Study of the correlation between serum antibodies against SARS-CoV-2 and quantitative viral load in a cohort of more than a thousand individuals

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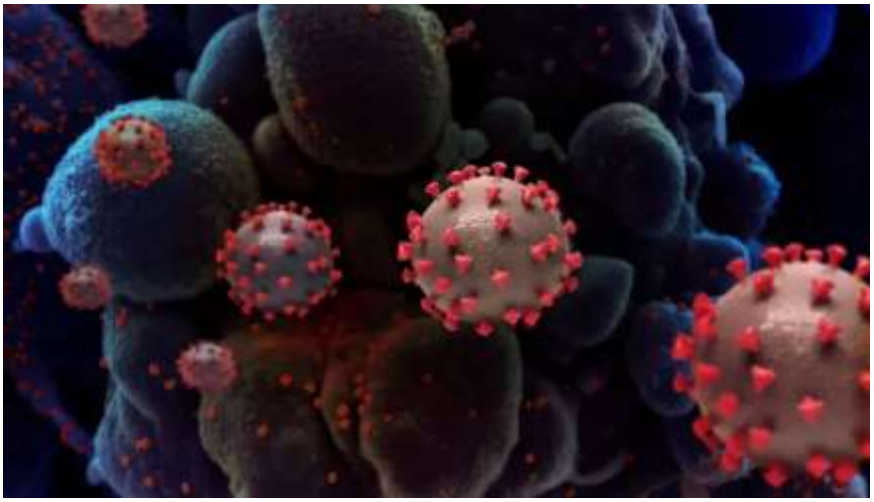
TREBALL FINAL DE GRAU BIOTECNOLOGIA



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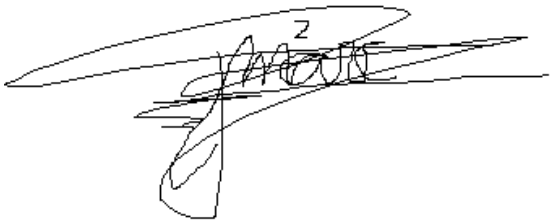
Juny 2020

Jo, Marc Molina Pocurull, amb DNI 49258328-X, sóc coneixedor de la guia de prevenció de plagi a la URV Prevenció, detecció i tractament del plagi en la docència: guia per a estudiants (aprovada el juliol 2017) i afirmo que aquest TFG no constitueixen cap de les conductes considerades com a plagi per a la URV.

Tarragona, 7 de juny del 2021.



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ELDINE PATOLOGIA

Eldine Patologia (www.eldinepatologia.com) is a Pathological Anatomy laboratory based in Tarragona dedicated to histological, cytological and molecular analysis, such as the study of tissue and cell samples extracted in surgeries and medical consultations. The 90% of the activity is based on the study for the diagnosis of cancerous pathology or infections by microorganisms. Reaching annual figures of more than 20,600 biopsies, 23,690 cytologies and 21,300 molecular tests.

2020 has been a year of innovation in Eldine and given the need to support the Public Health System, the laboratory has improved their facilities and services to offer the service of diagnostic tests for the detection of COVID-19 and specific SARS-CoV-2 antibodies.

Among the company's objectives are strict values of professional ethics and confidentiality, the importance of working with the highest quality scales, ensuring the process of samples, analysis, study, and delivery of diagnoses.

ABBREVIATIONS

cDNA: complementary DNA

COVID-19: coronavIrus disease 2019

CoVs: coronaviruses

Ct: cicle threshold

dNTP: deoxyribonucleotides

E protein: envelope protein

hACE2: human angiotensin-converting enzyme 2

HBB: human beta globin gene

HE protein: hemagglutinin-esterase dímer protein

Igs: immunoglobulin

IgA: immunoglobulin A

IgG: immunoglobulin G

IgM: immunoglobulin M

M protein: membrane protein

MALT: mucosa associated lymphoid tissues

MERS-CoV: middle east respiratory syndrome coronavirus

MHC I: class I major histocompatibility complex

MHC II: class II major histocompatibility complex

N protein: nucleocapsid protein

NK cells: natural killer lymphocytes cells

ORF1ab: open reading frame 1ab

PAMP: pathogen-associated molecular patterns

PPE: personal protective equipment

PRRs: pathogen recognition receptors

RBD: receptor binding domain

RdRp: RNA-dependent RNA polymerase

RDTs: rapid diagnostic test

RNA: ribonucleic acid

RT-PCR: reverse transcription polymerase chain reaction

SAC: sample adequacy control

SARS-CoV-1: severe acute respiratory syndrome coronavirus 1

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

S protein: spike protein

TLRs: toll-like receptor

TMPRSS2: transmembrane protease serine 2

UTRs: untranslated regions

ABSTRACT

On December 2019, an unknown respiratory disease appears in Wuhan (China). It was named COVID-19 (COronaVIrus Disease 2019) and caused by the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). It rapidly expanded throughout the world collapsing health services and the world economy causing 170.812.850 confirmed COVID-19 cases and 3.557.586 deaths.

In order to control this pandemic, diagnostic tests became key detection tools. Between July and October 2020, the Government of Morocco required a simultaneous serological test for antibodies against SARS-CoV-2 and a RT-PCR with negative result to all travellers who arrived in the country. Eldine Patología was one of the private centers that offered this service to travellers in that period. The main objective of this study is to analyse and correlate the results of both, the RT-PCR and serological test (including IgM and IgG) to explore and interpret the immune system response to the SARS-CoV-2 infection.

We collected simultaneous serological and RT-PCR data from 1.043 patients, of which 58 of them obtained at least one positive test.

From those patients, results indicated that 7 patients with positive RT-PCR were already infected in the moment of the sample collection, 5 patients have presented positives results for the IgM and, 52 patients presented positive results for the IgG.

As it was expected, the immune system responded in the presence of the infection producing IgM and IgG as part of the humoral immune response. Being the infection removed, the IgM proceeded to its degradation while the IgG stayed stable as to act as antibodies of memory.

Key words: SARS-CoV-2, RT-PCR, serologic test, IgM, IgG

1. INTRODUCTION

On December 21, 2019 a novel coronavirus disease was first reported when an outbreak of unknown respiratory disease occurred in Wuhan (China) after sequencing clinical samples from a cluster of patients with pneumonia (1). It was quickly identified as a novel betacoronavirus and officially named SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) by the International Committee on Taxonomy of Viruses on March 2, 2020 (2).

Coronaviruses (CoVs) are enveloped viruses belonging to the large family *Coronaviridae*, which can infect mammals and several other animals. Nowadays, seven CoVs are known to cause human diseases (1). Preceding this new finding, two coronaviruses have periodically crossed animal species to human populations over the last two decades, including SARS-CoV-1 and MERS-CoV (Middle East Respiratory Syndrome Coronavirus), which might cause severe respiratory disease in humans linked to sometimes fatal illness (3).

SARS-CoV-2 quickly spread throughout Southeast Asia, Europe and North America, up until now it has already expanded by practically the entire world. The disease caused by this agent was named COVID-19 (COronaVIrus Disease 2019). WHO officially declared COVID-19 a pandemic on March 11, 2020 (2). Nowadays, there are over 170.812.850 confirmed COVID-19 cases reported world-wide and 3.557.586 deaths along 216 countries (4).

The clinical manifestation depends on multiple factors, such as genetic background and the individual variability in environmental/personal risk factors (age, smoking, diet, physical activity, vaccination scheme, contact history with other coronaviruses) (2).

While in most patients (81%) the COVID-19 is mild, including a 35% of people who remains asymptomatic, most common clinical symptoms are fever and shortness of breath. Some patients also experience other signs such as sore throat, headache, myalgia, fatigue and diarrhea (3).

A few of them develope severe pneumonia, pulmonary edema, acute respiratory distress syndrome, along with organ damage and dysfunction. In most of cases, the cause of death is respiratory failure, septic shock, or several organ failures with a 2,3% mortality rate (3). Increasing to 20% in those who have more than 80 years (3) (5).

1.1 Structure of the SARS-CoV-2

SARS-CoV-2 consists of two basic components: genomic RNA and a protein capsid packaged forming a nucleocapsid (3).

SARS-CoV-2 is a positive-sense single-stranded non-segmented RNA (+ssRNA) virus with a genome of 29,881 bp in length and it is about 89% identical to bat SARS-likeCoVZXC21 and 82 % to human SARS-CoV-1 (2).

Its envelope consists of a spherical lipid bilayer (80–160 nm) derived from the cell membrane of the host and several structural proteins, which seems to have crown-shape spikes projecting from its surface, from which its name derived (3).

Approximately, two thirds of the genomic RNA encode for a large overlapping polyprotein, with an open reading frame ORF1ab (6). The result is a chain of 16 proteins that by post-translational modifications will give rise to the protein complex with transcriptase activity composed of RNA-dependent with RNA polymerase phosphatase activity (RdRp), a helicase and fourteen non-structural proteins associated with the synthesis of RNA or related to the modulation of the host response, as well it has two flanking untranslated regions (UTRs) (3)(6) .

The remaining genome encodes for five structural proteins: (Figure 1) (2)(3)(7)

- **Spike protein (S):** is a glycoprotein (comprising a S1 subunit and S2 subunit in each spike monomer) which attaches to cellular receptors on the host cell and mediates viral entry resulting in interspecies transmission and pathogenesis.
- **Nucleocapsid protein (N):** tethers the viral genome to replicase-transcriptase complex and package the encapsulated genome into viral particles to protect it from degradation.
- **Envelope protein (E):** is a transmembrane protein which facilitates assembly and release of the virus.
- **Membrane protein (M):** promotes membrane curvature and it binds to nucleocapsid.
- **Hemagglutinin-esterase dimer protein (HE):** together with the spike forms the phospholipid bilayer and it is thought to enhance S protein-mediated cell entry and virus spread through mucosa.

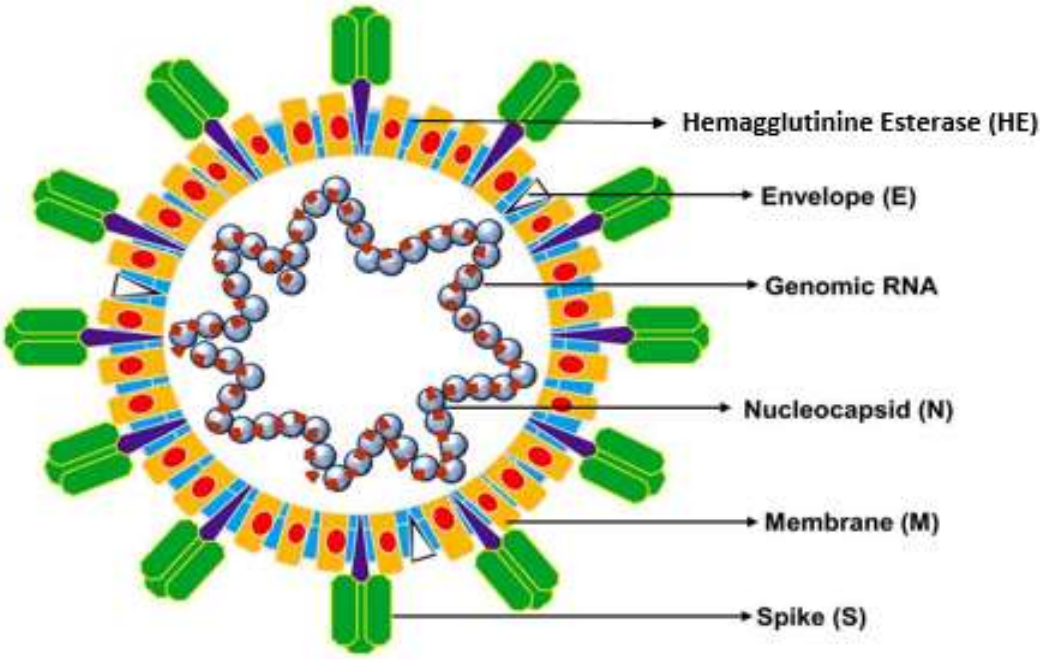

Figure 1 - Structure of SARS-CoV-2 (19).

1.2 Mechanism of infection

SARS-CoV-2 efficiently infects the epithelial cells in the lower respiratory system, entering for the nostril or mouth and its receptor recognition mechanism regulates its infectivity, pathogenesis, and host range.

The entry of SARS-CoV-2 is mediated by the interaction of the receptor binding domain (RBD), a short immunogenic fragment in the virus spike protein S1 subunit that binds to a specific endogenous receptor sequence as the human angiotensin-converting enzyme 2 (hACE2). This receptor is mainly expressed on the surface of the naso- and oropharyngeal epithelium cells, but also in some organs as kidneys and guts. The receptor transmembrane protease serine 2 (TMPRSS2) from the host promotes priming of the spike protein and facilitates its S2 subunit to initiate the viral-cell membrane fusion (7)(8).

Following the receptors binding, the virus can enter the cell cytoplasm via endocytosis. (Figure 2). This will cause a decrease of the pH by the internalization of hydrogen protons (H+) through the vesicle formed, which will modify the structure. At this moment, the nucleocapsid of the virus is released to the cytoplasm, where the capsid would be degraded to release the genomic material outside (9).

Then, the replication of the genome involves the synthesis of a full-length negative-strand RNA and serves as template for full-length genomic RNA to produce genomic and subgenomic RNA (+ senses). Following the genomic RNA, the ORF1ab, are translated into a protein using the viral transcriptase complex (phosphatase activity, RNA-dependent RNA polymerase (RdRp) and a helicase) by the human ribosomes. The newly synthesized viral genome associates with the N protein, forming the nucleocapsid and thus preventing its degradation while structural proteins S, E and M are translated and inserted in the membranes of the endoplasmic reticulum. In this compartment, structural proteins interact with the nucleocapsid to synthesize new viral particles. Viral progeny will later be transferred to the cell membrane by budding and the virus is released by exocytosis (1).

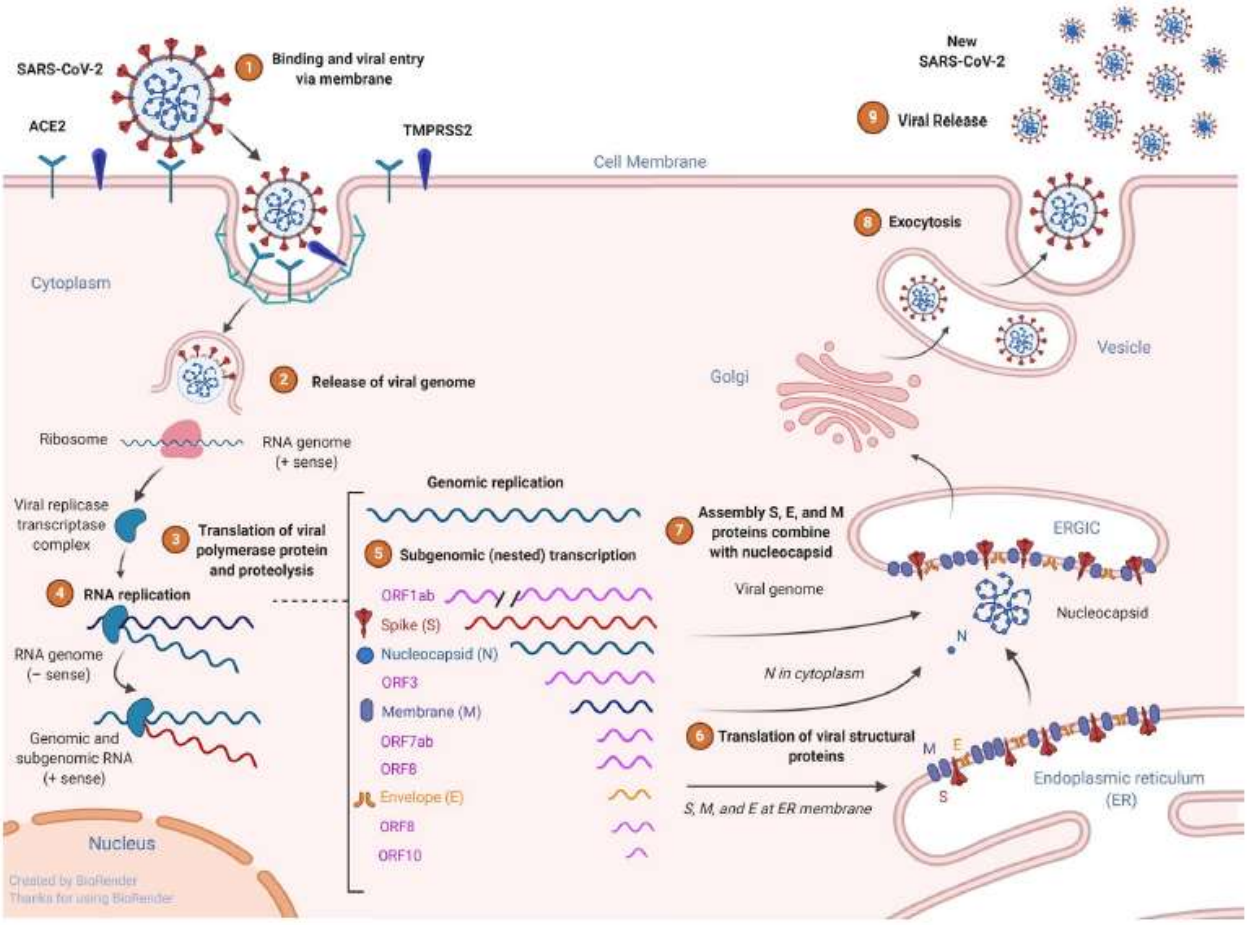

Figure 2 - Simplified representation of the mechanism of infection. From the binding of SARS-CoV-2 to the host cells until it released by exocytosis (1).

1.3 Immune system response

The current knowledge about COVID-19 indicates that the immune system has two different responses, the cellular and humoral response, which plays a crucial role in setting the severity of the disease.

1.3.1 Cellular immunity response

Mucosal surfaces, presenting the first line of defence, are protected against the virus via mucosa associated lymphoid tissues (MALT) with high presence of IgA (2).

Once the SARS-CoV-2 has infected the cell by internalizing its viral RNA forming endosomes, the innate response of the immune system mediates via recognition of the viral nucleic acids as pathogen-associated molecular patterns (PAMPs) by specific pathogen recognition receptors (PRRs) as toll-like receptors (TLRs) in the vesicle membrane or in the cytosol (2). This binding between receptors will activate several signalling pathways and transcription factors that promote the production of cytokines by respiratory epithelial cells and interferons, which are associated with interferon responsive genes and those allow a robust innate immune response to occur (10).

The dendritic and macrophages cells are the other first line of defence to start the immune reaction, producing cytokines as well during the early stage of infection and showing the viral peptides through class II major histocompatibility complex (MHC II) proteins to CD4+ T cells, the cells are going to induce the production of antibodies (Figure 3) (1).

These viral peptides came from the CD8+ cytotoxic T cells who lyse the virus-infected tissue cells, which were expressing class I major histocompatibility complex (MHC I), via multiple mechanisms including perforins and granzymes. However, SARS-CoV-2, similarly to other coronaviruses, restrains antigen presentation by downregulating MHC class I and II molecules, which inhibits the T cell mediated immune responses, suggesting that lymphocytes (particularly T lymphocytes) are likely target of SARS-CoV-2 (1).

In severe cases of COVID-19 disease, an increased number of neutrophils was detected, triggering an overactivation of the inflammatory immune response leading to a cytokine storm and subsequent immune exhaustion. Cytokine storm is induced by the activation of large numbers of white blood cells, including B cells, T cells, NK cells, macrophages, dendritic cells, and resident tissue cells, such as epithelial and endothelial cells, which

release high amounts of pro-inflammatory cytokines. These findings suggest that severe and uncontrolled inflammatory responses have a more damaging effect on COVID-19 induced lung injury than viral pathogenicity (1).

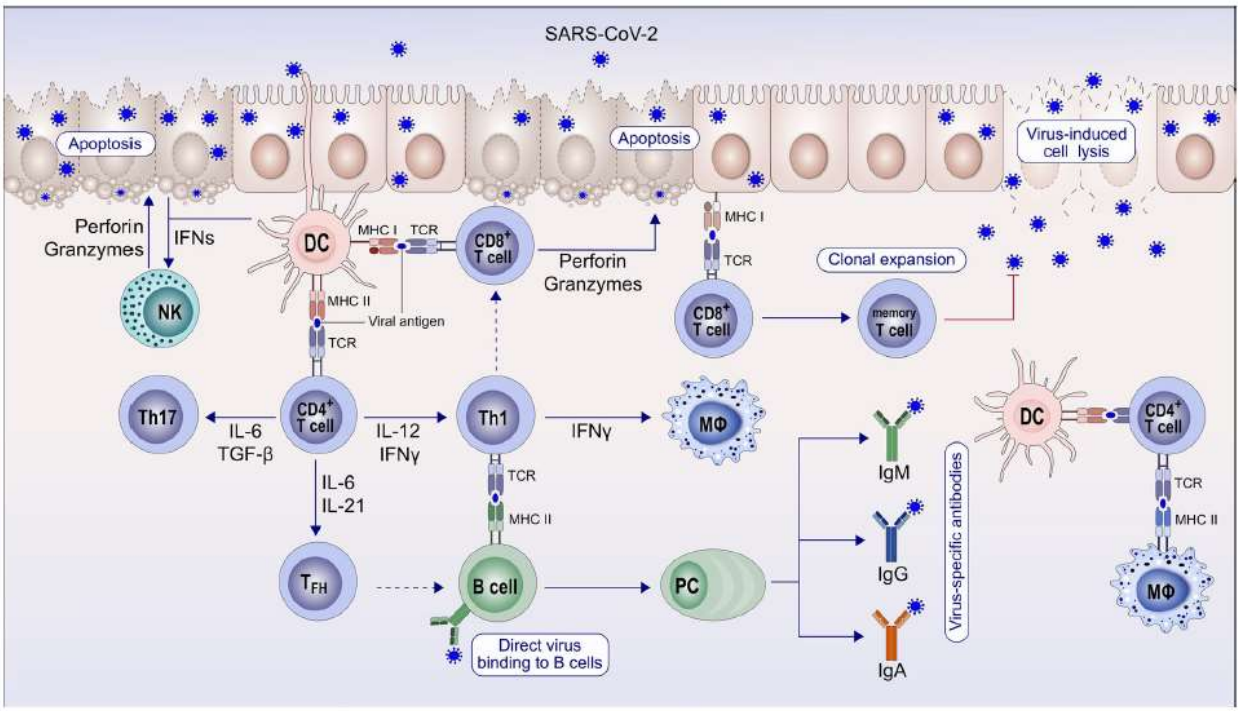

Figure 3 - Immune response and cellular differentiation in front of SARS-CoV-2 and other respiratory viruses (1).

1.3.2 Antibody immunity (humoral response)

The humoral response is the main mechanism of adaptive immunity, which consist in the production of proteins and antibodies. Antibodies or immunoglobulins (Igs) are glycoproteins produced by B cells lymphocytes which have recognize the viruses and get activated by them or have interact with CD4+ T cells so can block the viruses from entering the host cells, playing a critical role in the virus clearance. In fact, there are 5 isotypes of antibodies, but the IgM, IgA and IgG are the most clinically relevant (1) (11).

- **IgM**: appears and continues along the acute phase of the disease, after this it decreases to undetectable levels. It forms immune complexes with the respective antigen covering the pathogen's surface.
- **IgA**: plays a crucial role neutralizing or preventing the attachment of the viruses to the mucosal surfaces/epithelium and it mostly retains lifelong immunity.

- **IgG**: follows the early IgM response and is assumed to continue lifelong as a protective antibody.

Antibodies against SARS-CoV-2 have a neutralizing action over the virus, primarily targeting the S and N proteins, and preventing its binding to and the invasion of the host cells (1).

Early SARS-CoV-2 humoral response found that the median duration of IgM and IgA detection was 5 days after symptom onset, and IgG was detected at a median of 14 days after symptom onset (12) (Figure 4). In general, IgM is the first antibody made after infection with a new pathogen, and IgG is a more stable and longer lasting antibody present in the serum to help fight off infection. However, specific IgG levels can be found at already high levels in serum at the same time or earlier than IgM against SARSCoV-2 (1). Although IgA is the main antibody in the mucus membranes, considered a major effector molecule to defend the physical barriers against viruses and appears to be stronger and more persistent than IgM response (2).

Therefore, the combined induction of antibodies and complement system may provide optimal protective humoral immunity.

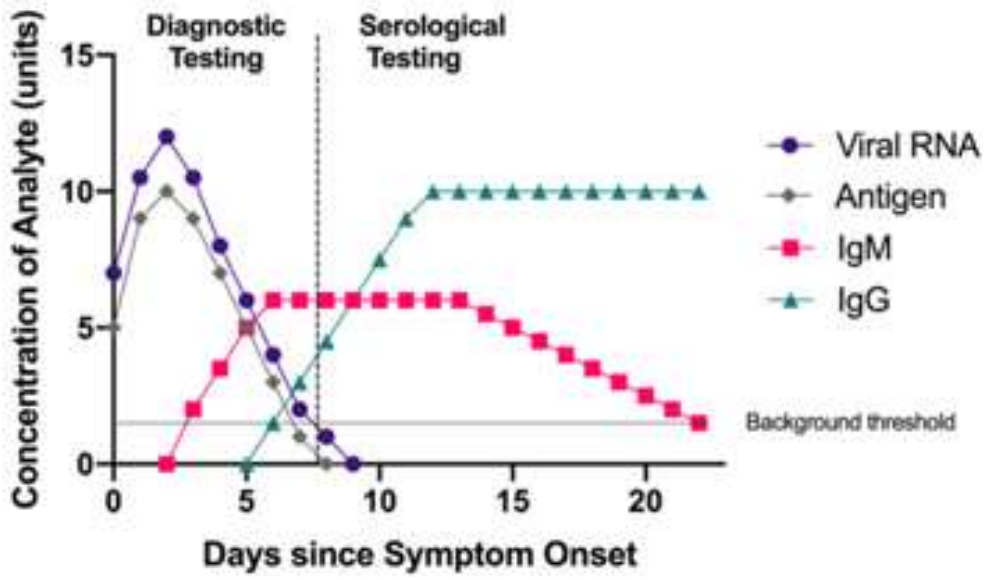

Figure 4 - Time course of approximate concentrations of viral RNA, antigen, and antibodies against SARS-CoV-2 after symptom onset for a hypothetical patient with COVID-19. In general, RT-PCR and antigen testing are effective to diagnose active infection when viral RNA or antigen is present. Serological assays are effective after about 5 days to detect IgM, with IgG rising afterwards (2).

1.4 Diagnostic test

Diagnostic tests are considered the principal tool for the control of the pandemic. Epidemiologists consider mass testing for SARS-CoV-2 the most practical way to control the current outbreak among both asymptomatic carriers and individuals showing signs of the disease (6).

Several diagnostic methods have been proposed to provide a rapid response in the combat against the pandemic, each of them having a different degree of specificity and based on different target molecules from SARS-CoV-2 or the host in response to infection. These methods include nucleic acid detection and serological tests (6).

1.4.1 Virus detection techniques

These techniques are used to detect whether the patient is infected by SARS-CoV-2 at the time of sample extraction. We can distinguish two main techniques:

1.4.1.1 Nucleic Acid-Based Tests

Nucleic acid detection has played an important role in early diagnosis of COVID-19. The molecular techniques utilize the genetic material of the virus and are based on the principle of the specificity of base pairing with homologous strands (6).

The molecular technique of reverse transcription–polymerase chain reaction (RT-PCR) is the most suitable method, as it allows for viral detection and quantification of the RNA of SARS-CoV-2.

In general, these tests involve three essential steps: (Figure 5) (6)

- **Preparation:** to detect the presence of the SARS-CoV-2 in the samples taken from the nasopharynx or oropharynx of the patient, the epithelial cells and the virus capsid will be broken using a lysis buffer. Therefore, the viral RNA will be released and targeted by a reverse transcriptase, which will synthesize complementary DNA (cDNA) from the RNA.
- **Amplification:** the small amounts of template cDNA and the specific primers are used in parallel for detection of specific gene/s of interest and control gens through heating and cooling steps. The primers bind to regions of SARS-CoV-2 viral genes as nucleocapsid (N) and using the 4 deoxyribonucleotides (dNTP) and the Taq polymerase enzyme will amplify the gene/s into millions of copies.

- **Results:** In this test, the primers are bound to fluorophores, which they release as they bind by complementation with the cDNA emitting fluorescence, as the dNTP do during the amplification. The thermal cycler has lasers which measure this fluorescence at the end of each cycle. The viral load of SARS-CoV-2 is normally defined through the cycle threshold (Ct) and, in a practical application, it is considered that a Ct value less than 40 is clinically reported as RT-PCR positive. Ct value is defined as the number of replication cycles in which the fluorescence of the sample exceeds a chosen threshold above the calculated background fluorescence.

The RT-PCR is considered the reference test to detect SARS-CoV-2 much earlier in clinical samples with a high specificity. However, the limitation of high prices and lengthy procedures difficult the use of these assays as mass testing (13).

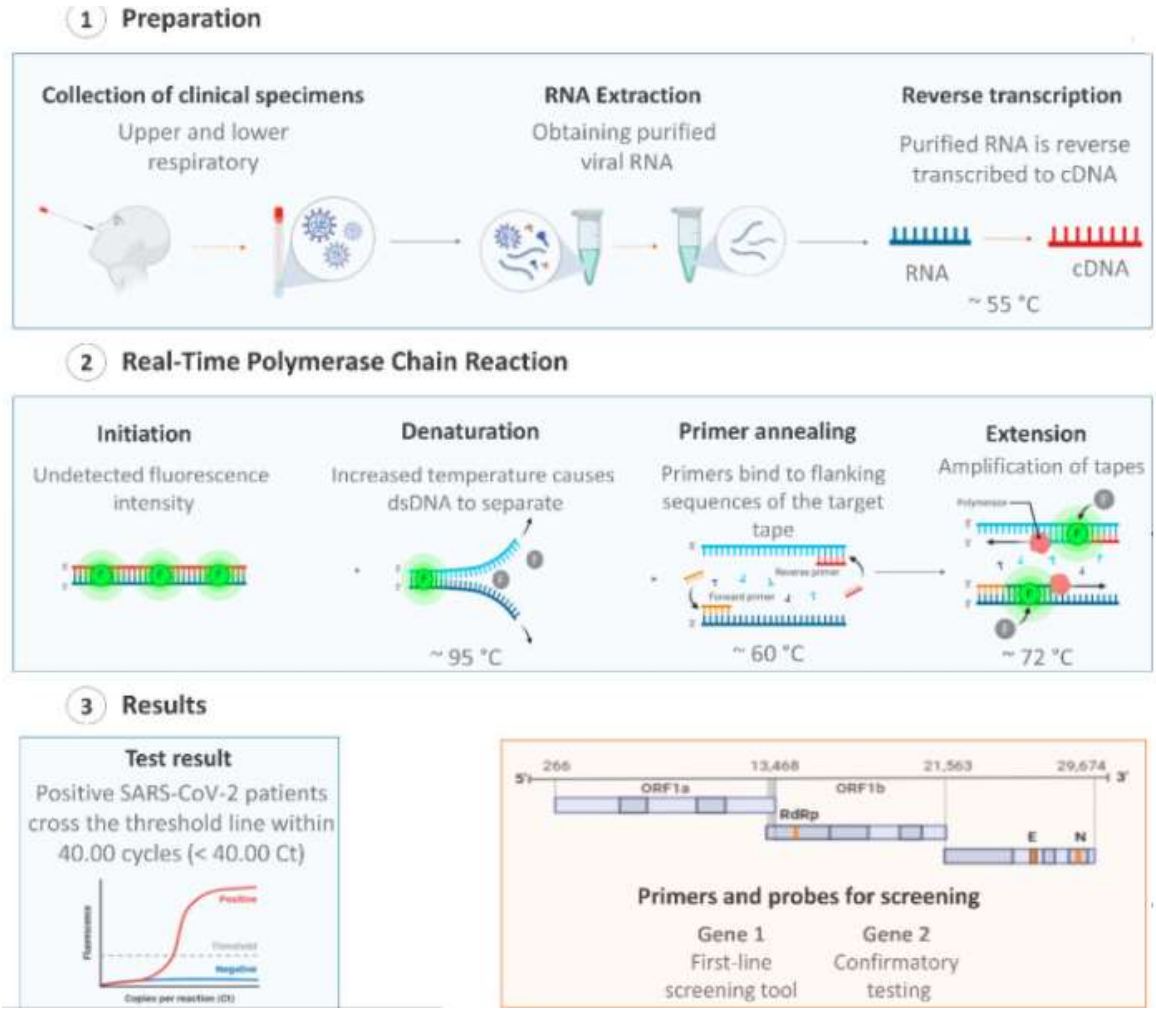

Figure 5 - Procedure of technique of quantitative reverse transcription–polymerase chain reaction RT-PCR with samples extracted from nasopharynx and oropharynx to detect SARS-CoV-2 (6).

Interpretation of the RT-PCR results: (Figure 6) (14)

Detection of specific SARS-CoV-2 RNA	Detection of universal SARS-like RNA	SAC HBB gene	Result	Interpretation
+	+	±	Positive	Both specific SARS-CoV-2 RNA and universal SARS-like RNA are detected. The tested individual could be infected. *Note: Positive specimens should be sent to the appropriate public health authority for confirmatory testing.*
+	-	±		The universal SARS-like RNA not detected might be caused by low viral load in the specimen or the accumulation of mutation over time. *Note: Positive specimens should be sent to the appropriate public health authority for confirmatory testing.*
-	+	±	Negative	The specific SARS-CoV-2 RNA not detected might be caused by low viral load in the specimen or the accumulation of mutation over time.
-	-	+		The sample does not contain detectable amounts of SARS-CoV-2 RNA.
-	-	-	Invalid	Specimen not collected properly, RT-PCR inhibition or reagent failure. Collect a new sample and Repeat testing.

Figure 6 - Possible RT-PCR results. In this case we have 3 lasers that measure fluorescence to detect specific RNA for SARS-CoV-2, RNA from the family Coronaviridae and the gene that codes for human beta-globulin (HBB). The first two lasers serve as a species control while the last one serves as a sample control (14).

Negative: The test control appears while the laser, specific por each canal, does not detect the RNA of the SARS-CoV-2. The laser which detects the RNA of the family *Coronaviridae* is not conclusive in the test results because, in this case, the patients would not be infected by SARS-CoV-2. In case of being positive of the family *Coronaviridae* would indicate that the patient could be infected by another Coronavirus.

- **Positive:** It appears the control of the test as well as the canal of detection of the specific RNA for the SARS-CoV-2. The laser to detect the RNA of the family *Coronaviridae* is not conclusive because, in this case, indistinctly of the result the patient would be infected by SARS-CoV-2. In the case of a negative result in the

canal of the *Coronaviridae* family, would indicate that the genome of the SARS-CoV-2 that has infected, accumulates mutations or a low viral charge.

- **Invalid:** The control of the test does not appear and because of that the other canals lack reliability. That indicates that the sample collection has not been done correctly or that the reaction of the amplification has been inhibited due to some external agent.

1.4.1.2 Antigen detection tests

Antigen assays were recently introduced as a diagnostic tool to screen the spread of the COVID-19 pandemic in the population. Antigen tests are based on lateral flow immunoassays that can detect the presence of a specific viral antigen of SARS-CoV-2, which implies an active viral infection (6) (15).

The test device consists of a membrane strip pre-coated with immobilized anti-SARS-CoV-2 antibodies that allow detecting the presence of viral N-protein present in the sample, and an anti-human epithelial antigen to ensure the correct sample acquisition (Figure 7).

Briefly, sample swab is lysated with a specific buffer and put into the test device. It will ascend by capillarity throughout the surface, binding to the antibodies conjugated with gold nanoparticles on the conjugated pad, which subsequently will reach the test and control lines where anti-antibodies are immobilized, allowing their detection by a colour change.

The higher efficiency of the test is on the first 7 days after onset of symptoms, when the viral load is higher, so a positive result in the test ensures us that the infection of SARS-CoV-2 is current (16).

Antigen test is a kind of rapid diagnostic test (RDTs) so the results will be ready in 15-20 minutes, allowing its use as a tool for mass testing, but the high margin of false negatives it does not allow it to be a decisive test, so in the case of negative results it is recommended to carry out subsequent tests with greater reliability.

Interpretation of the results: (15)

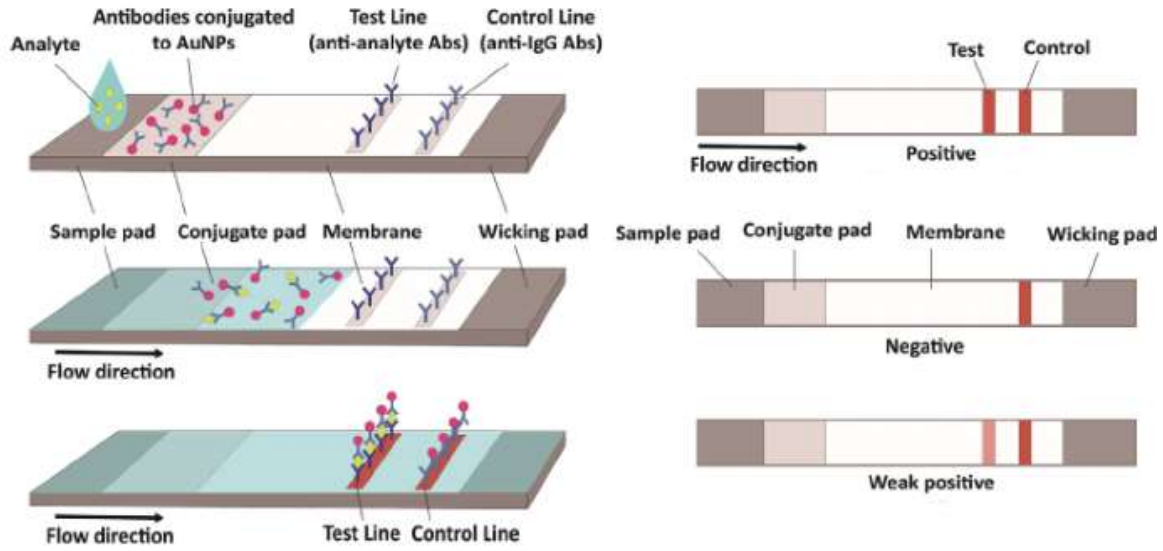

Figure 7 - Schematic conception and dipstick assay of the antigen lateral flow immunoassay test strips. The presence of an analyte is indicated by the appearance of coloured lines on the membrane, that can be analysed by naked eyes. The different possible results are shown in the image. The colouring of the control line (C) indicates the correct procedure in the extraction and its absence would render the test invalid. On the other hand, the test line (T) indicates the presence of SARS-CoV-2 in the sample and therefore its coloration would indicate the infection of the patient (20).

1.4.2 Serological tests

As previously described, the humoral response is in charge of making antibodies, which we will use for the development of immunological diagnostic methods.

Serological tests are a solid phase immunochromatographic assay for the rapid, qualitative, and differential detection of antibodies to SARS-CoV-2 in human whole blood, serum, or plasma. This lateral flow immunoassay works the same as the antigen test but using different reagents and it will detect antibodies instead of antigens. Via capillary action whereby the mix of the sample (often being a fingerprick blood test) and the buffer run over a nitrocellulose membrane that is pre-functionalized with capture and detect antibodies, mainly the IgM and IgG ones, and usually gold nanoparticles to generate coloured lines on the membrane if the analyte of interest is present (2) (Figure 8). The test uses anti-human IgM antibody (test line IgM), anti-human IgG (test line IgG) and rabbit IgG (control line C) immobilised on the nitrocellulose strip.

In contrast, the application of serological tests is recommended from the second week since the onset of symptoms. Antibodies are more likely to be detected in assays after 6 or 14 days since the onset of the first symptoms. For SARS-CoV-2, the IgG and IgM produced specific against the S and N viral proteins are of particular diagnostic interest (2) (6).

Serology-based antibody tests are also a type RDTs so the results would be ready in 20 minutes, which can promote an estimate of the SARS-CoV-2 incidence. They can complement the nucleic acid-based tests, as they can detect individuals with immunity against the disease by markers of the immune response. Moreover, they suppose a very useful tool for a better understanding of the population's immunity, given that they provide information about patients who have been infected and already recovered, especially asymptomatic patients who were never diagnosed (2) (6).

Compared to the RT-PCR for SARS-CoV-2 detection, serological tests are less expensive, the diagnostic time is shorter, and the steps involved in performing the tests are less complex, but they alone should not be used for diagnosing viral infection (6).

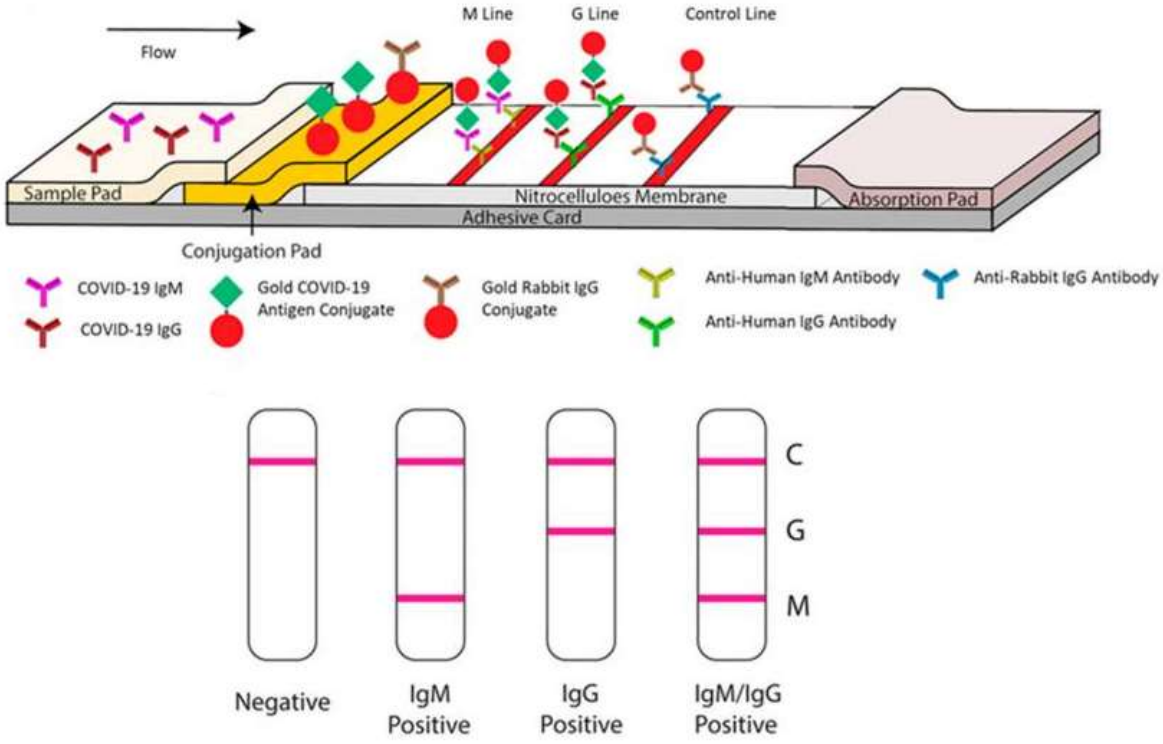

Figure 8 - Steps in serological lateral flow immunoassay based on COVID-19 diagnosis performed by using the blood sample collected from the individual with suspected COVID-19 infection. The conjugate pad contains all the reagents required to conduct a chemical reaction between antigen and antibody to detect the presence of IgM and IgG (21).

Interpretation of the results: (Figure 8) (17)

- **Negative:** The coloured line in the control line region (C) changes from blue to red. No line appears in the test line regions M or G, consistent with a no previous COVID-19 virus infection.
- **IgM Positive:** The coloured line in the control line region (C) changes from blue to red, and a coloured line appears in test line region M. The result is anti-COVID-19 IgM positive, consistent with an acute or recent COVID-19 virus infection.
- **IgG Positive:** The coloured line in the control line region (C) changes from blue to red, and a coloured line appears in test line region G. The result is anti-COVID-19 IgG positive, consistent with a recent or previous COVID-19 virus infection.
- **IgG and IgM positive:** The coloured line in the control line region (C) changes from blue to red, and two-coloured lines appear in test line regions M and G. The result is anti-COVID-19 IgM and IgG positive, suggesting current or recent COVID-19 virus infection.
- **Invalid:** Control line is still completely or partially red and fails to completely change from blue to red. Insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure.

2. HYPOTHESIS and OBJECTIVE/S

Understanding the answer and the working mechanism of the immune system in the presence of SARS-CoV-2 can help us control the actual pandemic.

The hypothesis in which is based the current study is that, behind an infection due to SARS-CoV-2, having positive results in the RT-PCR, we expect to observe (from some days onwards) a beginning of an IgM production followed of the production of IgG.

So as to validate or discard such hypothesis, the main objective of this study is to analyze and correlate the results of both, the RT-PCR and serological test (including IgM and IgG) in a cohort of patients simultaneously undergoing both tests, in order to explore and interpret the immune system response to the SARS-CoV-2 infection.

Secondary objectives:

1. To determine the positivity/negativity for SARS-CoV-2 nucleic acid detection in the study subjects.
2. To determine the positivity/negativity for IgM against SARS-CoV-2 detection in the study subjects.
3. To determine the positivity/negativity for IgG against SARS-CoV-2 detection in the study subjects.
4. To correlate and interpret the results of both RT-PCR and serological tests.

3. METHODOLOGY

3.1 Study subjects

Between July and October 2020, the Government of Morocco required a simultaneous serological test for antibodies against SARS-CoV-2 and a RT-PCR with negative result to all travellers who arrived at the country, in an attempt to minimize the spread of the virus. Given that Eldine is a laboratory of healthcare activity, the individuals included in this study correspond to travellers with Moroccan nationality who required both tests between the months described above. In total, 1043 patients were included.

3.2 Sample collection

On the one hand, for the RT-PCR test we obtained a nasopharyngeal scraping using a specific swab meeting the European Community directive requirements for medical devices: propylene handle and a viscose head. On the other hand, for the serological test we used the fingerprick test done employed a lancet. In all cases, samples were obtained and manipulated using Personal Protective Equipment (PPE), which consists of a biosecurity lab coat, protective lab glasses, FFP2 masks and gloves.

Figure 9 - Procedure for the extraction of a nasopharyngeal sample (22).

For the nasopharyngeal swab extraction, let the patient sit on a chair and bent the head back between 45º to 70º. Then use the swab to enter through the nostril, following a parallel trajectory to the palate without changing its inclination and force to avoid causing possible injuries or irritation, spinning slightly the swab to boost the secretion of mucus and lubricate the path till nasopharynx. After reaching the optimal zone leave it in place

for a few seconds, then slowly rotate it while it is being withdrawn and then, save it to conserve until the analysis.

For the blood sample extraction, let the patient sit on a chair, disinfect the tip of a finger with ethanol, and use a lancet to obtain one drop of blood.

3.3 RT-PCR

For the RT-PCR test, use a VitaPCR™ kit and instrument. It is a rapid molecular in vitro diagnostic test utilizing a real time reverse transcription polymerase chain reaction (RT-PCR) amplification technology for the qualitative detection of SARS-CoV-2 viral RNA in nasopharyngeal or oropharyngeal swabs

Firstly, we introduced each patient swab into 5 mL of lysis buffer containing guanidine salts. After vorting it for a few seconds, we incubated for 20 minutes. Later, 30 µl of the lysis buffer were mixed with the lyophilized reagents (4 deoxyribonucleotides (dNTP), specific primers for SARS-CoV-2 and HBB, monovalent and divalent ions, Taq polymerase) provided by the manufacturer for the targeted amplification. (Credd Diagnostics, PAIS). All these steps were done inside the type 2 laminar flow cabinet Telstar (Bio-II-A), keeping adequate sterility conditions. After vorting the reagent tube, we introduced it in the thermal cycler VitaPCR™. The time for analysis of a sample is approximately 20 minutes. The material used in this diagnostic test is discarded following the bio-waste disposal protocol.

The primers used in this kit are designed for the detection of specific regions of the SARS-CoV-2 N gene. In addition, primers for the detection of the conserved region SARS-like gene (including SARS-CoV-2, SARS-CoV, bat SARS-like coronavirus) were also included, which will bind also to the N gene but in a different sequence. It allows to reduce the risk misdetection for the virus due to the accumulation of mutations caused by its molecular evolution. Furthermore, primers for the detection of human Beta-globin (HBB) were also included as a sample adequacy control (SAC) and for monitoring the presence of inhibition factors in the amplification process.
The primers were labelled with quenched fluorophores, which will suffer a cleavage and release once the probe has hybridized with the target sequence. The detection of target sequences was achieved by real-time measuring of fluorescence coming from the cleaved fluorophores. Three different fluorophores were detected according to the three specific primers described above. Consequently, three fluorescent channels, including FAM™, VIC® and ROX™, are applied at the same time to detect specific SARS-CoV-2 RNA, universal SARS-like RNA and SAC, respectively.

3.4 Serological test

For this study we have used the COVID-19 IgG/IgM Rapid Test Cassette kit from the HEALGEN trading house. We deposited the blood drop in the device (sample well) and immediately added 3 drops of sample buffer to the buffer well, avoiding air bubbles in any case. The results should be read in 20 minutes and the positive ones may be visible as soon as 2 minutes.

4. RESULTS

4.1 Clinical data of the studied population

During the months of July and October 2020, we were able to simultaneously perform serological and RT-PCR tests on 1043 patients (Table 1). As a percentage, 75.84% of the patients turned out to be male while the remaining 24.16% turned out to be female, presenting a mean age between both of 45 years with a standard deviation of 15 years. Of the total number of patients, only 2 presented symptoms at the sample collection.

Table 1 - Clinical table that summarizes the information of the 1043 patients who participated in the study.

	n = 1043	
Age	45,40 ± 15,33	
Gender	Male: 791 (75,84%)	Female: 252 (24,16%)
Symptoms	2/1043 (0,19%)	

4.2 Tests results of the studied population

Of the total of 1043 simultaneous tests performed, 58 patients tested positive in at least one of the tests (5.56% of the total) (Table 2). The mean age of these patients was maintained with respect to the total cohort (45 ± 15.5 years). Regarding the sex of the positive patients, we observed a certain tendency towards the male sex, based on the fact that 75% of the patients in the total cohort were men, 49 of the 58 positive patients also turned out to be positive (84,48 %). None of the positive patients had symptoms at the time of sample collection.

Table 2 - Clinical table that summarizes the information of the 58 positive patients who participated in the study

	n = 58	
Age	44,98 ± 15,53	
Gender	Male: 49 (84,48%)	Female: 9 (15,52%)
Symptoms	0/58	

While 985 people were negative for both tests (94.44%), for the RT-PCR diagnostic test we obtained a total of 7 positive patients (0.67%), and for the serological test 5 people were positive for the M antibody (IgM) (0.48%) and 52 people tested positive for antibody G (IgG) (Table 3).

If we analyse the 7 patients who tested positive for RT-PCR, 6 of them tested negative for both antibodies, while the remaining patient tested positive for the 3 parameters.

On the other hand, the 52 patients who tested positive for the memory antibody (IgG), 47 of them tested negative in the other 2 parameters (RT-PCR and IgM). And if we consider the patient who had tested positive for all 3 parameters, the remaining 4 patients obtained a positive result for both antibodies while a negative result for RT-PCR.

It should also be considered that there are combinations that have not obtained representation as a patient exclusively positive for antibody M, a patient positive for RT-PCR and IgM but negative for IgG, or a patient positive for RT-PCR and IgG and negative for IgM.

Table 3 - Results table that summarizes the information on the total number of patients and breaks down the 58 patients who have tested positive for one of the 3 parameters that we analyse: RT-PCR, IgM and IgG.

RT-PCR	IgM	IgG	n
-	-	-	985 (94,44%)
+	-	-	6 (0,58%)
-	+	-	0
-	-	+	47 (4,50%)
+	+	-	0
+	-	+	0
-	+	+	4 (0,38%)
+	+	+	1 (0,10%)

A representative graphic example of the results obtained in both tests corresponds to the following image (Figure 10):

Figure 10 - Graphic representation of a positive RT-PCR test and a positive serological test for both antibodies. **A.** In the Y axis total fluorescence is indicated; X axis indicate the cycle threshold (Ct). The yellow colour indicates fluorescence for RNA SARS-CoV-2, red colour indicates fluorescence for RNA family *Coronaviridae* and green colour indicates fluorescence for human control sample. **B**. Reading it from top to bottom serologic test bands indicate positive for human control, IgG, and IgM respectively.

4.3 The results obtained from the study cohort are representative of the Catalonian population

During the months of July to October in which the present study was carried out, the cumulative incidence of SARS-CoV-2 in Catalonia was 83,03 positives / 10.000 inhabitants (0,83%) (18) (Table 4). Interestingly, we observe a similar percentage (0,67%), when tacking into account our patients with a positive RT-PCR (7 patients). Moreover, although these percentages differ in approximately 16%, it must be considered that the province of Tarragona presented the lowest cumulative incidence from all the Community, which make these data even more representative of our area of study. However, we haven't been able to recover the exact official data of the province for that period of time.

These results suggest that the sample size used in this study and the results obtained can be extrapolated to those of the general population of the Community at the time of the study.

Table 4 - Weekly incidence of COVID-19/10.000 hab. during the dates of 1st of July since 4th of October of 2020 in Catalonia.

Dates	Weekly incidence of COVID-19/10.000 hab.
29/06/2020 – 05/07/2020	17,72
06/07/2020 – 12/07/2020	37,92
13/07/2020 – 19/07/2020	70,82
20/07/2020 – 26/07/2020	86,75
27/07/2020 – 02/08/2020	86,86
03/08/2020 – 09/08/2020	82,38
10/08/2020 – 16/08/2020	86,53
17/08/2020 – 23/08/2020	97,28
24/08/2020 – 30/08/2020	102,79
31/08/2020 – 06/09/2020	93,19
07/09/2020 – 13/09/2020	85,77
14/09/2020 – 20/09/2020	102,29
21/09/2020 – 27/09/2020	93,19
28/09/2020 – 04/10/2020	118,86
Mean	**83, 03 (0,83%)**

5. DISCUSSION

The main concern of the paper was to analyse the immune system response to SARS-CoV-2 infection.

As we have explained previously, 35% of positive patients remained asymptomatic to SARS-CoV-2 infection (3), but in our case, all patients reported no symptoms. As 6 of the 7 patients who were positive for RT-PCR still did not present antibodies, this could indicate that these patients were still in the first days of the infection and, that therefore, the symptoms didn't have enough time to appear. It must also be taken into consideration that these tests were carried out as a request of the patient to be able to return to their country, so this factor could have caused that some relevant information had been hidden.

As we have commented in the introduction, the human body produces antibodies after SARS-CoV-2 infection in a sequential manner and it would generally be expected to see IgM appear before IgG (1) (11) (12). Our results are not conclusive in this fact, since in our cohort of patients there is no case of positive RT-PCR with only one of the 2 positive antibodies, so we don't have enough information to confirm that IgM is the first of the two antibodies to be produced across our patients.

We also observed that 6 of the 7 patients who tested positive for RT-PCR didn't yet present antibodies, which would indicate that these patients would have been infected recently, that is, less than 5 - 7 days ago. Otherwise, we would have already observed evidence of antibodies in the test. In fact, the positive patient for RT-PCR, IgM, and IgG doesn't offer us information about the order of antibodies production, but it does corroborate that after an infection the body generates IgM and IgG antibodies. In this case, the diagnosis of infection would indicate that this patient is in the final stretch of infection, since having IgG would indicate that he has been at least 10-12 days since he came into with the virus, but not more because he still had viral remains in its body.

On the other hand, 51 patients were positive in at least one of the parameters of the serological test but negative for RT-PCR. Although at the time of sample extraction these patients were no longer infected, the fact of presenting specific antibodies for SARS-CoV-2 demonstrates previous contact with this virus. 4 of the above patients tested positive for both antibodies, but negative for RT-PCR. It is a result like the one previously mentioned, but in this case the infection could have occurred more days ago, since the immune system would have had enough time to completely eliminate the pathogen.

Finally, we analysed the remaining 47 patients who only tested positive for the IgG antibody. This information is interesting because it indicates and corroborates that the IgM is finite and that after a certain time since the infection it ends up degrading, leaving the other antibody resident in the blood. A couple of weeks need to pass by for these patients to eliminate SARS-CoV-2 completely from their organisms. Now luckily, having the IgG present, would be able to trigger a much faster immune response if they had a reinfection by SARS-CoV-2, which is what the vaccine is intended to achieve.

There is also another important point to consider. Although it has been described that there is a higher hospitalisation and mortality in men from COVID-19 than in women, to date, there is not enough evidence to affirm that men have a higher incidence of infection than women (5). Accordingly, our results show that men have presented a higher incidence, since 75% of the tests have been carried out on men, 84% of the positive ones were also. Recent studies could give us the answer to this sex differences in COVID-19 response, indicating that circulating levels of ACE2 are higher in men as compared to women, allowing more SARS-CoV-2 entry into the cell. Moreover, the number and activity of innate immune cells, including monocytes, macrophages, and dendritic cells (DCs) as well as inflammatory immune responses in general are higher in females than in males (5).

5.1 Strengths and limitations

The present work has a series of strengths and limitations that should be mentioned:

5.1.1 Strengths

- The originality of our solution lies in the fact that we have a big cohort of patients with which we can compare and extrapolate the response to this infection to the Catalonian population.

- This information is valuable and exclusive since it isn't available in most public hospitals as these are exclusive requirements for traveling and, because of this, they aren't covered by social security. These two tests are not done at the same time, but if they were done, they would offer us additional information which would help us to better understand this pandemic.

5.1.2 Limitations

- The ideal method would have been to quantify the serological test and note the Ct of the RT-PCR, in order to correlate the viral load with the antibody concentration.
- Although the patient cohort is large, we have relatively few positives for some of the tests.
- We do not have all the possible combinations of the results, which can skew the general interpretation of them.

6. CONCLUSIONS

From the research that has been carried out, it is possible to conclude that:

- Our body produces IgM and IgG antibodies against SARS-CoV-2 infection as part of the humoral immune response

- The results obtained indicate that these antibodies have followed the expected behaviour since we have been able to observe how IgM tend to degrade, while IgG maintain their function of memory antibodies.

- From the outcome of our investigation, it is not possible to conclude that IgM is produced before IgG.

- The results in terms of viral positivity of our cohort are representative of the population of Catalonia in the period of study.

- Future research should focus on the duration of these memory antibodies (IgG). This could help us understand and explain the evolution of the pandemic, the effectiveness of the vaccines and if the end of the pandemic is near.

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8. SELF-ASSESSMENT:

After these last 4 years I can affirm that I did not make a mistake in choosing my career and that, therefore, currently, I am doing what I like. There has been a lot of knowledge learned, which this year, luckily, we have been able to put into practice.

I have met many people with whom I have been able to share good times, they have taught me to organize myself, to work as a team and to think like a biotechnologist.

This study has been a great opportunity to challenge myself and thus improve my skills within the laboratory, to improve my scientific writing and to know the world of research from within.

This year has been full of challenges, and I feel like I am more prepared than the last. Working in the COVID-19 field throughout the pandemic has been a great experience, but with a very high demand that has strengthened my communication skills by becoming a point of information for the people around me.