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Efficacy of COVID-19 vaccines in patients with chronic liver diseases

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"Diz-me e eu esqueço, ensina-me e eu relembro, envolve-me e eu aprendo."

Abstract

The COVID-19 pandemic has caused serious public health concerns since 2020. Several studies have shown that patients with chronic liver disease (CLD) present with inherent immune dysfunction which appear to associate with worse COVID-19 infection consequences, leading to higher risk of mortality. Vaccines that were approved for the general public lack insights on specific population groups, including patients with CLD, calling for the need of additional investigations of vaccine efficacy in these subgroups. This study aimed to assess the humoral immunity of CLD patients vaccinated against COVID-19 and the extension of protection that the vaccines may confer. To achieve this, a cohort of CLD patients were recruited from Centro Hospitalar Universitário Lisboa Norte, Portugal, regardless of etiology and disease stage. Blood sampling and processing allowed for the extraction of serum used in the analysis of IgGs ,IgMs and neutralizing antibodies (NAbs) by ELISA and neutralization assays, respectively. Results showed that antibody levels and NAbs percentage were significantly increased in patients with CLD following the vaccination protocol. Clinical characteristics such as older age, alcoholism and cirrhosis had a negative impact on the immune response of patients. Immune responses measured at 6-months following vaccination were significantly decreased, comparing with the previous time point. Altogether, our results indicate that patients with CLD, particularly those with cirrhosis and advanced age present with lower immune responses to COVID-19 vaccination. As such, this specific population should be prioritized for receiving booster doses no longer than 6 months after vaccination.

Keywords: Chronic Liver Disease; SARS-CoV-2; Vaccines; adaptive immune response; humoral immune response;

Resumo

A pandemia COVID-19 forçou o desenvolvimento de vacinas capazes de conferir imunidade face ao SARS-CoV-2. Porém, nos vários ensaios clínicos, bem como nos estudos efetuados após a admissão e administração da vacina á população geral, vários grupos de risco não foram tidos em conta, como é o caso dos doentes hepáticos crónicos. Estes doentes apresentam, tipicamente, respostas imunitárias deficientes às vacinas. E embora a eficácia clínica em doentes hepáticos immunocomprometidos seja desconhecida, a vacinação é fortemente recomendada. O desenvolvimento de anticorpos em resposta à infeção é só uma parte da reposta imunitária de um organismo. Existe a necessidade de estudar não só estes anticorpos, mas também a resposta das células do sistema imunitário - imunidade celular. No seu conjunto, com este trabalho pretende-se estudar a eficácia das vacinas contra o SARS-CoV-2 em doentes hepáticos crónicos.

Os objetivos delineados para este projeto são: avaliar a imunidade humoral de doentes hepáticos crónicos para compreender a extensão temporal desta proteção imune e de tal forma averiguar a sua eficácia. Adicionalmente entender se há a necessidade de reforço imunitário para além dos protocolos de vacinação estipulados para a população geral.

Para a recolha de dados desta população, uma coorte de doentes hepáticos foi selecionada do Hospital de Santa Maria que apresentam variedade na idade, género, raça altura, peso, comorbidades, estágio de doença e medicação metabólica. Esta tese visa caraterizar esta coorte de doentes hepáticos de um ponto de vista imunitário recorrendo á testagem de dois isótipos de anticorpo (IgG e IgM) juntamente com a testagem de anticorpos neutralizantes e assim determinar a eficácia das vacinas na proteção contra o vírus. Foi feito um acompanhamento regular em fases temporais específicas denominadas de "timepoints" nomeadamente T0 *(baseline*), T2 (2 semanas após a 2ª dose) e T3 (6 meses após o início da vacinação) onde foi feita uma recolha de sangue por um profissional de saúde e consequentemente o processamento da amostra de sangue em laboratório. No processamento das amostras foi utilizado um protocolo de isolamento usando centrifugação para obter o soro com anticorpos extraído passando a seguir para a análise dos anticorpos. Adicionalmente foram extraídas também células mononucleares do sangue periférico para a análise em futuros estudos. Foram

realizados testes aos dois isótipos de anticorpo através de um protocolo de ELISA para determinar a concentração de anticorpos nas amostras recolhidas face á estirpe selvagem e as variantes mais comuns (Delta e Omicron). Adicionalmente foram considerados resultados provenientes de testes de neutralização das mesmas amostras. Inicialmente foi organizada uma tabela que representava a coorte sendo composta por 43 homens e 20 mulheres dentro dos quais 28 dos pacientes eram cirróticos (44%). A grande maioria foi vacinada com Pfizer (49%) seguida pela Astrazeneca (14%) e finalmente Johnson & Johnson (11%). Observou-se um pico imunidade em T2 que quando comparado aos resultados de pré-vacinação demonstravam um crescimento significativo nas concentrações de IgG e IgM como também na percentagem de anticorpos neutralizantes, tendo o IgG resultados mais elevados que os outros devido a quantidade em circulação. Seguidamente foi feita uma análise com base nas caraterísiticas clínicas da coorte com base no principal anticorpo, IgG, em que foi observado um decrescer com o avanço da idade e/ou com a progressão de condições hepáticas como a fibrose, esteatose hepática e a cirrose. Condições como o consumo alcoólico excessivo também levaram a níveis inferiores de anticorpos circulantes sendo correlativo de uma imunidade mais baixas. Alguns dos resultados foram inesperados como o grupo de fumadores, pacientes com doenças autoimunes ou até sob medicação imunossupressora que podem ser explicados pela pequena amostra de indivíduos que representam estes grupos não havendo valor estatístico significante associado. Relativamente à eficácia das diferentes vacinas, estas permanecem em ordem crescente de eficácia esperada (J&J, Astrazeneca Pfizer) sendo a Moderna excluída pelo facto de só haver um individuo com dados sobre esta vacina. Foi feita uma distinção entre os pacientes mediante os valores do anticorpo IgG em T2 no qual foi observado uma diferença estatisticamente relevante. Foi feita uma distinção entre os indivíduos da população baseada na concentração de anticorpos face a vacinação em T2 criando dois grupos: "low responders" e "high responders". Foi observado uma diferença estatisticamente relevante entre os dois grupos. Finalmente procedeu-se á comparação do efeito da vacinação entre a estirpe selvagem e as variantes Delta e Omicron onde se determinou que há uma relação inversamente proporcional face á imunidade que as vacinas conferem mediante o número de mutações das diferentes variantes. Visto que a variante Omicron é a mais afastada em termos evolucionários da estirpe selvagem os resultados comprovaram que a concentração de anticorpos face a esta variante era substancialmente menor (p value = 0.0016). Conclui-se que a vacinação embora eficiente inicialmente, após os 6 meses observa-se um declínio da imunidade ao longo do tempo e tendo em conta a geração de novas variantes que sucessivamente tornam-se mais resistentes e evasivas aos mecanismos do sistema imune, o protocolo atual de duas doses não é suficiente .Isto leva á necessidade de um protocolo com uma maior frequência de doses de vacinação e com a possibilidade do método de vacinação heterogénea, que consiste essencialmente de vacinar indivíduos com base em um protocolo que utilize mais do que uma plataforma de vacinação. Tem-se vindo a provar mais eficiente que a utilização de uma só plataforma de vacinação. Futuramente é também vital compreender o papel da imunidade celular e das células T maturadas que intervém na defesa do organismo pois interagem diretamente com as células infetadas. Células mononucleares do sangue periférico extraídas neste estudo serão um bom exemplo para futuros estudos de forma a compreender melhor a interação destas células e que importância têm na defesa do organismo. Como tal a investigação deste sistema de imunidade poderá beneficiar na produção de novos tipos de vacina que impulsionem uma defesa mais rigorosa e duradoura que a proporcionada pelas vacinas atuais.

Palavras-chave: Doença hepática crónica; SARS-CoV-2; Vacinas; resposta imunitária adaptativa; resposta imunitária humoral.

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SARS-CoV-2: severe acute respiratory syndrome

RBD: Receptor-binding domain

NTD: N-terminal domain

ACE-2: Angiotensin-converting enzyme

MERS-CoV: middle east respiratory syndrome

TMPRSS2: transmembrane protease serin 2

ORF: open reading frame

ER: endoplasmatic reticulum

DMV: double membrane vesicle

MHC: major histocompatibility complex

J&J: Johnson and Johnson

MAFLD: Metabolic associated fatty liver disease

NAFLD: Non-alcoholic fatty liver disease

1.Introduction

1.1 Covid-19

Severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) was the seventh human coronavirus discovered in Wuhan, Hubei province, China in December of 2019. Since then, this virus became widespread at a global scale and, as of May 2022, 521 million cases had been confirmed with a total death toll of 6.2 million deaths. [1,2]

In this introduction, different aspects of this virus will be approached such as its origin, ability to infect human cells, epidemiology, and clinical pathological findings in the light of hepatic-diseased individuals, as well as information about vaccine development, its application and role in the defense against SARS-CoV-2.

1.2 Origin of SARS-CoV-2

1.2.1 How viruses emerge

Viruses are compact nucleic acid containers of either DNA or RNA associated with proteins and sometimes with lipids. They are not considered living organisms and can singlehandedly survive inside the cells of a host that are susceptible to its entry. These nonliving organisms can replicate viral nucleic acids and translate them into amino acids to build viral proteins. Viruses are therefore nonliving self-contained programs capable of manipulating a cell's system to produce more copies. New viral infections of human cells typically infer a host switching event. This event is defined as the transmission of a pathogen from one species to another, which is the case for most human viral and nonviral diseases such as measles, influenza, HIV, and others. Through these host-switching events viruses evolve, as observed in the cases of Influenza and rabies viruses. [3,4]Coronaviruses are RNA viruses that are distributed in numerous animal species all over the world. Those capable of infecting humans and causing disease are within two taxonomic subgroups named Alphacoronavirus and Betacoronavirus. Four endemic human coronaviruses of these subgroups cause mild self-limited upper respiratory tract infections. The more highly pathogenic coronaviruses, including SARS-CoV-2, are assigned to the Betacoronavirus genus. [5]

1.3 Structure of SARS-CoV-2

SARS-CoV-2, much like SARS-CoV, has a structure formed by four main components. The nucleocapsid protein (N) forms a container-like structure outside the genome, which is further packed by an envelope associated with three structural proteins: the membrane protein (M), spike protein (S), and envelope protein (E). Genome size is approximately 29.9kb. [6,7] SARS-CoV-2's entry into host cells is mediated by the spike glycoprotein. The S protein is composed of two functional subunits, the S1 and S2 subunits. The S1 subunit is divided into the N-terminal domain (NTD) and receptor binding domain (RBD). Its function is to bind to the receptor of the host cell. The S2 subunit carries out the fusion between the virus and host cell membranes. The RBD is a critical component due to its capability to recognize the angiotensin-converting enzyme 2 (ACE2) receptor in host cells.[8] Another major component within the structure of SARS-CoV-2 is the proteolytic cleavage site or polybasic

cleavage site located in the S1/S2 junction that can be triggered by the activity of either furin or cathepsin L proteases. This cleavage site was also observed in MERS and SARS-CoV. [9] Novel variants of the virus have conformational changes in the spike protein to have either better affinity for the ACE-2 receptor or increased immune evasion. A recent study has shown that up to 84 unique mutations have been discovered in the spike glycoprotein of the virus and are quite possibly linked to an increase in binding affinity[10]. It was also found that, together with these mutations, an alteration of surface polarity (an increase in positive charge) could destabilize the interaction between the epitope residue of the virus and SARS-CoV-2 antibodies, due to a dependence of electrostatic interaction, and could also increase binding affinity to human ACE2 receptors.[11] Additionally, in a *in silico* study, prions, which are proteins capable of conformation conversion ,were detected in the SARS-CoV-2 spike protein. Within the Coronaviridae family, these prion-like domains were uniquely found in SARS-CoV-2. A substantial increase in prion-like domains within the S1 region of the spike protein was observed across SARS-CoV-2 emerging variants, leading researchers to believe that prion-like domains have a key role in viral adhesion and entry.

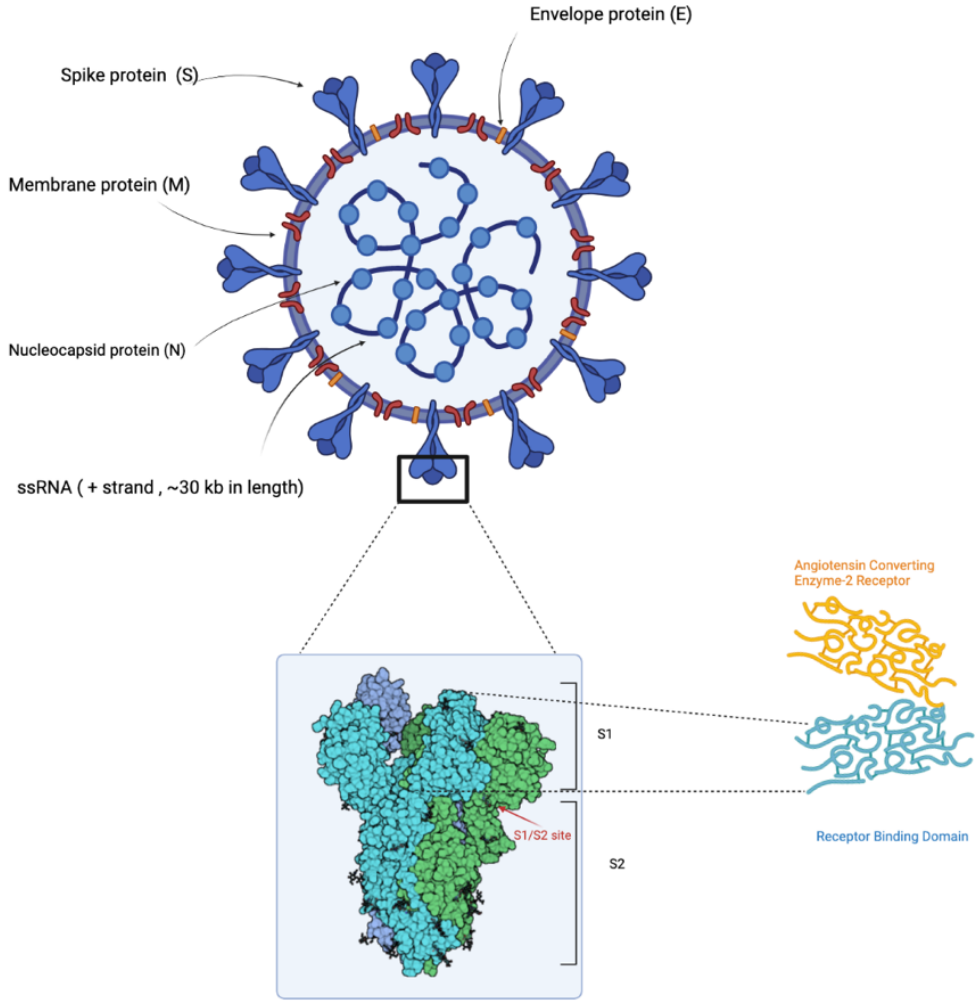

Figure 1.1- Structure of SARS-CoV-2. Main components are present as well as in more detail the S protein divided into its subsections S1 and S2. Created using the BioRender app.

1.4 Infection and Transmission of SARS-CoV-2

1.4.1 SARS-CoV-2 infection lifecycle

Even though SARS-CoV-2 is less lethal than SARS-CoV or MERS-CoV, it is undoubtedly more transmissible. Initially the virus contacts the host cell through the RBD that mediates the interaction with the ACE2 cellular receptor. For the virus to efficiently enter the host cell, the polybasic cleavage site, located in the S1/S2 spike protein junction must be excised. This is mediated by transmembrane protease serine 2 (TMPRSS2) which meets the spike protein in the surface of the host cell. Alternatively, cleavage can be mediated by cathepsin L or furin, which are proteases located in lysosomes and the Golgi apparatus, respectively. After the genome is released into the host cytosol, specific open reading frames (ORFs) are translated into viral replicase proteins that are consequently cleaved and modified into a RNA-dependent RNA polymerase. The replicase components proceed to rearrange the host endoplasmic reticulum (ER) into double membrane vesicles (DMVs) that allow a more precise viral replication of its genomic and subgenomic RNAs (sgRNAs). These sgRNAs are then translated into accessory and viral structural proteins that enable virus particle formation. SARS-CoV viruses target not only the endocytic pathway but also the autophagy process as means to rearrange the host's ER into DMVs, which then allow viral replication within the host.[12–15]

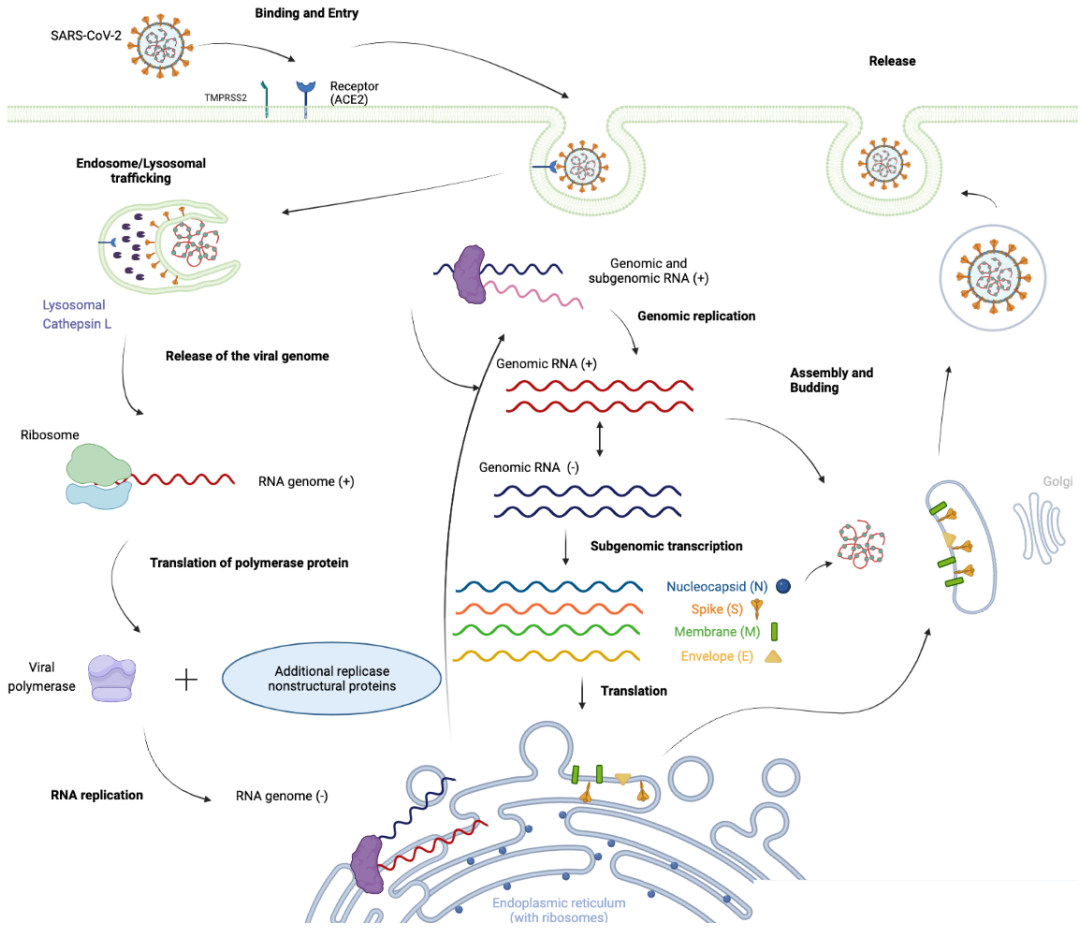

Figure 1.3- General view of SARS-CoV-2 infection lifecycle. Several steps comprise SARS-CoV-2 infection of a host cell: viral entry, translation of viral replication machinery, replication, translation of viral structure proteins, virion assembly and, finally, release of the virus. Created using the BioRender app.

1.4.2 Tissue Tropism of SARS-CoV-2

It is generally thought that SARS-CoV-2 cell/organ tropism is mainly associated with the distribution of the ACE2 receptor, given its key role in viral infection. These receptors are commonly found on type II pneumocyte cells in the airways. SARS-CoV-2 enters the host via the respiratory tract, airway and alveolar epithelial cells and alveolar macrophages.[16] Still, ACE2 expression has been described in other organs [17–19] indicating that SARS-CoV-2 infects not only the respiratory system (lungs and trachea for example) but also the kidneys, small intestine, pancreas, blood vessels and other tissues.[20] This could suggest that additional intrinsic factors, other than ACE2 receptor expression are at play and could lead to a more defined viral tropism.

Droplet transmission is thought to be the main route of transmission of SARS-CoV-2. Curiously, many SARS-CoV-2 patients were reported to have viral-associated gastrointestinal illness, due to the presence of ACE2/TMPRSS2 co-expression in the digestive tract. [18,21,22] This data indicates the possibility of a fecal-oral transmission route that may have a significant impact for health policy settings and change the way SARS-CoV-2 is perceived.

1.4.3 Transmission model of SARS-CoV-2

Human CoVs are transmitted through respiratory droplets, although direct contact with contaminated surfaces, aerosol and even fecal-oral transmission have been also reported.[23–26] The transmission of SARS-CoV-2 is very similar to that of SARS-CoV, being primarily transmitted via respiratory droplets (>5um in size) and aerosols (<5um in size), generated when breathing, coughing or sneezing, and that come in contact with the nose, mouth or eyes. [27],[28,29]Also known as "droplet nuclei", aerosols can remain in air currents and drift to considerable distances (>1m). These small particles can penetrate deep into the alveolar region of the lungs in an individual. On the other hand, larger droplets arise from the upper respiratory tract and most settle close to their source in a short period of time, being regarded as of lesser concern in viral transmission. [30,31] Fomites were heavily studied as a possible transmission vector for the virus, but no conclusive result has been obtained. Other environmental sampling studies also tested for viral RNA only, rather than viable virus. In this sense, studies in which the conditions are closer to real life SARS-CoV-2 exposure should be conducted. [32,33]

1.5 Pathology and symptomatology

Human coronaviruses are known to cause both mild and severe disease and SARS-CoV-2 is an example of the latter. However, for most patients (~ 80%), SARS-CoV-2 causes an asymptomatic infection or mild symptoms. [34,35]Some of the most common symptoms for PCR positive individuals are: fatigue, fever, chills, loss of appetite and persistent cough. Surprisingly, an habitual feature in SARS-CoV-2 infection is anosmia, which is the loss of taste and/or smell. [36] In severe disease cases, patients may present with blood clotting, respiratory compromise, renal damage and cardiovascular collapse. Most patients have lasting sequalae or persistent symptomatology, meaning permanent damage to affected areas.[37] In addition, a great percentage of infected individuals, including asymptomatic ones, display pulmonary ground glass opacity changes meaning that CT scans show a notable increased density in the lung. [38]

1.6 Variants

Modifications in genetic sequences are referred to as mutations. Such events create similar versions of a known sequence that are defined as variants. This is no exception for SARS-COV-2, and many different variants were identified throughout the course of the pandemic. These different variants had mutated genetic sequences in the receptor binding domain (RBD) and the N-terminal domain (NTD) to increase binding affinity to the ACE-2 receptor, and improving antibody escape by reducing binding affinity to monoclonal antibodies, respectively. According to the Center for Disease Control and Prevention, in April 2022, twelve variants were being monitored worldwide, with 5 variants being of concern: Alpha (B1.1.7), Beta (B.1.351), Gamma (P.1) , Delta (B.1.617.2) and Omicron (B.1.1.529). These variants were described to infect and spread more frequently than others and therefore required monitorization. The Alpha variant or UK variant was first identified in the United Kingdom in September 2020, followed by the Beta variant in South Africa in October 2020. In December 2020, the Gamma variant was discovered in Brazil and in the following month the Delta variant was detected in Japan. Lastly, Omicron was sequenced in November 2021 in Botswana. As variants chronologically followed each other, its transmissibility and ability to escape immune defenses increased, and the clinical outcomes in patients became more aggravated. The evolution and appearance of new variants is impossible to predict, which only underscores the need for the development of efficient vaccines. [39,40]

1.7 COVID-19 vaccines

To fight the SARS-CoV-2 spreading pandemic, COVID-19 vaccines were approved across different countries in late 2020 / early 2021. Even though there was some reluctancy in vaccination due to the surprisingly fast development of the vaccines, as of October 2022 11.69 billion vaccine doses had been administered globally with over 65% of the population receiving at least one dose of a COVID-19 vaccine. Most vaccines are developed in a very lengthy and thorough process that can lead up to almost a decade of research and experimenting. For COVID-19's vaccines, this process was significantly shortened, with human clinical trials starting just two months after the genetic sequencing of the virus was available. Nevertheless, there are many crucial steps for a vaccine to be approved and globally distributed. [41,42] After initial development, vaccines undergo a three-phased process to ensure their safety and effectiveness. Generally, these phases are done one at a time however, for time's sake, COVID-19 vaccines underwent overlapped phases. Involving a diverse population that covered most races, ages and ethnicities, thousands of people took part of the clinical trials. Results from the trials showed that COVID-19 vaccines were effective, especially against severe illness, hospitalization and death. After reviewing the followed protocol in the clinical trials, designated and authorized health care entities approved these vaccines to be distributed in the US and most European countries (Food and Drug Administration (FDA) and European Medicines Agency (EMA), respectively).At the moment of writing, eleven COVID-19 vaccines have been granted authorization to be marketed[43]. The range of technology platforms that were developed for these novel vaccines range from viral components, which include nucleic acids, virus-like particles, peptides, viral vectors (replicating and non-replicating), and recombinant proteins; to whole viruses either inactivated or attenuated (Figure 1.3). Each vaccine type may have specific advantages or less positive inherent aspects (Figure 1.4).

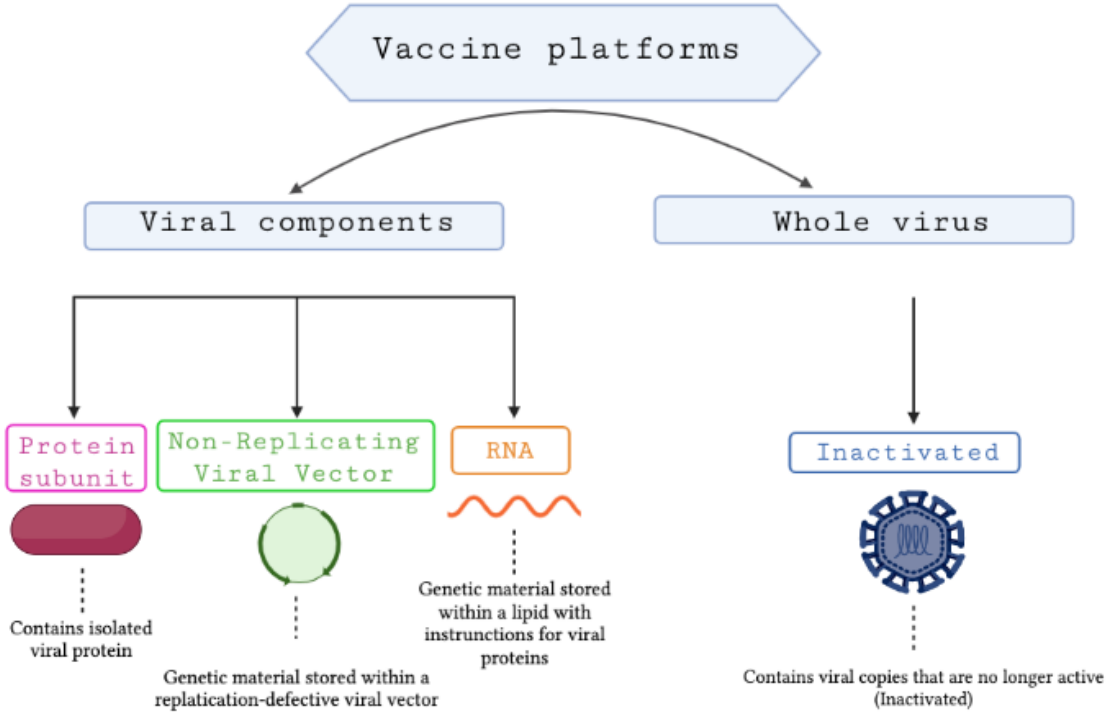

Figure 1.3- COVID-19 vaccine platforms approved by the World Health Organization (WHO). Although sharing the same goal, these vaccine platforms differ in methodology, by using distinct and unique approaches that take advantage of natural products or create new ones. Created using the BioRender app.

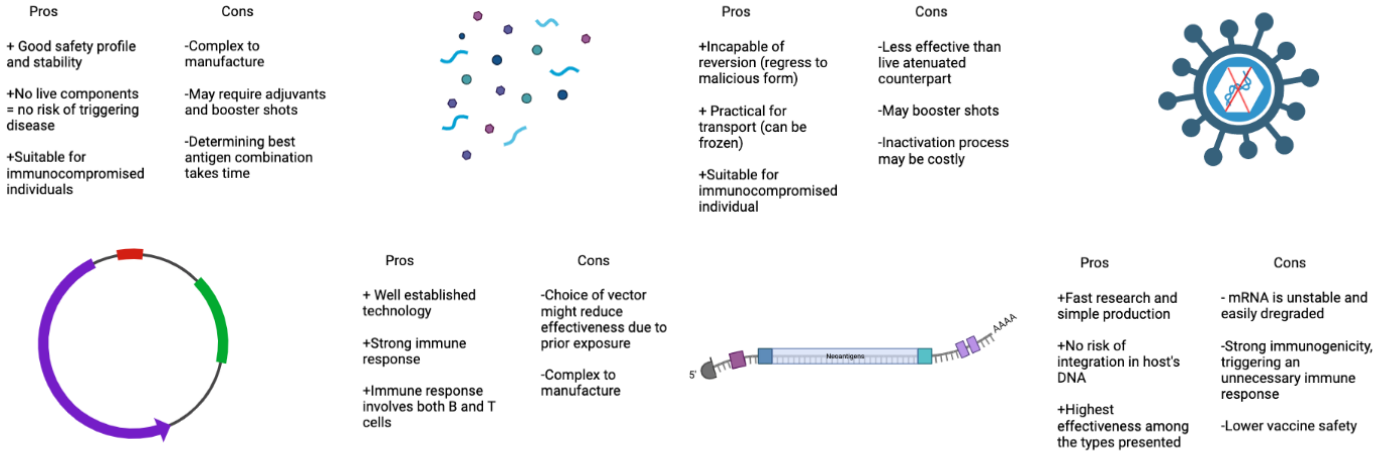

Figure 1.4- Advantages and disadvantages of different vaccine platforms of vaccination. A comparison is made between the different platforms of vaccination exposing the major differences among them. Created using the BioRender app.

1.7.1 Protein subunit vaccines

Subunit vaccines contain proteins (or parts of a protein) derived from a pathogen with immunogenicity that can trigger a response from the host immune system. [44] Immunogenicity of a subunit vaccine's antigen can vary based on the antigen used and in the case of COVID-19, the S protein was chosen as the better candidate due to being common among coronaviruses and to the fact that it remains exposed on the outer surface of the virus. [45] It was shown that a protein subunit vaccine exhibited a high efficacy against SARS-CoV-2 infection and that it even prevented severe COVID-19 symptomology in some cases. [46] Novavax is is a subunit vaccine that has been approved by the WHO and that exhibited 90.4% overall efficacy against COVID-19. [47] In general, this type of vaccine shows great promise and continues to do so in the experimental aspect, since novel subunit vaccines are under development using the RBD as antigen. These new vaccines appear to induce a specific and efficient response by the immune system when immunized into monkeys. [48]

1.7.2 Non-replicating viral vector vaccines

Viral vector vaccines work by using harmless, unrelated viruses as means to insert genetic material into cells; In the case of COVID-19 vaccines, researchers have switched specific parts of the DNA of the carrier virus with DNA encoding for the SARS-CoV-2 spike protein.[44] In contrast to protein subunit vaccines, this platform not only induces humoral immune responses but also cell-mediated immunity, since cells infected by these carrier viruses can express antigens in its major histocompatibility complexes (MHC) as they would if naturally infected. Presently, AstraZeneca's and Johnson & Johnson (J&J's) vaccines are best known to use this platform. A non-replicating chimpanzee adeno-viral vector vaccine is used to deliver the genetic material in AstraZeneca's case, since part of the population may have immune resistance towards the human adenovirus which could blunt the vaccine's effectiveness. J&J uses a modified version of a virus called adenovirus 26. As for results, Astrazeneca exhibits 79% efficacy against SARS-CoV-2 infection.[49] J&J's vaccine, in a comparative study with mRNA vaccines, also pointed to relatively high efficacy (76%) against COVID-19 infection. Although results from comparison studies point out that mRNA vaccines appear to confer a more efficient protection against COVID-19 infection, they also appear to lack the durability of viral vector vaccines, which was shown to be ~2- and ~4-months for Pfizer/Moderna (mRNA) and Astrazeneca/J&J vaccines, respectively. [50]

1.7.3 mRNA vaccines

mRNA-based vaccines revolve around the usage of a virus own genetic information being injected into host cells with the aid of proteins/sugars/lipids. After inserted, the mRNA can produce viral products and be marked for destruction by the host's immune system. In this manner, it achieves humoral and cell-mediated immunity that can last up to 6 months. Although the technology is novel and had its breakthrough due to the global health situation, it shows positive results in protecting against SARS-CoV-2 not only in the general population but also in specific populations, including patients with long-term hemodialysis or multiple sclerosis. [51,52] BNT162b2 or Comirnaty, produced by Pfizer; and mRNA-1273, produced by Moderna, are the leading candidates in the effort of worldwide vaccination against the virus showing efficacy results in the general population around 95%. [53–55]

1.7.4 Inactivated virus vaccines

The fundamentals behind this type of vaccine involves using whole pathogens that were cultured and later inactivated to dispose of malicious capacity to the host. However, some of its integrity is still intact to be recognized by the immune system and elicit an immune response. This platform technology was one of the first of its kind being developed as far back as the late 1800's for diseases such as cholera, plague or even typhoid fever.[42] Nowadays, it has proven useful against diseases such as influenza, hepatitis A, among others. [56] Even though some might consider inactivated pathogens an "old fashion" way of making vaccines, due to licensing issues and the past use of this technology, it is one of the forefronts against COVID-19. CoronaVac and Covaxin are two examples of inactivated virus vaccines that were approved by the WHO [57]. CoronaVac's phase 3 clinical trial in Indonesia alongside Covaxin's phase 3 clinical trials in India, respectively, have shown 65.30% and 77.80% overall efficacy against infection.[58,59] However, this type of vaccine usually needs support from adjuvants to create better immune responses. Vaccines are composed of multiple components as shown (Table 1).

	Function
Antigen	Molecule of foreign origin that can specifically bind to a certain antibody or T-cell receptor which triggers an immune response from the host.
Adjuvants	Allows for increased breadth, durability and magnitude in immune response stimulated by vaccination.
Stabilizers	Mantainance as main role in order to preserve a vaccine's effectiveness by keeping the antigen and other components stable during storage.
Surfactants	Type of emulsifier. Used to maintain particle suspension in a liquid, preventing settling and clumping by lowering the surface tension of the liquid.
Diluent	Usually sterille waters is used. A liquid used to dillute a vaccine to a proper concentration immediatly prior administration.

Table 1.1 - Overview of vaccine components along with their designated function. Vaccines are composed by molecules with distinct functions that provide chemical/physical support as means to elicit an immune response by the host's immune system. Created using the BioRender app.

1.8 Immune responses induced by COVID-19 vaccines

The immune system is compartmentalized in two major components. The innate immune system is composed of proteins and phagocytic cells that constitute the initial response against foreign entities such as viruses, capable of recognizing some conserved features from pathogens serving as a non-specific recognition system. Its existence is critical since the counterpart of this type of immune system takes some time to develop a response. Its purpose is divided into three functions: slow the rate of the infection, contain it in the location of infection while recruiting important inflammatory molecules such as interleukins or specific cells from its system like neutrophils and lastly priming its counterpart to effectively fight against pathogens. The adaptive immune system although very distinct from the innate immune system is just as important given that its response is the major factor against an infection. Its composed of virus-specific CD4+ T cells , CD8+ T cells and B cells (that produce

antibodies, also known as plasmoblasts) that are very efficient at neutralizing any infections, after being primed. CD4+ differentiate into multiple types of cell that mostly have helper functions such as enhancing B cells towards antibody production or priming CD8+ cells into cytotoxic cells that efficiently destroy infected cells. The extensive proliferation and differentiation of naive B and T cells into effector specific cells however takes time and resources hence the need for the innate immune system to regulate early infection on the host. The capability of evasion by the virus and the efficiency of the early innate immune response are two important variables that mediate the rate of infection and the severity of disease.[60]

Vaccines besides boosting antibody production and priming of T cells also generate long lasting memory B and T cells that outlast the protection provided by two-dose protocols with recent vaccines. Memory T cells for example are known to last years and it has already been proved that these SARS-CoV-2-specific memory T cells durably persist after vaccination or infection.[61,62]

In the case of SARS-CoV-2, it has been determined that this virus is particularly effective at avoiding or delaying the intracellular innate immune response that are usually associated to type I and III IFNs. Early viral recognition is vital for the later priming of the adaptive immune system.[63]Asymptomatic cases are linked to a delay early innate immune response. In most mild cases of COVID-19 early innate immune response is somewhat delayed however T cell and antibody responses when prompted act fast and control infection.[64] Severe COVID-19 cases are associated to the ineffective IFN innate immunity that fail to control primary infection allowing invasion of the host tissues and consequently a cytokine storm is unleashed due to the lack of control which leads to fatal outcomes.[65]

Humoral immune response incited by vaccination showed in several studies that IgG antibodies directed against SARS-CoV-2 S protein and its receptor binding domain (RBD) were produced, having rates of 95% and over in mRNA vaccination two-dose protocols such as Pfizer and Moderna's vaccines.[66] In the case of other types of vaccine such as AstraZeneca and Johnson & Johnson's vaccine a slightly lower antibody titer was measured (~55 - 95 %). [67–69] Additionally, neutralizations assays were performed results showed high titers of neutralizing antibodies that surpassed natural infection titers.[70,71] However regarding more recent variants such as Omicron antibody quantification assays show levels are lowered but still within range of conferring immune protection. [72]

Essentially most studies comprise data regarding adaptive immunity and test for antibody quantification, however when it comes to preventing spread of an infection, neutralizing antibodies are not as effective as T cell-mediated immunity that can kill infected cells and therefore ideally prevent spreading. In this regard T cell-mediated immunity due to recognizing more than the RBD or NTD domains maintained a high response (>80%) against infection even with different variants such as Omicron.[73] There is already some studies in which the presence of SARS-CoV-2-specific T cells are crucial for disease impact on macaques and humans.[74] One study provided results on cancer patients with B cell deficiencies where CD8+ T cells response correlated with milder disease in subjects. Another study showed failed vaccine results in macaques lacking Omicron-specific CD8+ T cells despite having reasonable levels of Omicron-specific NAbs.[75] This demonstrates the importance of T cell-mediated immunity in combination with humoral immunity.

1.9 Chronic liver disease (CLD)

Liver diseases (Table 2), such as hepatitis B and C, nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), cirrhosis and hepatocellular carcinoma (HCC), are some of the more common causes of illness and death worldwide. As an example, 2 billion people worldwide are affected by hepatitis B virus (HBV) infection and around 300 to 400 million are chronic carriers of the disease [76], whilst hepatitis C affects 150 million people over the world. ALD and NAFLD are also very common in developed countries affecting approximately 7.4% and 20% to 30% of adults, respectively. [77] Overall, chronic liver disease (CLD) affects around 1.5 million people worldwide. Noteworthy, some liver conditions such as liver fibrosis can progress to cirrhosis because of SARS-CoV-2 infection. [78]

Liver disease	Description & Origin
Hepatitis A	Hepatitis is a swelling of the liver due to damage to its tissues caused by viral infection.
Hepatitis B	
Hepatitis C	
Fatty liver disease	Unusual build up of fat in the liver, that can be caused by alcohol (alcoholic faty liver disease) or obesity (nonalcoholic fatty liver disease).
Cirrhosis	Scarred tissue in the liver caused by injury or long-term disease. Scarred tissue cannot perform same functions as healthy tissue.
Hepatocellular Carcinoma	Cancer of the liver. Multiple causes are pointed out : alcohol, obesity, cirrhosis and smoking are some.
Hemochromatosis	Excessive build up of iron due to increased absorption. Caused by either genetic inheritance or underlying blood diseases.
Wilson disease	Unrestrained accumulation of copper in the body that leads to copper in blood ciruclation. Caused by genetic inheritance.
Autoimmune Hepatitis	Non-contagious, chronic, inflammatory, autoimmune disease in which one's own immune system attacks healthy, normal liver cells. The cause of liver cell destruction in this disease is unclear, but suspected to be realated to an imbalance in immune cells.

Table 1.2- Description and origin of the most prevalent chronic liver diseases. Created using the BioRender app.

1.9.1 Etiology of CLD

CLD is a continuous process of inflammation, destruction, and regeneration of liver parenchyma, which leads to fibrosis and cirrhosis. The spectrum of etiologies is broad and includes toxins, alcohol abuse, infection, autoimmune diseases, genetic and metabolic disorders. Cirrhosis is a final stage of CLD.

Hepatitis – In severe cases, hepatitis can lead to end-stage cirrhosis or even liver cancer. It is estimated that 350,000 people die annually from HCV complications. It has a higher prevalence in the Middle East and Africa and about 150 million people are chronic carriers of the disease. Conventional or pegylated interferons, administered alone or with ribavirin, constitute one of the most recommended treatments. [79,80] HBV is another viral hepatitis variant with 300 million people chronically infected mainly being prevalent in Asia and Africa additionally this type of viral hepatitis is poorly sensed by the innate immune system and therefore can escape it at early stages of infection. [81] Current therapy for HBV is similar to HCV treatment using antiviral agents such as interferon therapy.[82]

NAFLD - Nonalcoholic fatty liver disease has wide range of causes and presents in different severity stages, from simple steatosis/fatty liver without inflammation to non-alcoholic steatohepatitis

(NASH) with inflammatory reactions and hepatocyte damage, which can further progress to fibrosis cirrhosis and, ultimately, liver cancer. The incidence of NALFD has risen alongside the rates of obesity, type 2 diabetes, insulin resistance and arterial hypertension, which constitute typical comorbidities of this disease. [83,84]

Cirrhosis - Long term CLD leads to cirrhosis. It is estimated that 1.5 billion people are affected by CLD and cirrhosis worldwide, with an annual death toll of about 2 million individuals.[85] Due to high chances of acute renal failure or gastrointestinal bleeding, cirrhotic patients need specific treatments in accordance to the degree of liver damage (whether they present with compensated cirrhosis which is an asymptomatic minor form of liver disease or decompensated cirrhosis that is considered a long term and severe form of liver disease) alongside different comorbidities such as hypertension, kidney disease, diabetes, among others. Common medication includes antihypertensive agents for blood pressure control, including beta blockers or vaptans as well as proton-pump inhibitors. Other more direct approaches involve abdominal surgery, endoscopy, and paracentesis. [86]

HCC - Hepatocellular carcinoma is probably one of the most complex liver diseases. 854,000 liver cases were recorded in 2015 and 810,000 cancer-related deaths in that same year. The incidence of HCC is increasing every year but at a slower rate than other liver diseases.[85] Regarding treatment, the best option would be liver transplantation the reason being that the removal of tissue that is at risk of developing cancer has the best chances at eliminating the disease. However, organ donation is not sufficient and therefore other treatments are considered such as hepatic resection, which consists in surgically isolating and removing a portion of the liver affected by cancer. Another common procedure - when resection is not viable - is ablation, which consists in the chemical or physical destruction of cancerous liver tissue.[87]

1.10 COVID-19 and liver disease

COVID-19 infection tends to worsen the state of underlying chronic diseases [88] ranging from pancreatic conditions such as diabetes to neuronal diseases such as dementia or Alzheimer's.[89,90] Chronic liver diseases are no exception; in fact, it was reported that COVID-19 infection in CLD patients increases their mortality rates (NAFLD, hepatitis B, autoimmune hepatitis, cirrhosis). [91] A meta-analysis looking at NAFLD patients underscored that 60% developed a severe course post COVID-19 infection with a mortality rate of 18%. [92]As for autoimmune liver diseases, some studies have suggested that COVID-19 infection is more severe and lethal in these patients; still, other studies failed to find such association. In addition, a study conducted in northern Italy reported that SARS-CoV-2 infection is no likelier to happen in autoimmune hepatitis patients comparing with the general population, and that the probability of severe development of this underlying condition after infection was low.[93] In sum, and in general, it appears that COVID-19 infection in liver disease patients leads to an aggravation of the disease.

1.11 COVID-19 vaccination in patients with CLD

Many recent studies have been reporting the effects of COVID-19 vaccination in particular groups of patients, including patients with CLD. Still, patients with CLD present with heterogenous clinical parameters as well as liver disease severities. For instance, liver transplant patients were hypothesized to present with lower levels of humoral and cell mediated immunity after COVID-19 vaccination when compared to healthy controls, due to being in a regimen of immunosuppression treatments. In fact, LT patients were shown to present with a lower long-term immune response after vaccination, comparing with non-LT patients. [94]

Regarding cirrhotic patients there is data from different studies suggesting that, as opposed to LT-patients, this subgroup of liver disease patients shows humoral and cellular immune responses that are comparable to healthy controls.[95] In one of the studies, seroconversion of all cirrhotic patients was achieved and also more than half of them had spike-specific T-cell responses.[96] In another study, the humoral immune response was poorly induced in only a quarter on CLD patients (with or without cirrhosis), in contrast with more than half of LT recipients [97] It should be noted that many of the studies conducted on CLD patients had reduced sample sizes and data on vaccine efficacy and clinical outcomes in these subgroups is still limited due to the small number of participants. [95]

2. Objectives

Patients with CLD are at higher risk of developing more severe COVID-19 and display higher mortality rates, compared to non-CLD patients. This has been suggested to be attributed, at least in part, to cirrhosis-associated immune dysfunction (CAID), a distinctive spectrum of immune alterations associated with the course of end-stage liver disease.[98] In addition, patients with CLD could also display lower immunity to COVID-19 vaccines. Moderna/NIAID mRNA-1273 and BioNTech-Pfizer BNT162b2 mRNA vaccines, as well as the AstraZeneca/University of Oxford ChAdOx1 adenoviral vectored vaccine, have each reported excellent safety profiles, marked efficacy in preventing symptomatic COVID-19 (62–95%), and have all gained rapid regulatory approval. Despite the high number of study participants, only a few patients with underlying liver disease were included in the trials. As such, it is essential to examine the effect of COVID-19 vaccines in patients with CLD. The aim of this Thesis was to evaluate humoral responses to SARS-CoV-2 vaccination in patients with CLD, at 2-weeks and 6-months after two-dose vaccination. Predictors of low and high response to vaccination were identified, and humoral immunity of CLD patients to the novel SARS-CoV-2 B.1.617 (Delta) and B.1.1.529 (Omicron) variants was assessed.

3. Materials and Methods

3.1 Study population

Non-pregnant adult patients (≥18 years) with CLD vaccinated against COVID-19 were recruited at Centro Hospitalar Universitário Lisboa Norte, Lisbon, Portugal. Definition of CLD was based on clinical, radiological, or histological evidence of liver disease. Patients with previous liver transplantation were excluded. Patients with CLD were extensively characterized at enrolment (anthropometric, clinical and biochemical data). Liver disease stage was categorized according to the Child-Pugh class into CLD without cirrhosis, cirrhosis Child-Pugh class A, cirrhosis Child-Pugh class B, and cirrhosis Child-Pugh class C. Additionally, comorbidities (type II diabetes, obesity, others) were also recorded.

3.2 Registry and samples collection

Blood samples and clinical data were collected between February 2021 and February 2022. Data was stored in the HEPCOVIVac Registry using a de-identified format in an electronic case report form, using "Research Electronic Data Capture" (REDCap™) hosted at the "National Center for Data Registries in Gastroenterology (CEREGA), Sociedade Portuguesa de Gastroenterologia" (SPG; https://www.spg.pt), a non-profit Scientific and Medical Society focused on Gastroenterology research.

The HEPCOVIVac Registry consists of a prospective, international, multicenter and observational registry, not interfering with the usual clinical routine and treatment of patients. Case report forms

included general information about the patient (e.g., gender, age, demographics, etc.), liver disease etiology and severity, comorbidities and risk factors, clinical parameters and therapy, and SARS-Cov-2 infection (symptoms, date of onset and resolution of symptoms, history of PCR testing, other) and vaccination details (type of vaccine, date of administration, side effects, other). For the analysis included in these Thesis, patients were grouped according to the etiology of liver disease into:

- Patients with viral hepatitis - including patients with hepatitis B and C.

- Patients with autoimmune and/or cholestatic liver disease - including patients with primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC) and/or autoimmune hepatitis (AIH).

- Patients with metabolic associated liver disease (MAFLD) - including patients with non-alcoholic fatty liver disease (NAFLD) and/or heavy alcohol consumption.

- Patients with hereditary liver disease - including patients with Wilson disease and hemochromatosis.

Patients were further divided according to pharmacology into:

- Immunosuppressive treatment (Prednisone, Tacrolimus, Azathioprine)

- Antiviral therapy (Tenofovir, Entecavir)

- Metabolic therapy (Ursodeoxycolic acid, Fibrates, Metformin, GLP-1 antagonists, insulin, statins, simvastatin, penicillamine, testosterone).

The study protocol consisted of collecting blood samples and clinical data from patients with CLD at different timepoints. In this Thesis, we analyzed samples collected at T0 (baseline; prior to vaccination); T2 (two-weeks after two-dose vaccination); and T3 (six months after the start of vaccination). Case report forms and venous blood samples were filled/collected, respectively, at each visit. Before enrolment, all participants gave written informed consent and a disclosure form according to the EU personal/patient data act. The HEPCOViVac Registry Study protocol and informed consent were reviewed and approved by the Ethic Committee of the Faculty of Pharmacy, Universidade de Lisboa (Code: 02/2021) and the Lisbon Academic Medical Center (Code: 24/21), as coordinating Centers, prior to study implementation.

3.3 Blood Processing

Venous blood samples were collected at the above mentioned timepoints by trained nurses, using appropriate personal protective equipment. The quantity of blood collected varied between 10 to 20 mL per individual. Following blood collection in EDTA tubes, 1mL of blood was taken to a 1,5mL tube and centrifuged at 2000 g for 15 min at 4ºC. The serum was withdrawn and stored at -80ºC for analysis of vaccine-induced immune responses resulting from antibody-antigen interactions. To analyze for cellular immunity, in future studies, the remaining blood was used to isolate peripheral blood mononuclear cells (PBMCs) as follows:

1. Blood was diluted with phosphate-buffered saline (PBS) 1x in a 1:1 ratio.

2. Ficoll was added to conical tubes in a proportion of 6mL per 5 mL of blood. The solution of blood and PBS was gently added to the conical tube using an electronic pipettor with minimum dispensing speed to prevent blood from mixing with Ficoll

3. After a centrifugation step at 800 g for 20 min, PBMCs, serum and blood are separated into different layers.

4. Plasma was collected into three eppendorfs and stored at -80ºC

5. PBMCs were transferred into a new tube containing PBS/FBS (97%/3%) to wash the cells.

6. After washing, cells were resuspended in PBS/DMSO (90%/10%) and stored at -80 ºC.

3.4 ELISA Assays

A 384-well ELISA plate was coated on the first day with the the SARS-CoV-2 nucleocapsid protein, the SARS-CoV-2 reference Spike S1 protein (Acro Biosystems), the SARS-CoV-2 B.1.617 Spike RBD protein (SinoBiological) or the SARS-CoV-2 B.1.1.529 Spike RBD protein (SinoBiological) by adding 50µL/well, except for the wells that contain the calibration curve. The plate was covered and incubated at 4ºC overnight. On the second day, the coated plate was washed with 90µL/well of wash buffer, composed of 0.05% Tween® 20 PBS. Afterward, the plate was blocked by adding 25µL/well of Blocking Buffer (0.10% Tween® 20, 3% bovine serum albumin (BSA) PBS) and incubated at room temperature for 2 hours. Each well was washed 3 times with 90µL of wash buffer. Twenty-five microliters of diluted samples were added to the wells. The assurance of duplicated wells for all samples is recommended, as well as the presence of control samples and empty wells on every plate. The plate was incubated at room temperature for 1 hour and, once again washed 3 times with 90µL of wash buffer. A highly specific secondary antibody, namely goat anti-human IgG Fc HRP conjugated for IgG detection was added to the plate in a volume of 25µL. The plate was incubated at room temperature for 1 hour and washed 3 times with 90µL of wash buffer. The addition of 25µL/well of TMB substrate was followed by 10 minutes of incubation in the dark, at room temperature. To stop the reaction, after 10 minutes, 25µL/well of H2SO4 was added. A schematic of the process is shown in Figure 3.1. The absorbance was measured at 450 nm in a Varioskan LUX Multimode Microplate Reader®. It is important to guarantee that there are standard controls, positive controls, and negative controls on the same plate. The standard curve is obtained through successive serial dilutions of a known concentration of the analyte. These dilutions are performed considering the expected values of the unknown concentrations present in the samples. The positive controls are samples that are known to have the substance that is expected to be detected, and the negative controls are, in contrast, the ones that do not contain this substance. The washing buffer is added between steps to ensure that no unbound components or debris remain on the plate. In this regard, the cut-off point - to establish positivity - was calculated using a mean value of the concentration of a cohort of 45 pre-pandemic negative controls, plus three times their standard deviation. Values for the experiment's optimal cutoff value, specificity, and sensitivity for this assay were calculated using the operator received curve (ROC) in GraphPad Prism Version 9.0. The optimal cutoff value was 7nM, with sensitivity and specificity of 100% and 95,45%, respectively.

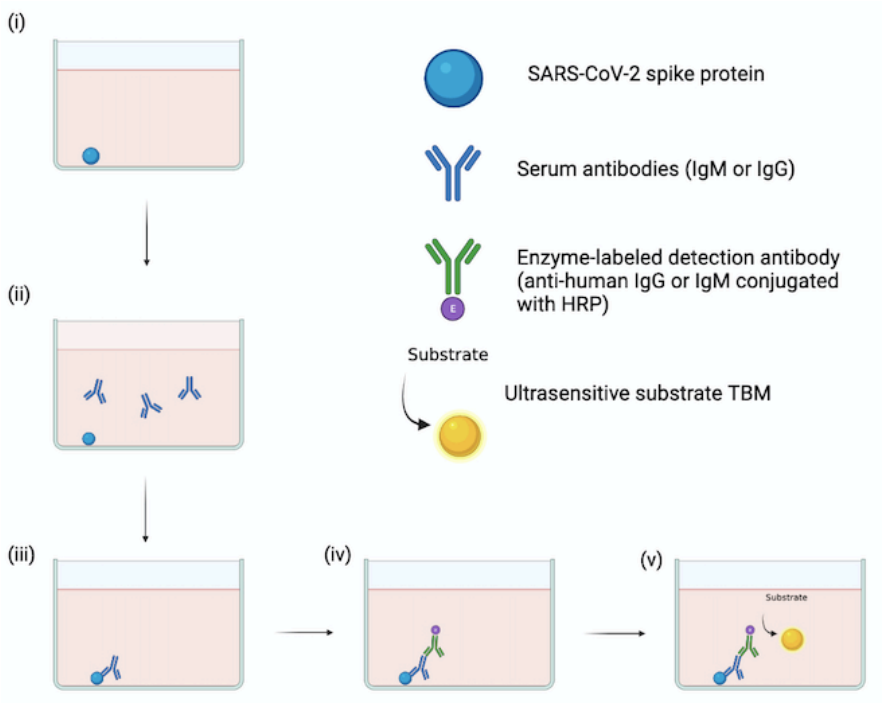

Figure 3.1- Indirect ELISA to detect spike protein-specific IgG/IgM. (i) Attachment SARS-CoV-2 spike protein to solid phase; (ii) incubation with serum antibodies (IgG/IgM); (iii) washing of unsecured serum antibodies out; (iv) incubate with enzyme-labeled detection antibody; (v) Incubation with substrate which is then converted by HRP (Horseradish Peroxidase) into a detectable luminous signal.

3.5 Surrogate neutralization assay

For the detection of neutralizing antibodies against SARS-CoV-2 RBD, AlphaLISA® technology was used, which allows detection of SARS-CoV-2 binding antibodies on human plasma samples, with the ability to block or inhibit the viral entry into cells through cellular receptor Angiotensin-Converting Enzyme 2 (ACE2). This procedure was conducted by a third party.

3.6 Statistical analysis

Comparison analysis was done using non-parametric Kruskal Wallis test and parametric one-way ANOVA test using GraphPad Prism (version 8.4.3). To directly compare two different groups, a non-parametric two-tailed Mann-Whitney U-test was employed. Comparisons were made between patient antibody levels of different timepoints, considering clinical characteristics and different SARS-CoV-2 strains.

4. Results

4.1 Patient adherence to the study

As part of my work during this Thesis, I was responsible for contacting patients and make appointments for the collection of samples in the T3 timepoint (twelve months after the start of vaccination). Patients were contacted by telephone and/or email. Of the 58 patients that were listed to be contacted, 11% were unreachable, 51% were scheduled to collect blood, 36% declined to continue to participate in the study and 2% reported that they were recently infected with COVID-19 (Figure 4.1).

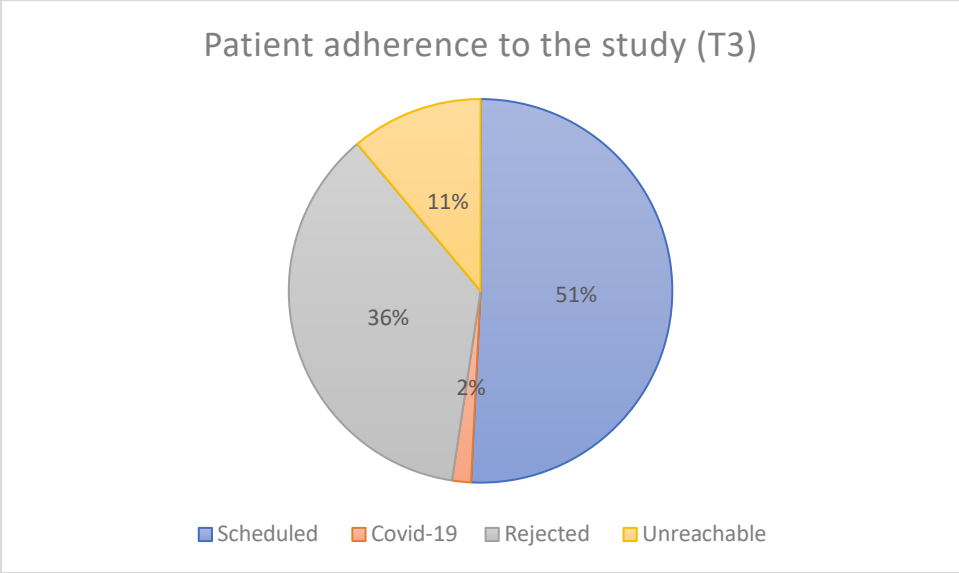

Figure 4.1- Pie chart regarding telephonic scheduling of patients for the fourth and last harvest. Successfully scheduled patients appear in blue, patients that rejected blood harvesting are in yellow, in orange are patients who got infected mid study and in grey are the patients that were unreachable. Created by using Excel.

4.2 Characteristics of the study population

A total of 63 patient samples were collected for this study. The different timepoints are composed by an uneven number of patients due to reasons explained in the previous chapter. Table 3 presents the demographical and clinical characteristics of the study population who had available samples at both T0 and T2. Among these, 28 patients presented with cirrhosis. Table 4 shows the demographical and clinical characteristics of patients with available samples at the T0 and T3 timepoints.

	All patients (n=63)	Cirrhotic patients (n=28)
Age, mean ± SD	54.5 ± 11.4	60 ± 11.2
Sex, female	20 (32)	6 (21)
BMI, mean ± SD	29.0 ± 5.0	28.6 ± 5.0
Race (n,%)		
Black	3 (5)	1 (4)
Caucasian	59 (93)	27 (96)
Others*	1 (2)	-
Vaccine (n,%)		
Moderna	1 (2)	-
Pfizer	31 (49)	12 (43)
Astrazeneca	9 (14)	5 (18)
Johnson & Johnson	7 (11)	5 (18)
Unknown	15 (24)	6 (21)
Etiology of liver disease" (n,%)		
Alcoholic	18 (29)	17 (61)
NAFLD	19 (30)	2 (7)
Autoimmune and/or cholestatic liver disease	12 (19)	2 (7)
Viral hepatitis	17 (27)	9 (32)
Other liver diseases**	6 (10)	-
Liver Disease Severity (n,%)		
Child-Pugh A score	-	22 (78.6)
Child-Pugh B&C score	-	6 (21.4)
Fibrosis score: F0-F2	31 (49.2)	-
Fibrosis score: F3-F4	32 (50.7)	28 (100)
Comorbidities" (n,%)		
Type 2 diabetes mellitus	15 (24)	9 (32)
Hypertension	8 (13)	2 (7)
Obesity	12 (19)	4 (14)
Other comorbidities ***	14 (22)	8 (29)
Immunosuppressant medication&	6 (10)	1 (4)
Metabolic drugs € (n,%)	33 (52.4)	13 (46.4)

Table 4.1- Demographic and clinical characteristics of patients with CLD (all versus cirrhotic). Others*: Hispanic or Asian. Other liver diseases**: Wilson's disease, Haemocromatosis, IgG4 cholangiopathy and cholestatic disease. Other comorbidities***: Hypertriglyceridemia, Hypercholesterolemia, Renal Insufficiency, Smoker. ": Numbers may not add up since some had more than one risk factor. Immunosuppressant medication&: Prednisone, Tacrolimus and Azathioprine. Metabolic drugs€: Fibrates, GLP-1 agonists, statins, simvastatin, penicillamine and testosterone.

Results showed that cirrhotic patients tended to be older, of male sex and with a lower BMI when compared to all patients. Patients were fully vaccinated (T2) with eitherChAdOx1 (14%; AstraZeneca; Cambridge, UK), mRNA-1273 (2%; Moderna, Cambridge, MA, USA), BNT162b2 (49%; Pfizer, Mainz, Germany) or vector-based vaccine JNJ-78436735 (11%; Johnson & Johnson, New Brunswick, NJ, USA). 24% of patients were fully vaccinated yet the information regarding the developer was not recorded. The most frequent underlying liver disease etiologies were non-alcoholic fatty liver disease (non-alcoholic fatty liver disease , 30%) followed by, alcohol (29%) viral hepatitis (27%) and lastly autoimmune and/or cholestatic liver disease (19%). More than half of cirrhotic patients were under heavy alcohol consumption (61%). Most patients presented with early stages of

liver fibrosis (49.2%); however, those in an advanced stage (50.7%), presented all cases of cirrhosis. Among cirrhotic patients, most presented with Child-Pugh A score (78.6%). Regarding comorbidities, the most prevalent was type 2 diabetes (24%) followed by obesity (19%) and finally hypertension (13%). Nearly a third of cirrhotic patients were diabetic (32%). A low percentage of patients were under the use of immunosuppressive medication (10%) but more than half of the population was prescribed metabolic drugs (52.4%). Results presented on Table 4.2 showed to be similar to those on Table 4.1.

	T0 & T3 patients (non cirrhotic) (n=14)	T0 & T3 patients (cirrhotic) (n=14)
Age, mean ± SD	50.5 ± 10.7	61.3 ± 11.6
Sex, female	8 (57.1)	5 (35.7)
BMI, mean ± SD	29.8 ± 5.2	27.1 ± 5.2
Race (n,%)		
Black	-	1 (7.1)
Caucasian	14 (100)	13 (92.9)
Others*	-	-
Vaccine (n,%)		
Moderna	1 (7.1)	-
Pfizer	9 (64.3)	8 (57.1)
Astrazeneca	2 (14.3)	3 (21.4)
Johnson & Johnson	1 (7.1)	2 (14.3)
Unknown	1 (7.1)	1 (7.1)
Etiology of liver disease" (n,%)		
Alcoholic	-	8 (57.1)
NAFLD	6 (42.9)	2 (14.3)
Autoimmune and/or cholestatic liver disease	4 (28.6)	1 (7.1)
Viral hepatitis	5 (35.7)	-
Other liver diseases**	1 (7.1)	4 (28.6)
Liver Disease Severity (n,%)		
Cirrhosis	-	14 (100)
Child-Pugh A score	-	12 (85.7)
Child-Pugh B&C score	-	2 (14.3)
Fibrosis score: F0-F2	12 (85.7)	-
Fibrosis score: F3-F4	2 (14.3)	14 (100)
Comorbidities" (n,%)		
Type 2 diabetes mellitus	2 (14.3)	6 (42.9)
Hypertension	3 (21.4)	2 (14.3)
Obesity	4 (28.6)	2 (14.3)
Other comorbidities ***	1 (7.1)	2 (14.3)
Immunosuppressant medication&	1 (7.1)	1 (7.1)
Metabolic drugs € (n,%)	7 (50)	4 (28.6)

Table 4.2 - Patients demographic and clinical characteristics separated by disease severity (Non cirrhotic or cirrhotic) within the pool of samples collected on the fourth timepoint (T3). Categories were divided into race, type of vaccine administered, the etiology of liver disease, any known comorbidities and usage of metabolic drugs. *- Others: Hispanic or Asian. **- Other liver diseases: Wilson's disease, Haemocromatosis, IgG4 cholangiopathy and cholestatic disease. ***- Other comorbidities: Hypertriglyceridemia, Hypercholesterolemia, Renal Insufficiency, Smoker. "- Numbers may not add up since some had more than 1 risk factor. &- Immunosuppressant medication: Prednisone, Tacrolimus and Azathioprine. €- Other metabolic drugs: Fibrates, GLP-1 agonists, statins, simvastatin, penicillamine and testosterone.

4.3 IgG antibody analysis based on demographic and clinical characteristics (T2)

The T2 time-point was chosen given that previous studies had shown a peak response in antibody levels 2 weeks after the second dose administration. [99] Results on IgG testing according to age showed a decrease in antibody levels in older individuals, in accordance with vaccination data in other studies, proving once again that in older individuals the antibody count is lower most likely due to a weaker immune system (Figure 4.2). High consumption of alcohol associated with lower antibody levels (p-value = 0.0565) which is corroborated by the increase in severe cases of SARS-CoV-2 infections within this population; the lower antibody titer likely translates into a less effective immune defense against viral infection.[100] However, results in smokers are contrary to what has been described in the literature and this may be because of the low number of subjects with smoking habits in this study. Patients with autoimmune disease showed a statistically significant higher antibody count compared with other patients, which was unexpected, and possibly due to lack of sample amount to consider it meaningful. Viral hepatitis individuals showed less antibody production because, similarly to autoimmune disease patients, the immune system in these patients is known to be disrupted by the viral infection.[101] NAFLD patients exhibited a slightly higher antibody level, comparing with individuals without NAFLD, which is in line with some clinical results inferring that this disease is associated with an excessive activation of the immune system causing it to produce relatively more antibodies than other patients that do not have that disease.[102] Although with a reduced number of individuals classified, the distinction made by Child-Pugh classification showed that Child-Pugh A patients present with higher antibody levels comparing to Child-Pugh B or C. This was somehow expected, since the former classification is for patients in less severe stages compared to the latter. Fibrosis progression in patients also showed that patients in later stages (F3-F4) produced less antibodies. Although it has been reported that fibrosis *per se* appears not to hamper the adaptive immune response (antibody production), the outcome of excessive fibrosis leads to an impairment of the whole immune system. [103]Patients with ongoing treatments showed atypical results, possibly because of the lack of statistical samples in the three different treatment groups. Our results also showed that cirrhotic patients display a lower antibody count compared with patients without cirrhosis. However, these results were not statistically significant, possibly due to the low number of patients in the study. Finally, the categorization of the administered vaccinations showed a significant difference in antibody levels between patients vaccinated with the Pfizer vaccine and patients vaccinated with the J&J vaccine. These results corroborate current data that shows improved immune responses for Pfizer, AstraZeneca, and J&J in that order of efficacy.[104] Overall, similar trends were found when performing the same comparisons for IgM and NAb levels (Supplementary Figures 7.1 ;7.2).

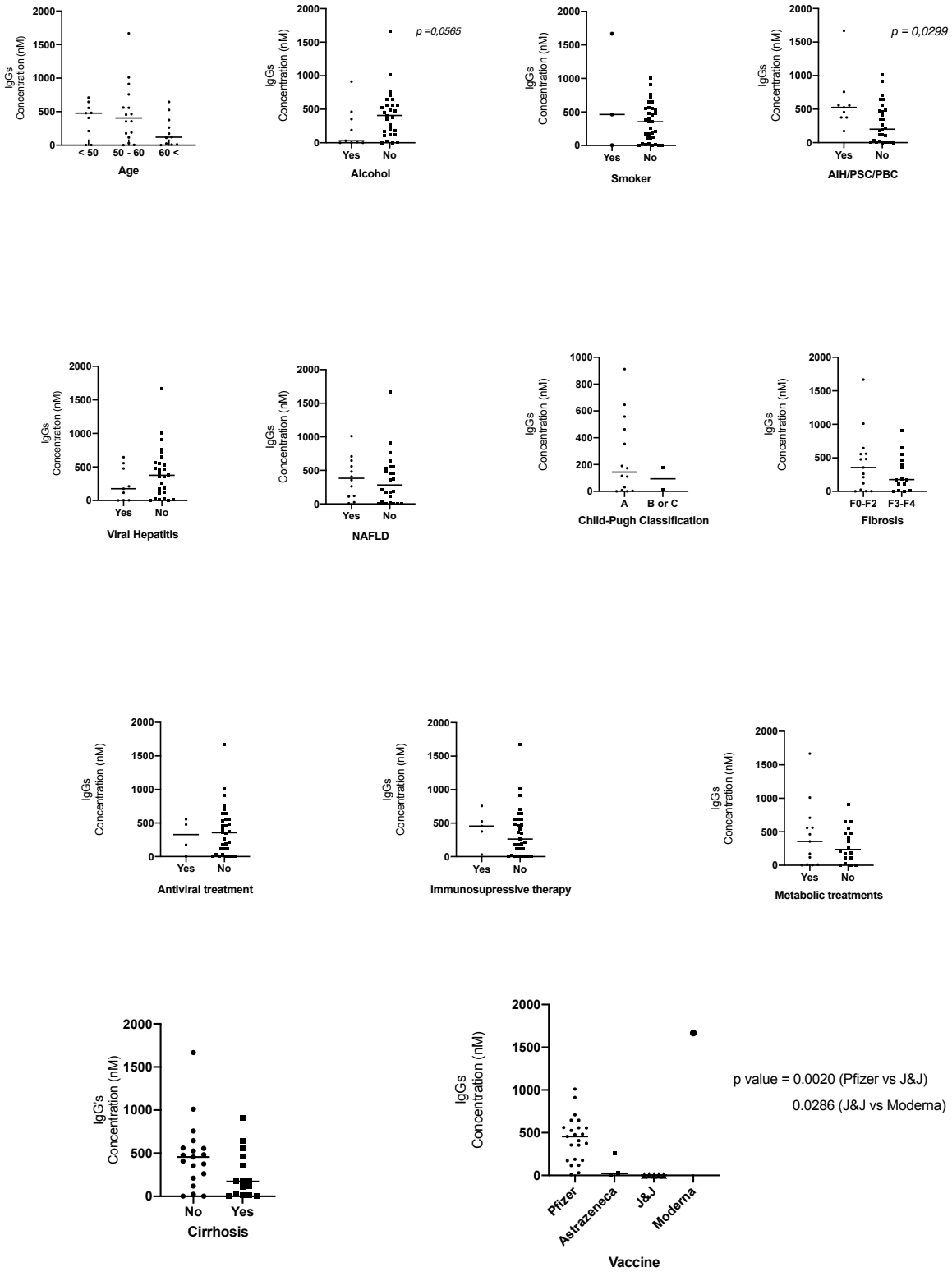

Figure 4.2– T2 IgG levels after SARS-CoV-2 vaccination in patients with CLD according to demographic and clinical characteristics, etiology of the disease, type of vaccine and pharmacology. Levels of spike-specific IgG antibodies were determined by ELISA.

4.4 Comparative analysis of antibody results T0/T2 & T2/T3

At two weeks post second dose vaccination (T2), all patients with CLD showed a significant increase in IgG, IgM and NAb levels ($p < 0.0001$) (Figure 4.3). Of note, measurement of IgG antibody levels showed a more distinctive result likely due to its higher amount in circulating blood when compared to IgM, as reported in the literature.[105] Regarding the follow up at 6 months after the start of vaccination (T3), it was observed that IgG levels decayed to a certain extent. This can be interpreted as waning immunity that withers over time; however, no statistical significance was found. No statistically significant differences were found when comparing humoral immunity between patients with CLD and a cohort of healthy volunteers (Supplementary Figure 7.2).

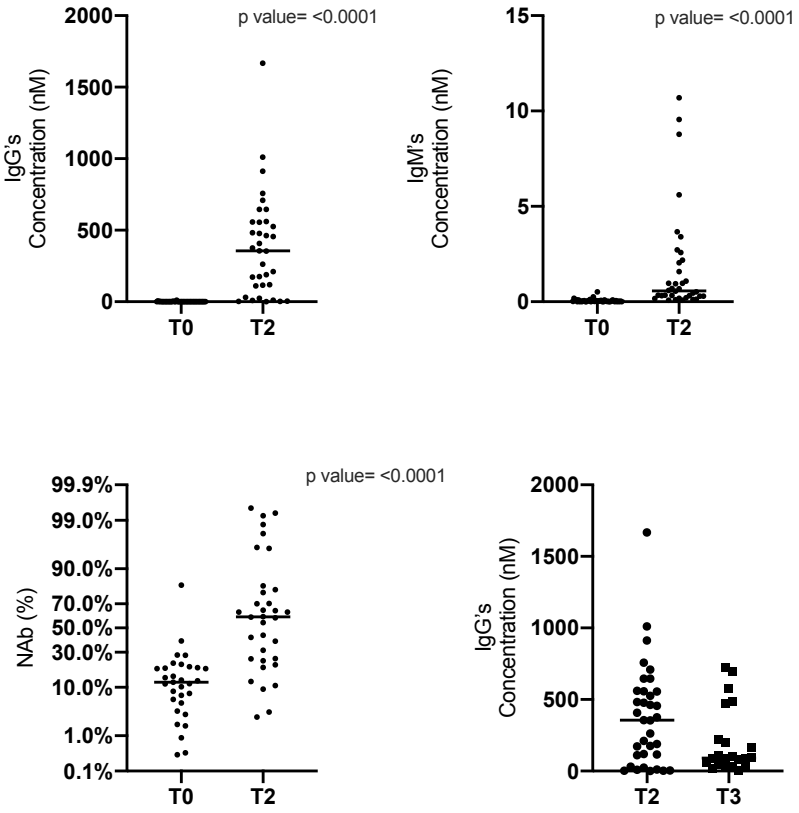

Figure 4.3 – IgG, IgM and NAb levels in patients with CLD before SARS-CoV-2 vaccination (at baseline -T0); 2 weeks after the second dose (T2); and 6 months after the start of vaccination (T3). Levels of spike specific IgG, IgM and NAb were determined by ELISA testing. Comparison using a two tailed non-parametric Mann-Whitney U test resulted in a p-value of <0.0001 in the first three graphics.

4.5 Antibody analysis to define high and low responders.

We next divided our cohort of patients with CLD as high and low responders according to their IgG median at T2 (356.1 nM; Figure 4.4, top). Both IgM and NAb results confirm the statistical significance between high and low responders. This can be interpreted as evidence proving the wide response in CLD patients to COVID-19 vaccination, that is, some individuals are found to be in less favorable conditions that consequently weaken the immune system and show poorer results whereas others, although chronically diseased, can mount a higher humoral response to vaccination.

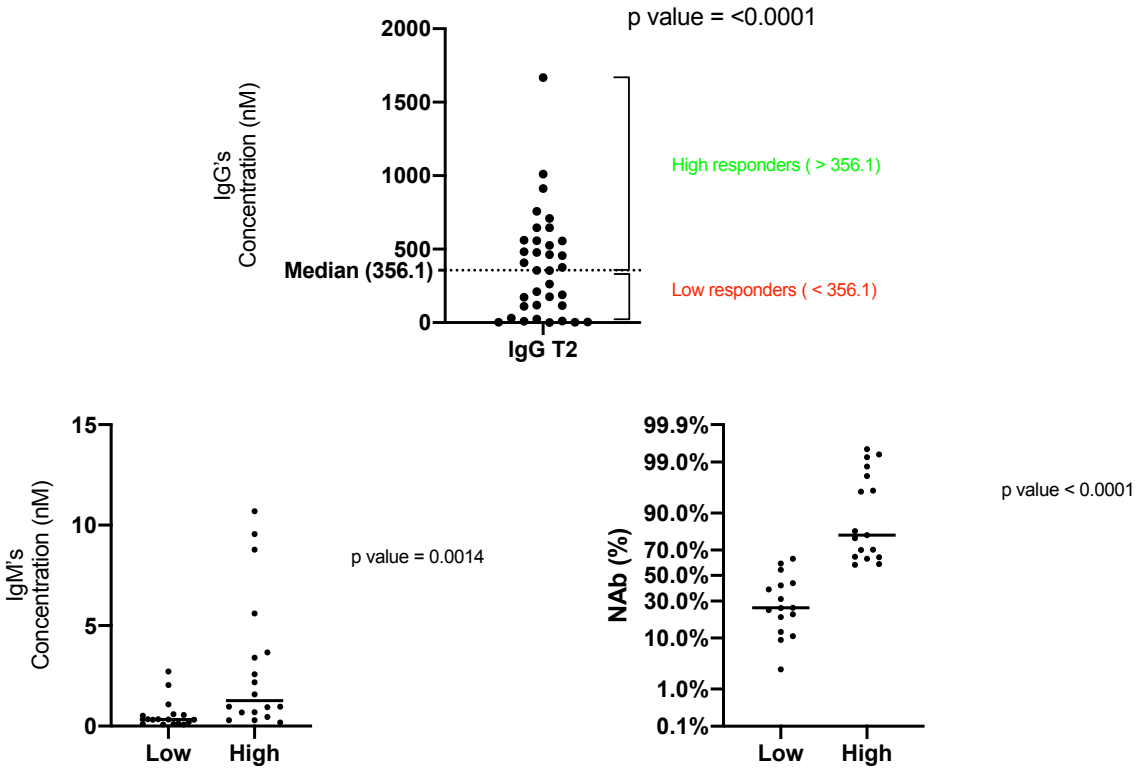

Figure 4.4- IgG, IgM and NAb levels in patients with CLD two weeks after second dose vaccination (T2), divided as high and low responders. Levels of IgG, IgM and NAb were determined by ELISA testing. Comparison using a two tailed non-parametric Mann-Whitney U test was used.

4.5.1 Comparison of the demographic and clinical characteristics between low and high responders

	Low responders (n=17)	High responders (n=18)
Age, mean ± SD	56.3 ± 11.4	52.4 ± 11.2
Sex, female	4 (23.5)	7 (38.9)
BMI, mean ± SD	29.0 ± 5.1	29.0 ± 5.0
Race (n,%)		
Black	-	-
Caucasian	17 (100)	18 (100)
Others*	-	-
Vaccine (n,%)		
Moderna	-	1 (5.6)
Pfizer	7 (41.1)	16 (88.9)
Astrazeneca	3 (17.6)	-
Johnson & Johnson	4 (23.5)	-
Unknown	-	1 (5.6)
Etiology of liver disease" (n,%)		
Alcoholic	6 (35.3)	2 (11.1)
NAFLD	5 (29.4)	7 (38.9)
Autoimmune and/or cholestatic liver disease	1 (5.9)	7 (38.9)
Viral hepatitis	6 (35.3)	3 (16.7)
Other liver diseases**	1 (5.9)	1 (5.6)
Liver Disease Severity (n,%)		
Cirrhosis	11 (64.7)	4 (22.2)
Child-Pugh A score	9 (52.9)	4 (22.2)
Child-Pugh B&C score	2 (11.8)	-
Fibrosis score: F0-F2	7 (41.1)	12 (66.7)
Fibrosis score: F3-F4	10 (58.8)	6 (33.3)
Comorbidities" (n,%)		
Type 2 diabetes mellitus	5 (29.4)	7 (38.9)
Hypertension	2 (11.8)	5 (27.8)
Obesity	5 (29.4)	6 (33.3)
Other comorbidities ***	6 (35.3)	7 (38.9)
Immunosuppressant medication&	1 (5.9)	4 (22.2)
Metabolic drugs € (n,%)	6 (35.3)	9 (50)

Table 4.3-Demographic and clinical characteristics of patients with CLD divided as low and high responders. Categories were divided into race, type of vaccine administered, the etiology of liver disease, any known comorbidities and usage of metabolic drugs. *- Others: Hispanic or Asian. **- Other liver diseases: Wilson's disease, Haemocromatosis, IgG4 cholangiopathy and cholestatic disease. ***- Other comorbidities: Hypertriglyceridemia, Hypercholesterolemia, Renal Insufficiency, Smoker. "- Numbers may not add up since some had more than 1 risk factor. &- Immunosuppressant medication: Prednisone, Tacrolimus and Azathioprine. €- Other metabolic drugs: Fibrates, GLP-1 agonists, statins, simvastatin, penicillamine and testosterone.

Given the results shown before between low and high responders, a more profound analysis into the characteristics described before could reveal the explanation to the humoral response difference between groups. Table 4.3 shows the demographic and clinical characteristics of patients with CLD divided as low and high responders. Overall, no specific characteristic was found to associate with a higher or lower humoral immune response of patients to COVID-19 vaccination. Still, higher responders were slightly younger than lower responders, in line with what was already described in the literature. Also, the percentage of patients on immunosuppressive or metabolic drug treatments was bigger in the higher responders group, although the meaning of this potential association remains to be elucidated and may actually result from the relative low number of patients in this study. In addition, there appeared to be a trend for a higher response associating with the type of vaccine.

4.6 Humoral response of patients with CLD to SARS-CoV-2 variants

We next compared wild-type (WT), Delta and Omicron IgG levels in patients with CLD, two weeks after second dose vaccination. As shown in Figure 4.5, IgG levels against the Delta variant were lower comparing with the WT and further decreased for the Omicron variant. The distinction is higher between wild type and Omicron, which likely relates with the evolutionary gap between them. When analyzing the results stratified according to vaccine developer, results were like those obtained for the WT variant (Figure 4.5), but with much lower antibody titers, particularly for the Omicron variant. All this data points to the evolutionary divergence of the virus throughout the pandemic and works as evidence that although plenty effective at first, COVID-19 vaccines do not transpose great results when assessed for different variants of the virus. Cross-reaction although existent may not confer the necessary protection against more recent variants.

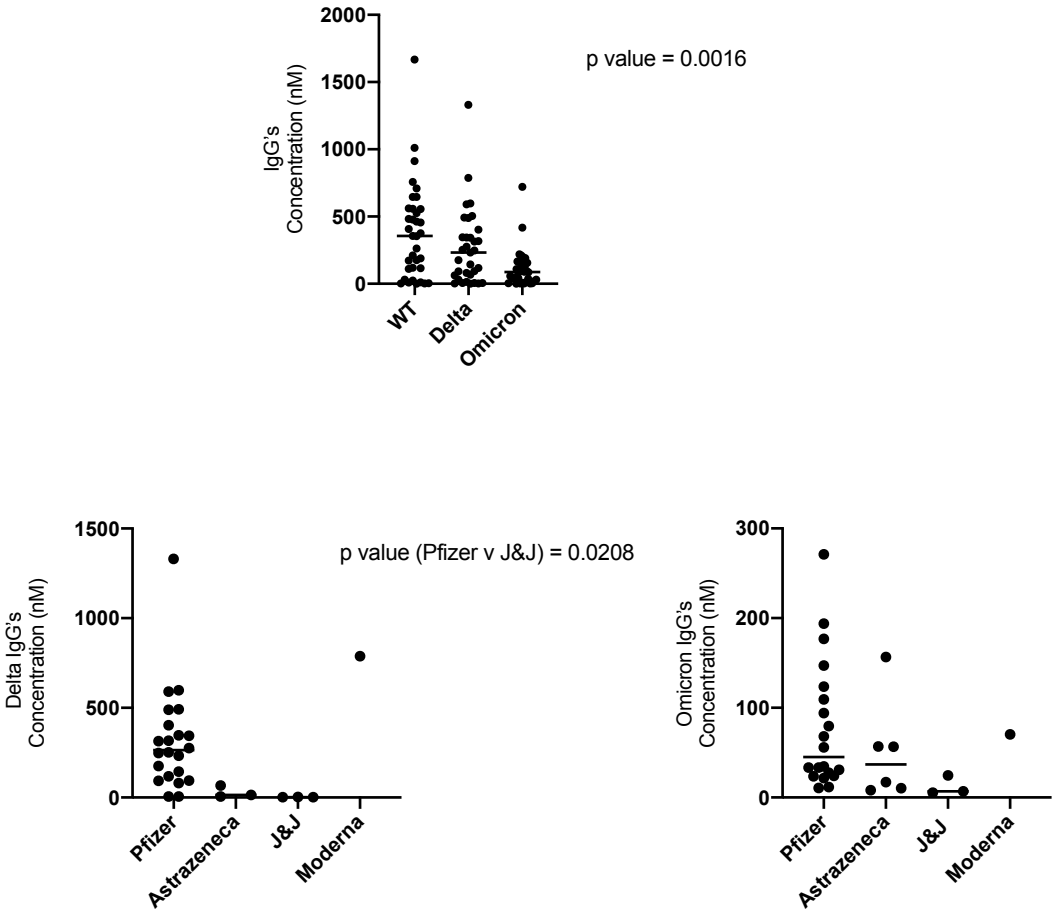

Figure 4.5- - Wild-type (WT), Delta and Omicron IgG levels in patients with CLD two weeks after second dose vaccination (T2) (top) and stratified according to vaccine developer (bottom).Levels of variant-specific IgGs were determined by ELISA testing. Comparison using a two tailed non-parametric Mann-Whitney U test was used.

5. Conclusion

SARS-CoV-2 has become in recent times an important risk to global health and safety, for which it becomes imperative that it is better studied, in parallel with the efficacy and safety of COVID-19 vaccination. Research and education on the matter are required to prevent the virus from reoccurring or at the very least contain it. The development of vaccines was critical for a chance to retaliate and immunize the world population against the virus and so, its efficacy becomes a genuine concern that should be investigated. The production of various types of vaccines not only helped in dissemination of protection, but also allowed research to progress and come out with innovative technologies such as the mRNA vaccines. This study aimed to assess the efficacy of the different vaccines in patients with CLD with different etiologies. The repertoire created for this study was found to be lacking in much information for various reasons, one of them being the several checkups over a long period of time that some individuals were bound to skip or give up entirely on the process lowering the number of subjects in the results. Some of the information from patients was missing and with the lack of evidence made it obligatory to pull out test subjects, again lowering the sample pool. In other cases, some of the samples from patients turned out to be spoiled and so results were not trustworthy. Overall results from this study, regardless of not presenting statistical significance in most cases, corroborated evidence found in the studies regarding efficacy of these vaccines in patients with CLD. The lack of statistical substance is mainly due to the lack of subjects in the study after basic filtering. From our data and recent literature, we can conclude that vaccination against SARS-CoV-2 is, in most cases, efficient, although the levels of antibodies produced by vaccination lower up to 6 months after. As such, to prevent future outbreaks, it is recommended that patients with CLD receive booster doses, preferably on a 6 month to a year basis. SARS-CoV-2 is expected in the future to become a similar case to the influenza-virus where there will be season outbreaks from time to time and so vaccination in this context can work as a containment against widespread infection.

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7.Supplementary data

Figure 7.1- IgM results based on characteristics (T2)

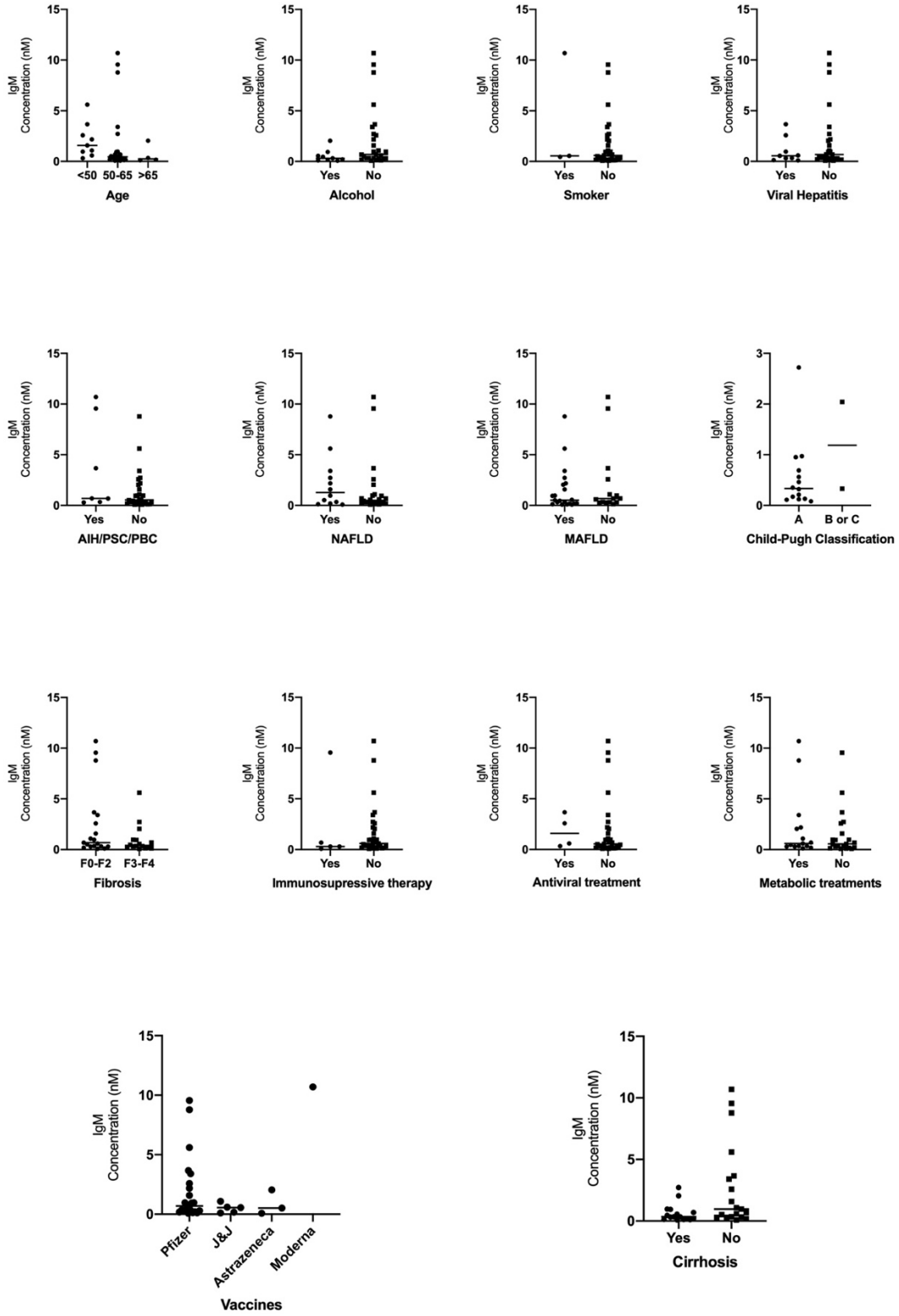

Figure 7.1 - T2 IgM antibody levels after SARS-CoV-2 vaccination in patients with CLD according to demographic and clinical characteristics, etiology of the disease, type of vaccine and pharmacology. Levels of spike-specific IgM antibodies were determined by ELISA and neutralization assay, respectively.

Figure 7.2- NAb results based on characteristics (T2)

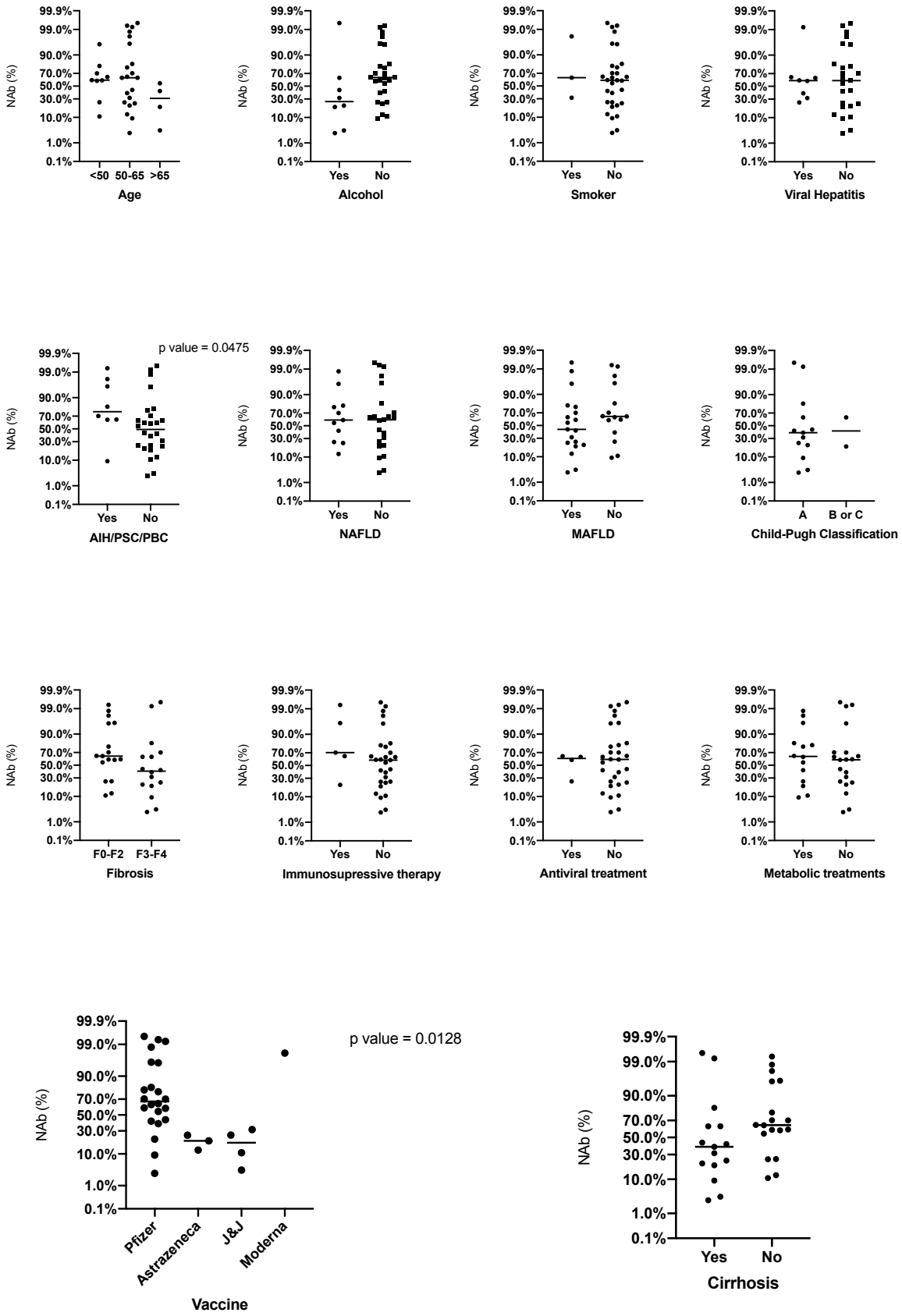

Figure 7.2 - T2 NAb antibody levels after SARS-CoV-2 vaccination in patients with CLD according to demographic and clinical characteristics, etiology of the disease, type of vaccine and pharmacology. Levels of spike-specific NAb were determined by ELISA and neutralization assay, respectively.

Figure 7.2– T2 sample comparison between CLD patients and healthy controls

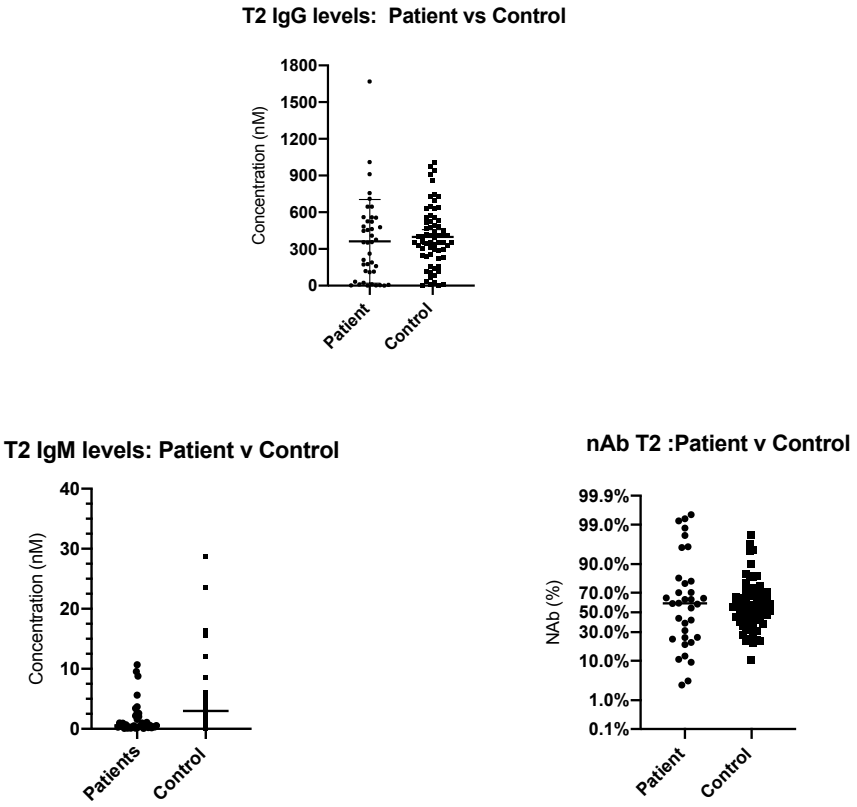

Figure C- T2 IgG,IgM and NAb levels comparison between CLD patients and healthy controls two weeks after vaccination protocol.