

# Serological Determinants of COVID-19

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## Research

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# Abstract

**Background:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection spreaded rapidly worldwide, as far as it has become a global pandemic. Therefore, the introduction of serological tests useful for the determination of IgM and IgG antibodies, it has become the main diagnostic tool, useful for tracking the spread of the virus and consequently its containment. In our study we compared point of care test (POCT) lateral flow immunoassay (FIA) vs automated chemiluminescent immunoassay (CLIA), in order to assess their specificity and sensibility against COVID-19 antibodies detection.

**Results:** We find that different specificities and sensitivities for IgG and IgM tests. Notably IgM POCT FIA method vs CLIA method (gold standard) has a low sensitivity (0.526), while POCT FIA method vs CLIA method (gold standard) test has a much higher sensitivity (0.937); further, with respect of IgG, FIA and CLIA could arguably provide the same information.

**Conclusions:** FIA method could be helpful in assessing in short time, the possible contagiousness of subjects that for work reasons cannot guarantee “social distancing”.

## Background

Coronavirus disease 2019 (COVID-19) is a novel coronavirus pneumonia caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)<sup>1,2</sup>. Emergence of new infectious diseases poses serious clinical issues<sup>3-9</sup>, this new disease was first encountered in December 2019 in Wuhan, Hubei Province, China, and then spread worldwide taking on the appearance of health emergency of international concern. Starting from February 2020, the COVID-19 outbreak spreaded in Europe, particularly interesting northern Italy and Spain<sup>10-12</sup>. World Health Organization (WHO), on 11th March 2020 declared COVID-19 disease a global world pandemic. SARS-COV-2 belongs to the beta coronavirus family along with other human pathogens known as SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-Cov)<sup>13</sup>. As COVID-19 was identified as a health emergency by WHO, large-scale population testing proved to be of crucially importance to identify and isolate symptomatic and asymptomatic case, in the global efforts to contain its expansion.

In December 2019, SARS-COV-2 was firstly transmitted to humans through human-animal contact at live animals market in Wuhan (China)<sup>14</sup>.

SARS-CoV-2 belongs to the subfamily of the Coronavirinae, which is part of the order Nidoviralescoronaviruses. It is a single-stranded RNA-enveloped virus, containing 4 structural proteins (from the 3'end open reading frames (ORF)) and 16 accessory proteins (nsp 1 to nsp 16) from the 5'end ORFs. The viral envelop contains structural proteins E and M, while the N protein nucleocapsid binds the viral RNA. The S glycoprotein is the key player for the interaction with ACE2 on the host cells (Fig. 1)<sup>15</sup>. The interaction between angiotensin-converting enzyme 2 (ACE2) and the S glycoprotein was conserved also in the SARS-CoV, the virus responsible of the SARS outbreak of 2002–2003. The S protein binds to

the receptor to target host organism cells. The virus uses also other host cell receptors such as the type 2 transmembrane serine, protease, TMPRSS2, to trigger the endocytotic process employed to access the cells<sup>16</sup>. Viral polyproteins are expressed in the host cell, RNA can be synthesized via its RNA-dependent RNA polymerase and new viral particles can be produced and released.

Cleavage at the S1/S2 and the S2' site of the S protein by the proteases of the host cell is necessary for membrane fusion<sup>17</sup> (Fig. 2). Cleaved S protein is therefore the activated form ready to enter the cell. This proteolytic step can also occur in the constitutive secretory pathway of infected cells by endosomal cathepsins B and L and furin<sup>18</sup>. When on the viral membrane the S protein is cleaved (primed) in two segments (Fig. 2). The N-terminal S1 segment is responsible for the interaction with the host cell receptor, as it contains a signal peptide and the receptor binding domain (RBD). The S2 segment anchors the S protein to the viral membrane, contains the fusion peptide which mediates the fusion of the viral membrane with the plasma membrane of the target cell. The proteases responsible for the S protein activation represents promising drug targets for the treatment of the disease, following failure of first attempts, such as hydroxychloroquine<sup>19</sup>.

Many mutations in the SARS-CoV-2 virus have been observed. One among the most prevalent is the D614G, at the C terminal region of subunit S1 of the Spike protein, which is the region in which subunit S1 associates with S2 (Fig. 2b). How and from where this mutation spread is not clear, however it appears to give the virus a decisive transmission advantage over the non-mutated variant<sup>20</sup>.

SARS-COV-2 infection displays a broad spectrum of symptoms ranging from asymptomatic forms, mild to moderate symptoms, up to severe respiratory symptoms and lung abnormalities which require intensive care including assisted oxygenation<sup>10,21</sup>. The most frequently symptoms are: fever, dry cough, upper tract respiratory symptoms, myalgia, anosmia, ageusia and headache<sup>22,23</sup>. Other fearsome complications are represented by Acute Respiratory Distress Syndrome (ARDS), respiratory failure and liver injury, acute myocardial injury and acute kidney injury, septic shock and multiple organ failure<sup>24</sup>. Recently, the alteration of the intestinal microbiota has been described in patients with COVID-19, as occurs in chronic non-communicable diseases (CNCDs)<sup>25,26</sup>. In the future, the possible understanding of the mechanisms underlying the alterations of the intestinal microbiota following SARS-CoV-2 infection could represent a new diagnostic biomarker and therapeutic target for the fight against COVID-19. The incubation period ranges from 0 to 24 days<sup>27</sup>.

SARS-COV-2 infection mainly affects the geriatric population (subjects aged over 65 years) and subjects with altered immune systems or with chronic diseases (such arterial hypertension, chronic kidney disease, chronic obstructive bronchopathy, etc.)<sup>28,29</sup>.

Serological tests, for the determination of IgG and IgM are one of the most important components of the public health response to COVID-19, along with viral diagnostic tests, for the contact tracing and the lockdown. However, given the simplicity of the method of serological tests, especially those performed through a point of care test (POCT) method, able to detect simultaneously the presence of IgM and IgG,

their use could probably reduce the extent of the shielding required to obtain a better reduction of COVID-19 transmission, in order to allow a considerable number of individuals to return to social and economic interactions<sup>30</sup>.

Accurate and rapid diagnostic tests will be critical for achieving control of COVID-19. OMICs approaches and data integration have facilitated identification of biomarkers for many diseases<sup>31–35</sup>. Similarly, production models have been proven as useful tools<sup>36,37</sup>, however serology represents a critical step in the COVID-19 control. Diagnostic tests for COVID-19 fall into two main categories: molecular tests that detect viral RNA, and serological tests that detect anti-SARS-CoV-2 immunoglobulins. Reverse transcriptase polymerase chain reaction (RT-PCR), a molecular test, is widely used as the reference standard for diagnosis of COVID-19; however, its limitations include potential false negative results<sup>38,39</sup> that affect diagnostic accuracy over the disease course<sup>40</sup>, and precarious availability of test materials<sup>41</sup>. Serological tests have generated substantial interest as they represent an alternative or complement to RT-PCR in the diagnosis of acute infection. Serological tests might be cheaper and easier to implement in the POCT. A clear advantage of these tests over RT-PCR is that they can identify individuals previously infected by SARS-CoV-2, even if they never underwent testing while acutely ill. Serological tests could be deployed as surveillance tools to better understand the epidemiology of SARS-CoV-2 and potentially inform individual risk of future disease. Many serological tests for COVID-19 have become available in a short period, including some marketed for use as rapid (POCT).

Aim of this study is to compare two different diagnostic laboratory methods, rapid lateral flow immunoassay (FIA) vs automated chemiluminescent immunoassay (CLIA), in order to assess their specificity and sensibility against COVID-19 antibodies detection. For the assessment of COVID-19 and evaluation of its spread, it should be advisable to develop a rapid laboratory test for its serological early-diagnosis.

## Results

Table 1 shows the confusion matrix for the IgM tests, while Table 2 shows the confusion matrix for the IgG tests. In Table 3 the summary statistics is presented. As it can be seen, the specificity of both COVID-19 IgM FIA and COVID-19 IgG CLIA tests were 1, i.e. no false positive results were recorded for neither of the two tests. Conversely a difference in terms of sensitivity was registered the IgM and IgG tests: while the COVID-19 IgM FIA test registered a sensitivity as low as 0.526 (high ratio of false negative results), the COVID-19 IgG FIA test registered a much higher sensitivity equal to 0.937. The overall accuracy was also significantly different: 0.878 (CI: 0.782–0.943) vs 0.973 (CI: 0.906–0.997) for IgM and IgG respectively.

Table 1  
Confusion matrix for the classes relative to IgM detection.

IgM classes		Covid-19 CLIA	
		Negative	Positive
Covid-19 FIA	Negative	55	9
	Positive	0	10

Table 2  
Confusion matrix for the classes relative to IgG detection.

IgG classes		Covid-19 CLIA	
		Negative	Positive
Covid-19 FIA	Negative	42	2
	Positive	0	30

Table 3  
Summary table of the statistical measures for FIA vs CLIA test.

Statistics	IgM (FIA vs CLIA)	IgG (FIA vs CLIA)
Accuracy	0.8784	0.973
Accuracy 95% CI	(0.7816, 0.9429)	(0.9058, 0.9967)
McNemar's test p-value	0.007661	0.4795
Sensitivity	0.5263	0.9375
Specificity	1	1

The McNemar test p-values were also very different from IgM and IgG tests. Relatively to the IgM, the very significant McNemar test p-value = 0.00076 indicates that the FIA and CLIA tests do convey different information and are not interchangeable, with a very high significance. Relatively to the IgG, the McNemar test p-value = 0.48 indicates that we cannot reject the hypothesis that the FIA and CLIA tests are statistically equivalent.

With the FIA method, no significant differences were observed between results obtained from capillary blood tests and results obtained from venous blood test.

## Discussion

The aim of this study was to evaluate whether a POCT could be able to screen correctly viral IgM or IgG antibody against SARS-CoV2. In our study we tested an analytical method (FIA method) of rapid detection of viral IgM/ IgG antibodies which was compared with a gold-standard method (CLIA method). The antibody response follows the invasion of the pathogen into the host and it is characterized by the production and secretion of antibodies from B lymphocytes (adaptive immune system)<sup>42</sup>. IgM are the first antibody response against pathogens, subsequently IgG are produced and they represent immunological memory.

Recently, many commercial rapid tests (among these POCT FIA) have been developed and CE-marked<sup>43</sup>. The results of many studies showed that their global sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were equivalent to the ELISA IgG/IgM or the CLIA IgG/IgM tests<sup>44</sup>. Similarly, to previous studies, we found accordance between the two analytical methods. In fact, the results showed a good sensitivity (88.6%) and specificity (90.6%) with the rapid test. Moreover, we obtained similar results on both venous blood and capillary blood samples. The FIA method is a rapid serological test that can be performed in the laboratory or used as POCT<sup>45</sup>. In our study we focused on the sensitivity and specificity of the qualitative-quantitative detection of the IgG with the two methods compared, as previous studies have highlighted the risk of obtaining false positive results with tests for IgM, due to their potential crossreactivity with common cold coronaviruses (such as HKU1, NL63, OC43, 229E)<sup>46</sup>. In fact, in our study protocol, subjects who presented positivity for IgM antibodies underwent to oropharyngeal swab in order to verify the real positivity to the SARS-CoV2. The latter modality is able to provide accurate results within 10 minutes with equivalent sensitivity and specificity, as confirmed by our data, both quantitatively and qualitatively, with respect to automated immunoassays. In particular, the results of our study suggest that, due to its ease of implementation, the use of the FIA method might be advantageous where the rapidity of the test results is a key factor, i.e. the FIA test can prove useful in monitoring subjects that must be reintegrated into the workplace, ensuring workers health surveillance. In a wider perspective, this analytical method could be applied in different contexts such as facilities hosting communities, like assisted health residencies, convents, army barracks and prisons, for the purpose of applying a health surveillance model in epidemic areas.

A valid example of health surveillance model in epidemic areas was realized in the municipality of Vo, Padua (Italy)<sup>47</sup>. In this rural city, researchers performed a global screening of resident population that allowed accurate tracking of the viral transmission. In particular, this model should be applied both in subjects asymptomatic, potentially infectious, and in patients who have already manifested the disease. For this reason, the systematic use of health surveillance through a POCT might be a key factor in monitoring the epidemiological situation related to viral transmission, developing good socio-political strategies, with low cost, against the expansion of the epidemic. A further field of application of POCT, related to the detection of antibodies to Sars-CoV-2, could be that of sport. In fact, in some disciplines “social distancing” is not possible (for example football, rugby, martial arts etc.) therefore it is essential to evaluate whether the athletes are positive or not for the Sars-CoV-2 virus<sup>48,49</sup>. Therefore, this antibody

screening could also be carried out to the public who goes to attend sporting events or other mass events, such as concerts, theatrical performances etc.

Further advantages of FIA method are represented by simultaneous diagnosis of IgG and IgM (antibody) in 10 minutes both on serum and on whole blood (by capillary sampling). Although it requires the constant presence of the operator during the entire analytical phase (comparison with CLIA fully automated method), however it allows the possibility of carrying out other biochemical investigations in 10 minutes such as C Reactive Protein (CRP), troponin, procalcitonin<sup>50,51</sup>. Moreover, the opportunity to test outside of the clinical laboratory by lateral flow assay, it allows to reach larger population groups without saturating the capacity of the laboratories. POCT may play an important role in large-scale testing in order to evaluate herd immunity against SARS-CoV-2. However, mistakes in the interpretation of results in situations that are not under the control of trained staff must be taken into consideration. For this reason, the development of automated reader devices could help to reduce human errors and increase sensitivity. In addition, such a device could support the transmission of the results to a public health institution to provide real-time information about seroprevalence at the population level.

Finally, the FIA method also proves to be safer than oral swab sampling. In fact, the latter could cause reaching and cough, increasing the risks of operator exposure to the virus. The results of this study show a good reliability, in terms of sensibility and specificity, of POCT FIA method to check accurately the population screening for the antibodies SARS-CoV-2 research.

## Conclusions

FIA method could be helpful in assessing in short time, the possible contagiousness of subjects that for work reasons cannot guarantee “social distancing” in order to avoid the spread of COVID-19 by symptomatic but, above all, by asymptomatic subjects. However, the development of an automated FIA would ensure greater sensitivity associated with a relative decrease in the workload by the operator.

## Methods

### Design of the study and diagnostic methods

To assess the concordance between FIA and CLIA methods, a group of 100 subjects (49 males, 51 females, mean age  $49,7 \pm 4,5$  years) have been selected to be tested with both techniques. The subjects were recruited from two different centers: the COVID Unit of the University Hospital Policlinico Tor Vergata (PTV), Rome, Italy and the Artemisia Lab-Alessandria (ALA), Rome, Italy.

In each subject blood samples were taken from antecubital vein, collected into vacutainer tubes and subsequently they were centrifuged and processed with both methods. In particular, we tested anti-Sars-CoV-2 antibody of all enrolled patients. Among these, 30 samples were collected from COVID-19 positive patients (determined by CLIA methods), belonging to Laboratory of Clinical Microbiology, University

Hospital PTV, 30 COVID-19 negative samples (assessed by CLIA methods) were taken from ALA and subsequently re-analyzed in double blind with FIA method. In addition, 40 samples from subjects with COVID-19 suspected, were analyzed with both laboratory methods at ALA. In order to avoid biases of sampling methods, we performed the same sampling procedures in both diagnostic methods. The study protocol complied with the declaration of Helsinki and was appointed by the Ethical Committee of University Hospital PTV. All subjects were > 18 years and they all signed a full informed consent before the enrollment into the study. Exclusion criteria were: immunosuppression for specific causes; clinical conditions inducing immunosuppression such as neoplastic, solid or hematological, HIV and autoimmune diseases in the active phase; pregnancy and breastfeeding.

The blood serum samples, collected into tubes contain spray-coated silica and a polymer gel for serum separation (Vacutainer, BD, Plymouth, UK), were used to perform the venous sampling. In order to guarantee operator safety, samples have been subjected to direct viral inactivation with dry heat, without preparing secondary aliquots, since this strategy has already proved an effective workload management<sup>52</sup>.

The tubes were transported from the University Hospital PTV to the ALA in a container for biological material transport with dry ice. The samples analyzed in ALA first underwent a 37° C dry-heat treatment, then were centrifuged for 20 minutes at 3,500 rpm. Subsequently, anti-Sars-CoV-2 antibodies were analyzed with two methods: FIA method "AFIAS COVID-19 Ab- Boditech Med Inc.'s Technical Services" and CLIA method SARS-CoV-2 Snibe Diagnostic with the MAGLUMI instrumentation. Both the samples were processed sequentially with the two devices.

The first one is MAGLUMI™ 800 (New Industries Biomedical Engineering Co., Ltd [Snibe], Shenzhen, China). It is an automated CLIA, featuring high throughput (up to 100 tests/h). According to the manufacturer's inserts (271 SARS-CoV-2IgM, V2.0, 2020-03 and 272 SARS-CoV-2 IgG, V1.2, 2020-02), the SARS-CoV-2 IgM cut-off is 1.0 AU/mL, while the SARS-CoV-2 IgG cut-off is 1.1 AU/mL. Manufacturers claimed that the calculated clinical sensitivities of IgM and IgG were 78.65% and 91.21%, respectively, while specificities of IgM and IgG were 97.50% and 97.3%, respectively<sup>10,53</sup>.

The procedure of MAGLUMI test occurs in this sequence: the sample, buffer, magnetic microbeads coated with anti-human IgM or IgG monoclonal antibody are mixed thoroughly and incubated, forming immune-complexes. After precipitation in a magnetic field, the supernatant is removed and wash cycle is performed. Then SARS-CoV-2 recombinant antigen labeled with ABEI is added and incubate to form complexed. After precipitation in a magnetic field, the supernatant is removed, and then another wash cycle is performed. Subsequently, the Starter 1 + 2 are added to initiate a chemiluminescent reaction. The light signal is measured by a photomultiplier as relative light units (RLUs), which is proportional to the concentration of SARS-CoV-2 IgM present in the sample.

The AFIAS COVID-19 Ab sandwich immunoassay that uses the AFIAS-automated fluorescent immunoassay system direct immunofluorescence technique from Boditech Med Incorporated. This test

uses a sandwich immunodetection method; fluorescence-labeled conjugates in a dried detection buffer binds to antibody in sample, forming antibody-antigen complexes, and migrates into nitrocellulose matrix to be captured by the other immobilized-anti-human IgG & anti-human IgM on test strip. The presence of antibodies in sample, forms the antigen-antibody complex and leads at an increase fluorescence signal on detector antigen, which is processed by to show concentration of anti-nCoV IgG and IgM in sample respectively <sup>22</sup>.

To ensure the correspondence between results obtained from capillary blood tests and these obtained from venous blood test with the FIA method, both types of sampling were analyzed. Subsequently, the same sample was analyzed with the CLIA Snibe method with the automatic MAGLUMI tool.

To evaluate intra-series consistency and repeatability for both FIA test and CLIA test, 5-fold repeated test-retest was performed <sup>24</sup>.

The production lots used to perform the tests described, were the following: Covid-19 FIA, AFIAS COVID-19 Ab. Boditech Med Inc.'s Technical Services Lot WHQDA12G EX 2021/12/16; Covid-19 IgM-CLIA MAGLUMI SARS-CoV-2 Snibe Diagnostic Lot 271200501 Ex2021/03/17; Covid-19 IgG-CLIA MAGLUMI SARS-CoV-2 Snibe Diagnostic, lot 2722000501 Ex 2021/03/17.

## Statistical analysis

For both IgM and IgG analysis, we conducted two class of analysis. Specificity, sensitivity and accuracy of the Covid-19 FIA (prediction set) were evaluated with respect to Covid-19 CLIA (ground truth). The sensitivity is the proportion of positive cases in COVID-19 FIA test out of the number of cases, which were positive in the COVID-19 CLIA test. Conversely, the specificity is the proportion of negative cases in COVID-19 FIA test out of the number of cases, which were negative in the COVID-19 CLIA test. Accuracy is the sum of true positive and the true negative Covid-19 FIA test over the total cases. The accuracy's 95% confidence interval (CI) were also calculated.

Further, McNemar's test was performed to test whether the row and column marginal frequencies are equal – i.e., if the COVID-19 FIA results and the COVID-19 CLIA results significantly disagree one with each other. Statistical analysis was conducted using R software <sup>54</sup> and caret software libraries <sup>55</sup>.

## Abbreviations

ACE2: Angiotensin-converting enzyme 2; ALA: Artemisia lab Alessandria; ARDS: Acute respiratory distress syndrome; CI: Confidence interval; CLIA: Automated chemiluminescent immunoassay, CNCDs: Chronic non-communicable diseases, COVID-19: Corona virus disease-2019, CRP: C reactive protein; FIA: Flow immunoassay; MERS-Cov: Middle east respiratory syndrome coronavirus, NPV: Negative predictive value; POCT: Point of care test; PPV: Positive predictive value; PTV: Policlinico Tor Vergata; RBD: Receptor binding domain; RT-PCR: Reverse transcriptase polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; WHO: World Health Organization.

# Declarations

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## Authors' contributions

Annalisa Noce and Nicola Di Daniele conceived and designed the experiments; Maria Luisa Santoro and Cartesio D'Agostini performed the experiments; Andrea Duggento analysed and interpreted the data; Annalisa Noce, Maria Luisa Santoro, Giulia Marrone, Ivano Amelio and Manfredi Tesauro wrote the paper.

The author(s) read and approved the final manuscript.

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None.

## Availability of data and materials

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

## Ethics approval and consent to participate

The protocol was approved by the independent ethics commitment of University Hospital Policlinico Tor Vergata.

## Consent for publication

All authors approved this publication.

## Competing interests

The authors declare that they have no competing interests.

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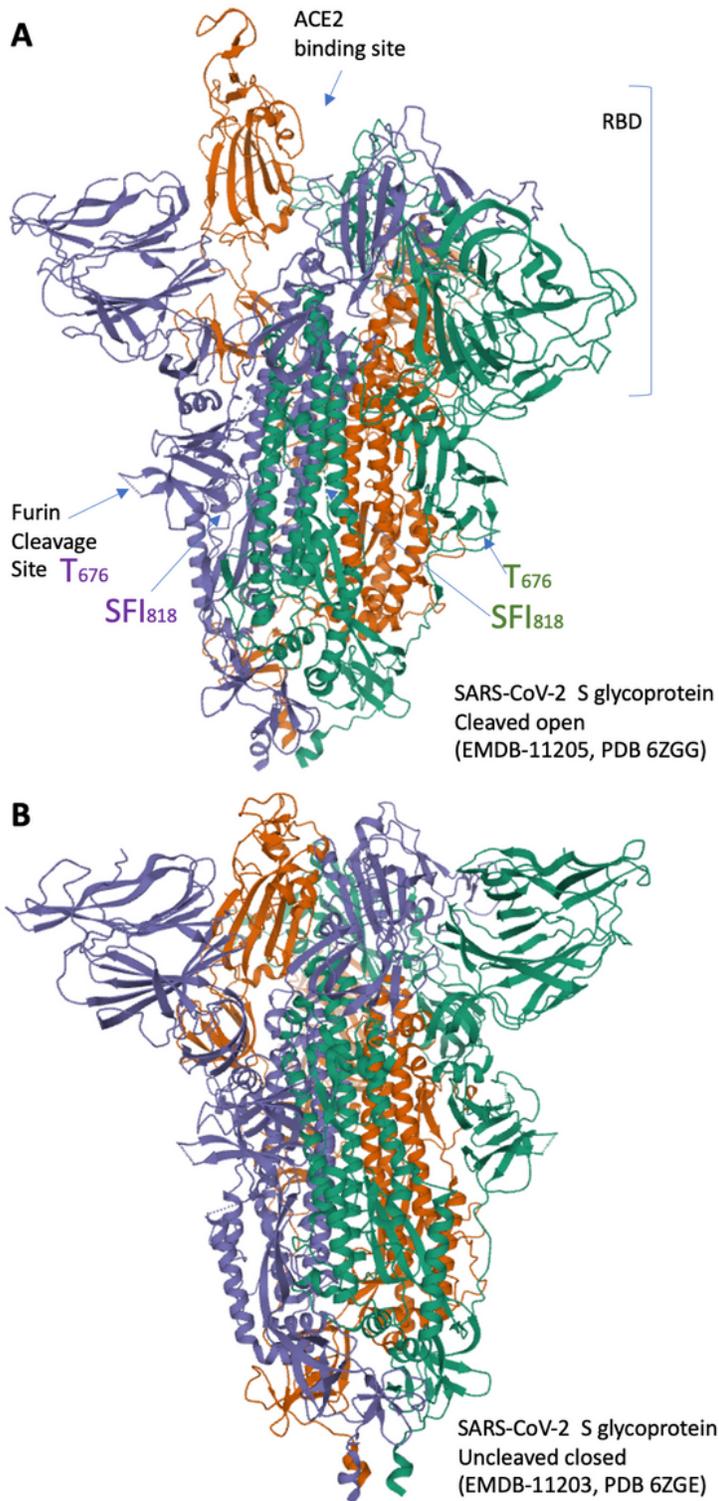
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## Figures



**FIGURE 1**

**Figure 1**

Spike protein of the SARS-CoV-2. a,b) 3D structure of the Spike protein in the cleaved (a) or uncleaved (b) conformations (EMDB-11205, PDB 6ZGG or EMDB-11203, PDB 6ZGE respectively). Panel "a" also indicates Furin cleavage site.

FIGURE 2

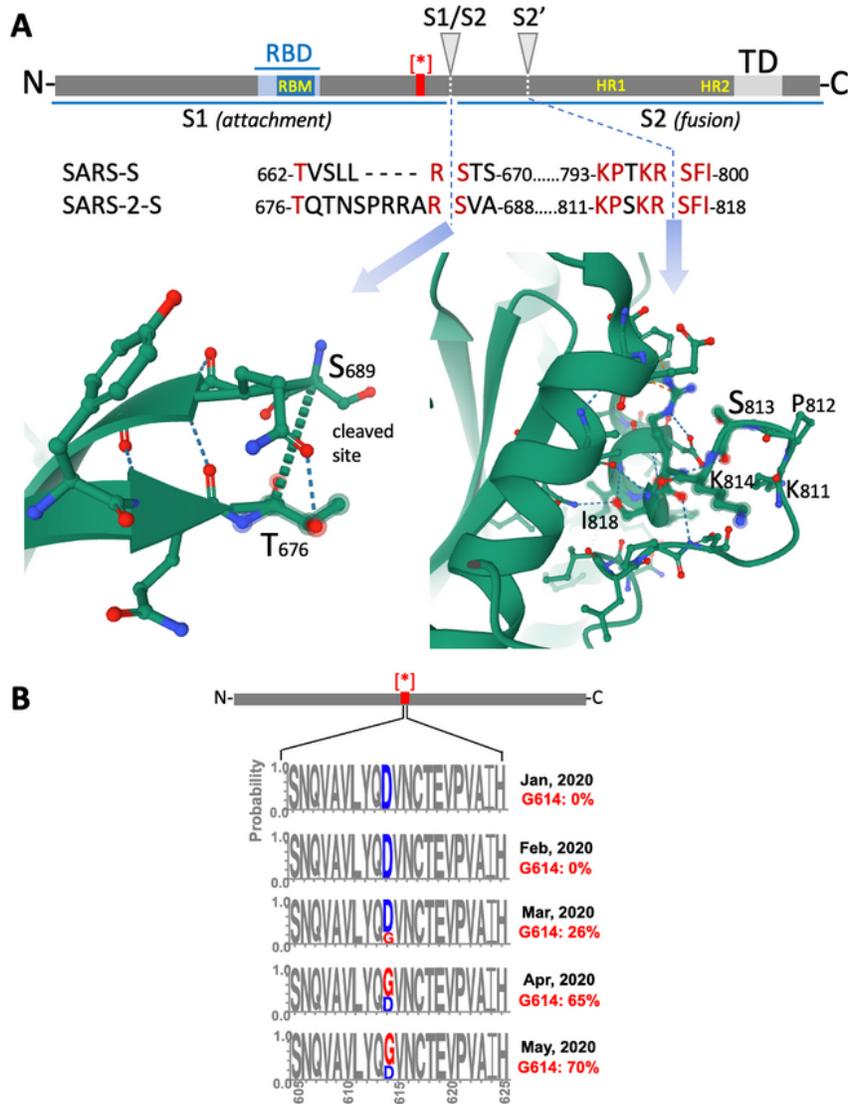


Figure 2

Structure and domain organization of the Spike protein of the SARS-CoV-2. a) The S1 subunit includes the receptor binding domain (RBD), which is responsible for the interaction with the ACE2 receptor on the host cell membrane. The subunit S2 includes the membrane fusion complex (fusion peptide, heptad repeats HR 1 and HR2), anchors the S2 subunits to the viral membrane with its transmembrane domain,

and interacts with the viral ribonucleoprotein complex through its endodomain. b) D614G mutation in the Spike protein and frequency across the time.