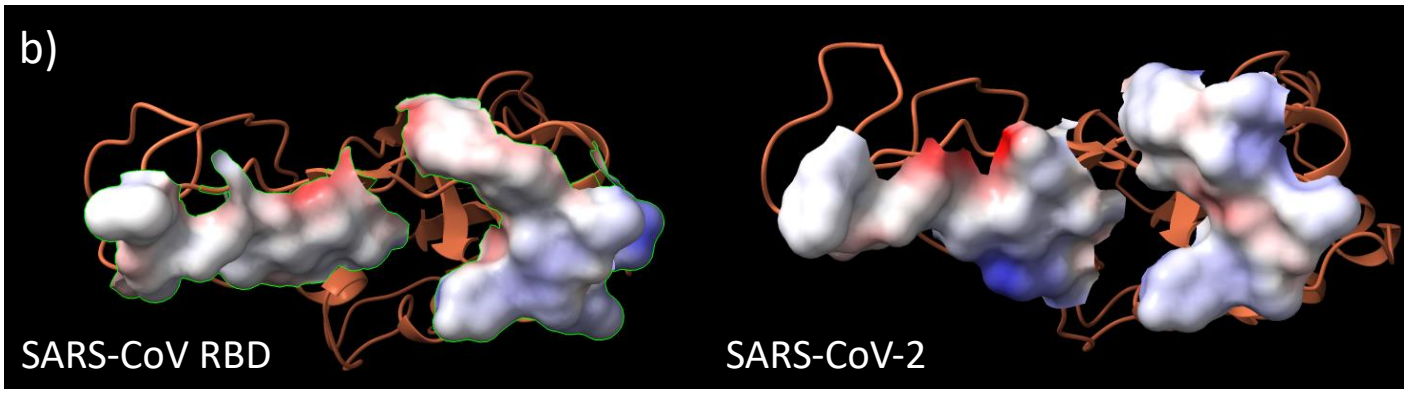

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Figure 5

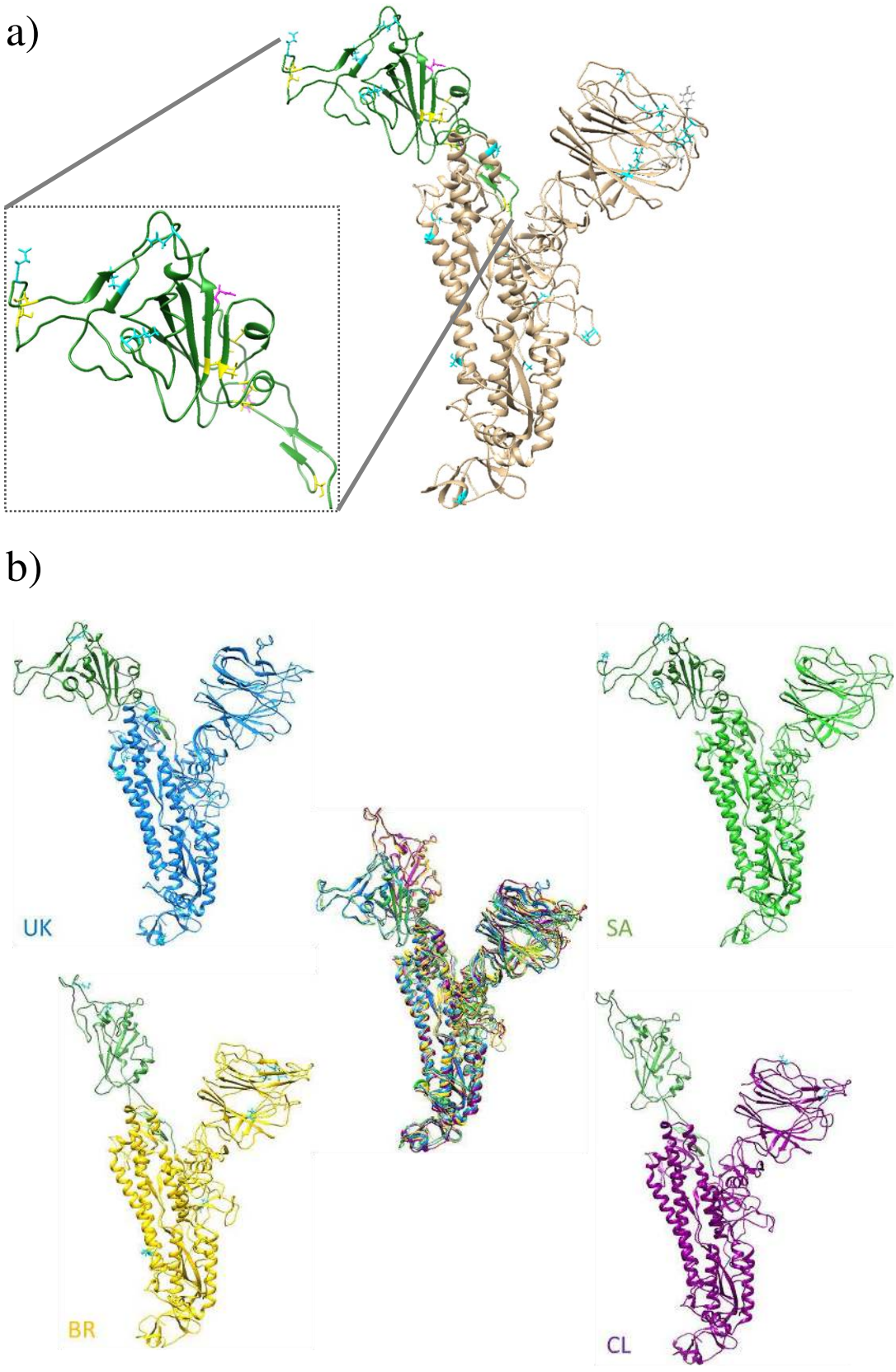


Table 1

Conserved a.a.		Selection according to neutrality
449	Y	*Neutral*
453	Y	*Neutral*
487	N	*Positive*
489	Y	*Neutral*
500	T	*Positive*
502	G	*Negative*
505	Y	*Neutral*

a.a. code	Polar

Table 2.

SARS2-S		SARS-S		Selection according to neutrality
417	K			*Positive*
446	G	433	T	*Negative*
455	L	442	Y	*Negative*
456	F	443	L	*Positive*
475	A			*Negative*
486	F	472	L	*Positive*
493	Q	479	N	*Positive*
496	G			*Negative*
498	Q	484	Y	*Positive*
501	N	487	T	*Positive*

a.a. code	Non-polar	Polar	Polar positive

Table 3.

						Selective pressure	
Position	SARS2	UK/ B.1.1.7	SA/ B.1.351	BR/ P.1	CL/ CAL.20C	Whole protein	RBD
13	S				I	*Neutral*	
18	L			F		*Positive*	
20	T			N		*Positive*	
26	P			S		*Negative*	
69	H	*					
70	V	*					
138	D			Y		*Positive*	
144	Y	*					
152	W				C	*Negative*	
190	R			S		*Negative*	
417	K		N	T			*Positive*
452	L				R		*Negative*
484	E		K	K			*Positive*
501	N	Y	Y	Y			*Neutral*
570	A	D				*Positive*	
614	D	G	G	G		*Negative*	
655	H			Y		*Positive*	
681	P	H				*Negative*	
701	A		V			*Positive*	
761	T	I				*Neutral*	
982	S	A				*Neutral*	
1027	T			I		*Neutral*	
1118	D	H				*Negative*	

a.a. code	Non-polar	Polar	Polar positive	Polar negative

* Deletions

Table 4.

SARS2-S	Conserved with ACE2 binding sequences	a.a. substitution
17	No. Only SARS2-S branch	T
61	Yes	
149	No. Only SARS2-S branch	T
165	Yes	
234	Yes	
282	Yes	
331	Yes	
343	Yes	
603	Yes	
616	Yes	
657	No. Only SARS2-S branch	D
709	yes	
1098	yes	
1134	yes	

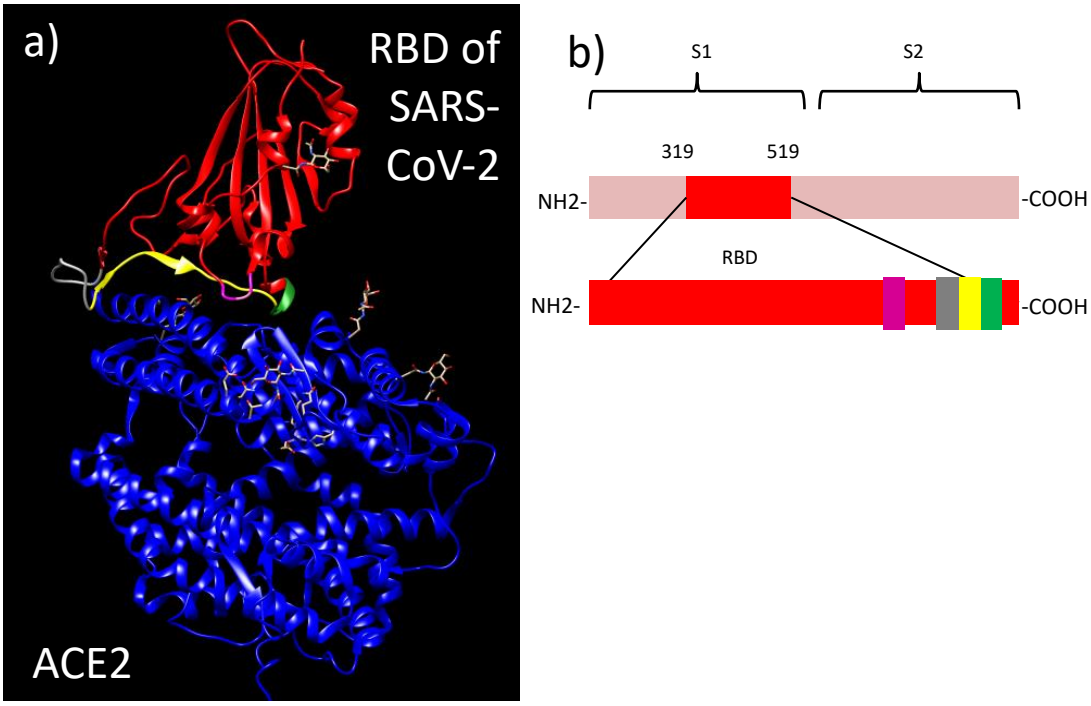
Supplementary information

Table S1.

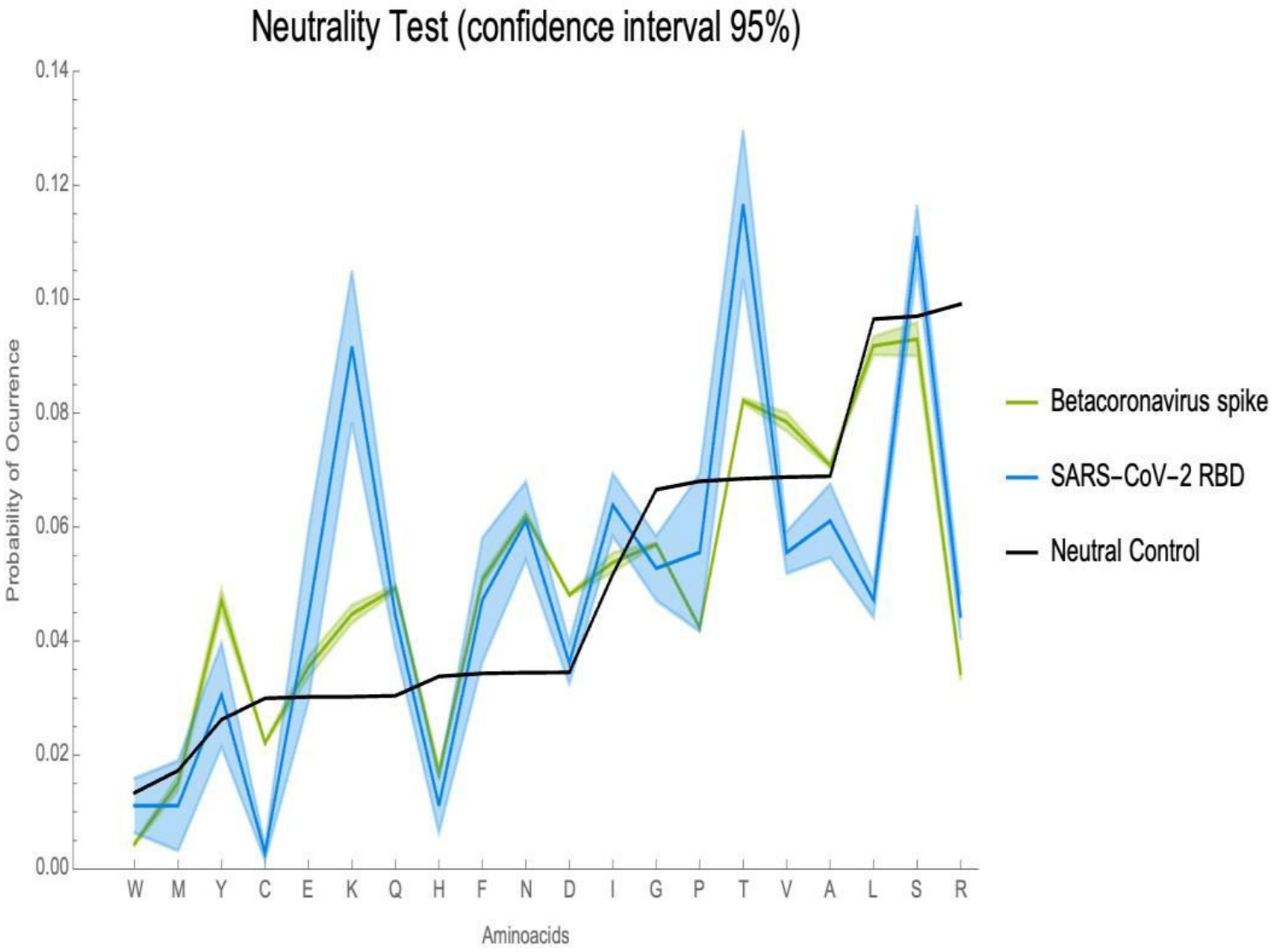
	ACE2 binding CoVs		SARS- CoV2			Chemical changes/ characteristics
	Position	a.a.	Position	a.a.	Selective pressure	
	335	P	348	A	*Negative*	Polarity
	341	E	354	N	*Positive*	Charge
	359	T	372	A	*Negative*	Polarity
	360	F	373	S	*Positive*	Polarity
	371	A	384	P	*Negative*	Polarity
	380	S	393	T	*Positive*	Polar
	389	V	402	I	*Positive*	Non- polar
	390	K	403	R	*Negative*	Same charge (+)
	393	D	406	E	*Neutral*	Same charge (-)
	404	V	**417**	**K**	*Positive*	Polarity and charge
	417	M	430	T	*Positive*	Polarity
	421	L	434	I	*Positive*	Non- polar
	425	T	438	S	*Positive*	Polar
	426	R	439	N	*Positive*	Charge
loop 1	428	I	441	L	*Negative*	Non- polar
loop 1	430	A	443	S	*Positive*	Polarity
loop 1	431	T	444	K	*Positive*	Charge
loop 1	432	S/Q	445	V	*Negative*	Polarity
loop 1	**433**	**T**	**446**	**G**	*Negative*	Polar
	439	K	452	L	*Negative*	Polarity and charge
	442	**Y/S**	**455**	**L**	*Negative*	Non- polar
	443	**L**	**456**	**F**	*Positive*	Non- polar
	445	H	458	K	*Positive*	Same charge (+)
	446	G	459	S	*Positive*	Polar
	447	K	460	N	*Positive*	Charge
	449	R	462	K	*Positive*	Same charge (+)
	457	N	470	T	*Positive*	Polar
	458	V	471	E	*Neutral*	Polarity and charge
	459	P	472	I	*Positive*	Polarity
	460	F	473	Y	*Neutral*	Polarity
	461	S	474	Q	*Positive*	Polar
loop 2	462	P	**475**	**A**	*Negative*	Polarity
loop 2	463	D	476	G	*Negative*	Charge
loop 2	464	G	477	S	*Positive*	Polar

	465	K	478	T	*Positive*	Charge
	467	T	481	N	*Positive*	Polar
	469	P	482	G	*Negative*	Polar
	470	P	483	V	*Negative*	Polarity
			484	E	*Neutral*	Insertion
	471	A	485	G	*Negative*	Polarity
	472	**L**	**486**	**F**	*Positive*	Non- polar
loop 3	476	W	490	F	*Positive*	Non- polar
loop 3	**479**	**N**	**493**	**Q**	*Positive*	Polar
loop 3	480	D	494	S	*Positive*	Charge
loop 3	**484**	**Y**	**498**	**Q**	*Positive*	Polarity
loop 4	485	T	499	P	*Negative*	Polar
loop 4	**487**	**T**	**501**	**N**	*Positive*	Polar
loop 4	489	I	503	V	*Negative*	Non- polar
loop 4	505	N	519	H	*Negative*	Charge

Figure S1



Figures



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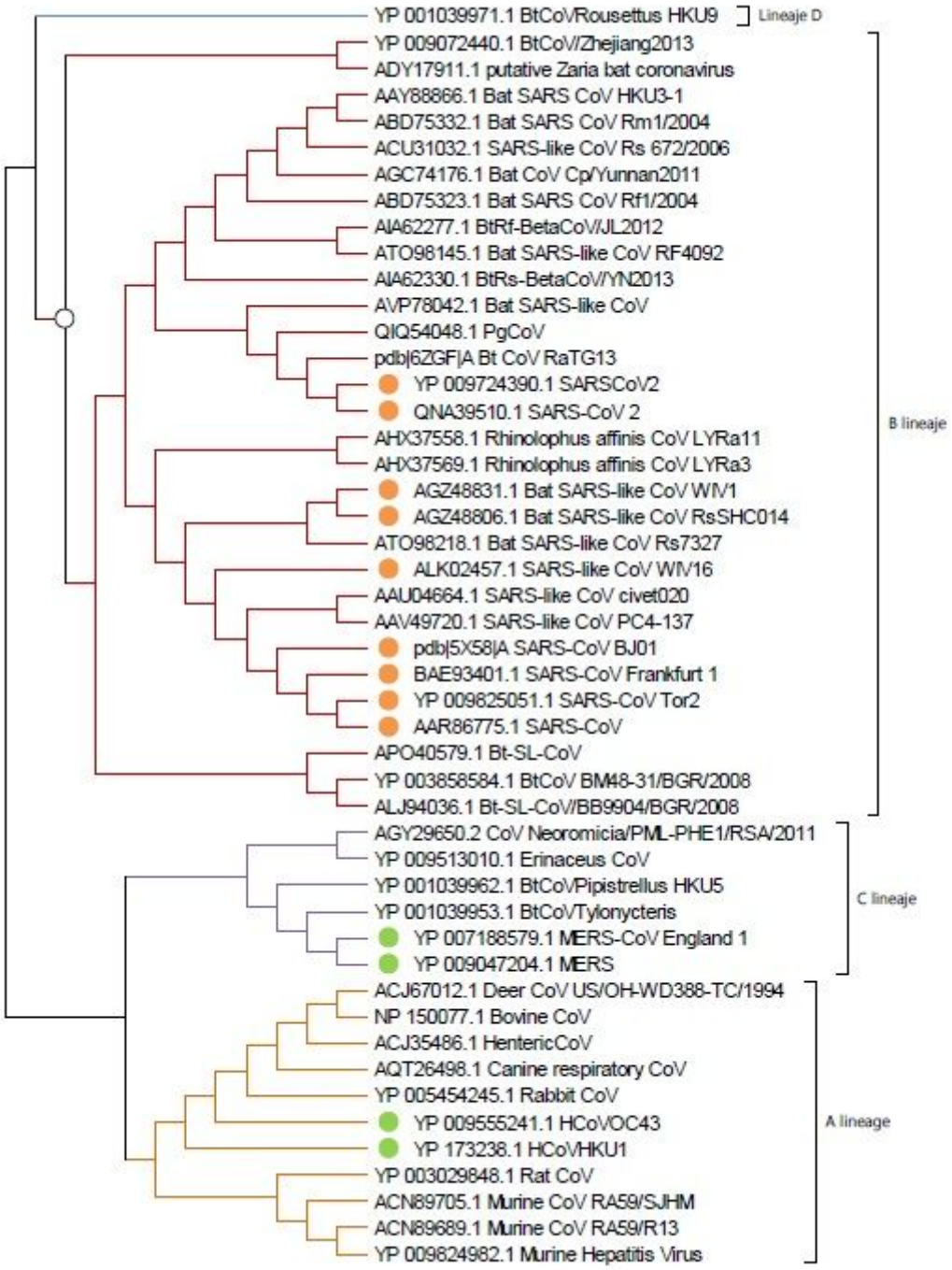

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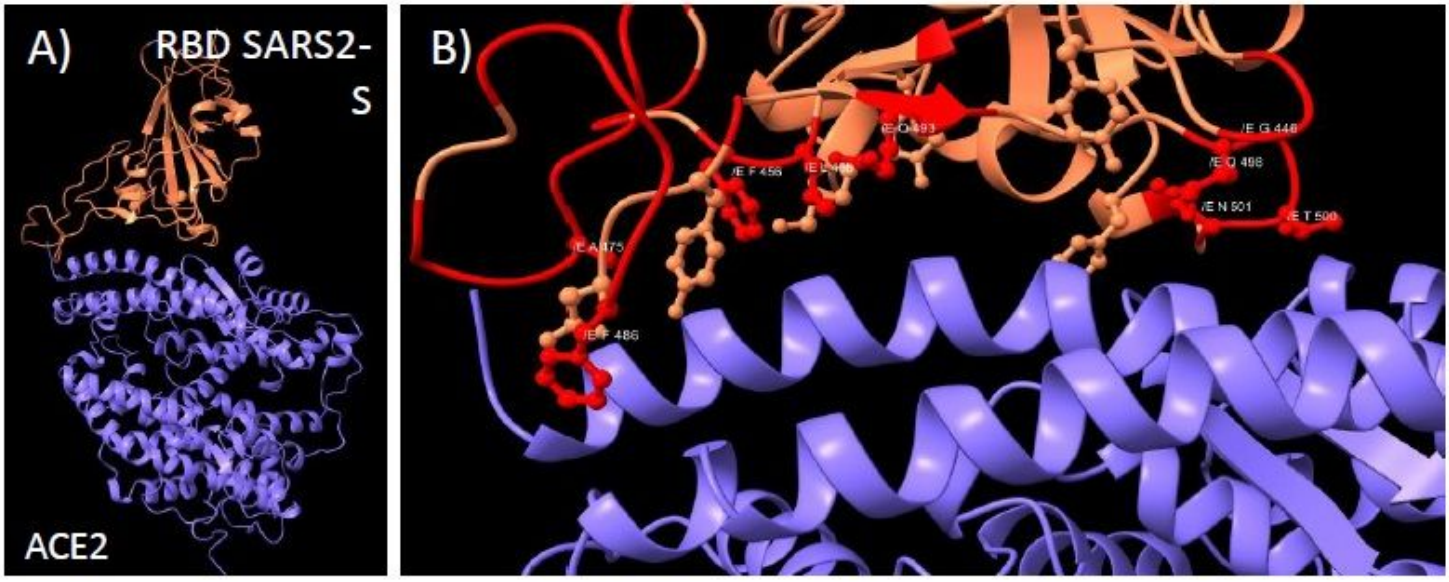

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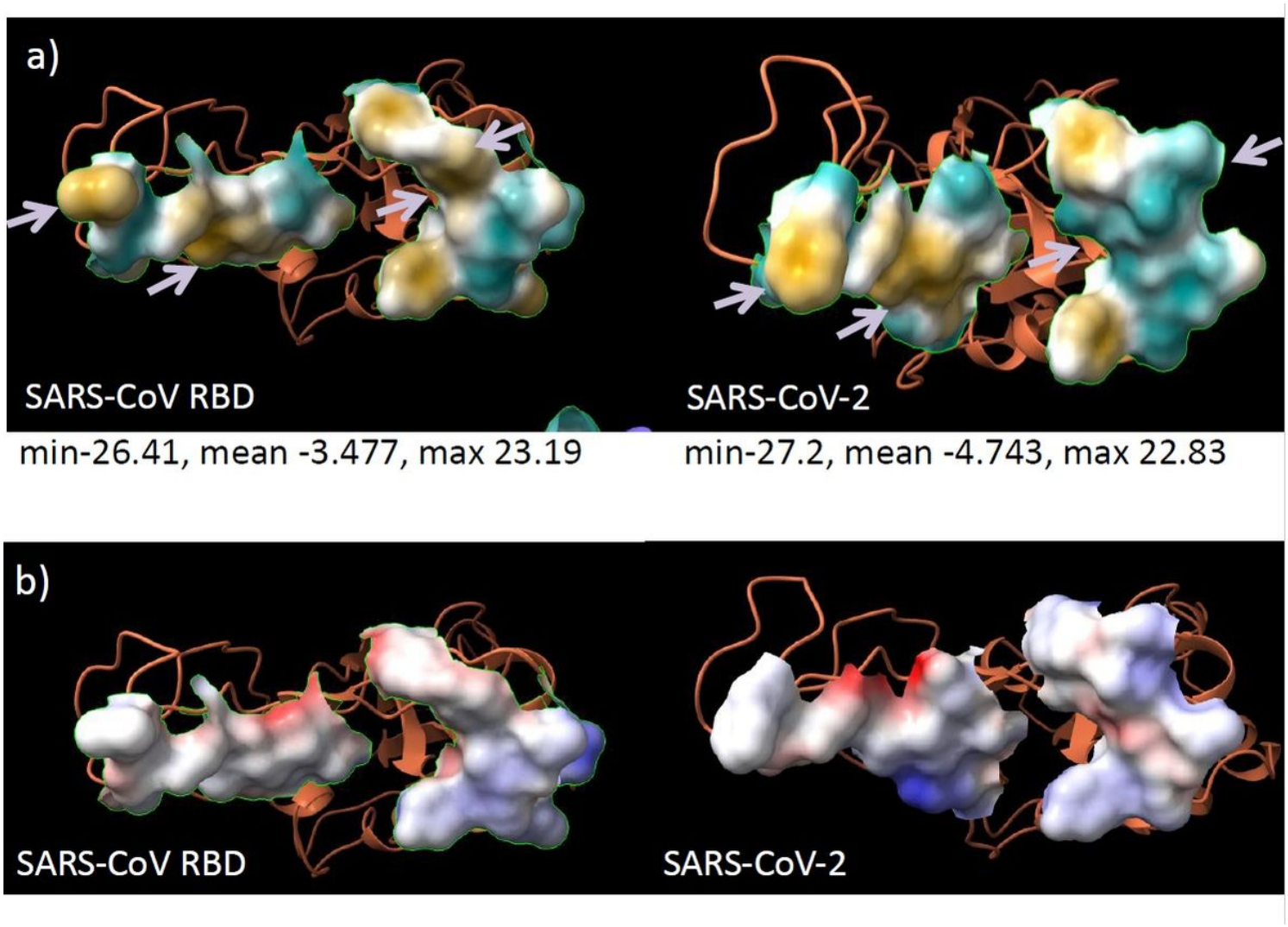

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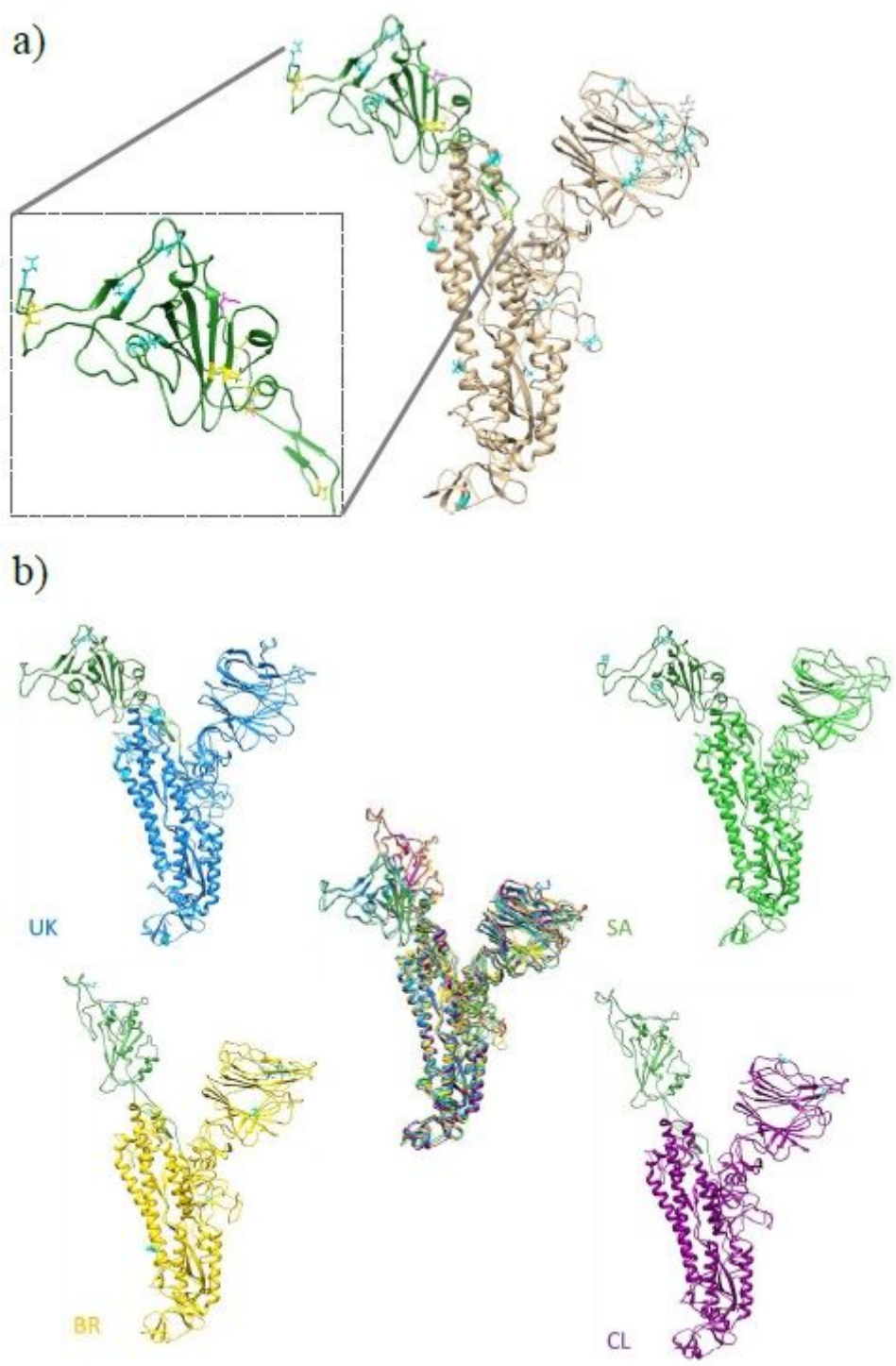

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