Universitat de Girona
Facultat de Medicina

HUMORAL RESPONSE TO SARS-COV-2 VACCINE IN MULTIPLE SCLEROSIS PATIENTS TREATED WITH ANTI-CD20

FINAL DEGREE PROJECT

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Girona, November 2020

I would like to show my gratitude to my methodological tutor, Dr. Xavier Castells, for his support and collaboration in the elaboration of this work.

I would also like to express my gratitude to my clinical tutor, Dr. Lluís Ramió, and all the members from "Unitat de Neuroimmunologia i Esclerosis Múltiple de l'Hospital Santa Caterina" whose knowledge and help let me learn a lot and enjoy this experience.

Also, I would like to thank Marc Sáez, Dúnia Pérez del Campo and Anna Marull for their advices.

Thanks to Arnau Casals. Your help, patience and moral support have been vital for me to carry out this project.

I'm very grateful to my team from Girona. This six-year road would be nothing without you.

Finally, and above all, I would like to express my gratitude to my parents.

1 ABSTRACT

Background: SARS-CoV-2 is a viral infection that causes COVID-19 disease, a pandemic or public health emergency situation we currently live forces the rapid development of a vaccine by compressing developmental stages and optimizing its safety and efficacy data in human use. For its part, Multiple Sclerosis is the most common chronic autoimmune disease of Central Nervous System (CNS). The patients who are receiving immunomodulatory treatment such as anti-CD20 therapy whose response to the vaccine is predicted to be blunted become the epicentre of concern in this protocol. These assumptions are based on previous experience with other vaccines and on the mechanism of action itself. The thin line between spacing out disease control treatment to aid the repopulation of B cells to create a competent immune response against the SARS-CoV-2 vaccine becomes a challenge in the management of such patients.

Objective: The aim of this study is to study the dynamics of humoral response against SARS-CoV-2 vaccine in Multiple Sclerosis patients who are receiving anti-CD20 therapy. The long-term goal is to optimize the management of their disease by offering them the possibility to develop immunization against SARS-CoV-2.

Methodology: The study will be a prospective cohort conducted in the Santa Caterina's Hospital using a consecutive method of sampling for a comparison of two cohort groups formed by patients attending to Neuroimmunology and Multiple Sclerosis Unit (cohort 1) and to the International Health Unit (cohort 2). All the participants must fulfil the inclusion criteria and have none of the exclusion ones. Determinations of SARS-CoV-2 antibody in blood pre- and post-vaccine administration will be performed during a follow-up period of 5 months. Once we collected the data a statistical analysis will be performed using Chi-square and Fisher's Exact test to assessing the relation between dependent and independent variables. A p-value <0.05 will be considered as statistically significant. The association between the dependent and independent variables will be adjusted using co-variables-controlled logistic regression in order to avoid confusion.

Keywords: Multiple sclerosis, anti-CD20 therapy, Immunoglobulins G (IgG), SARS-CoV-2 vaccine.

2 LIST OF ABBREVIATIONS

Ab: Antibody

Ag: Antigen

CEIC: "Comité de Ética i Investigación Clínica"

CMIA: Chemiluminescent Microparticle ImmunoAssay

CNS: Central Nervous System

CSF: Cerebrospinal fluid

DMT: Disease Modifying Therapies

EDSS: Expanded Disability Status Scale

ELISA: Enzyme Linked ImmunoSorbent Assay

HUDJT: Hospital Universitari Dr. Josep Trueta

IS: Immune System

LP: lumbar puncture

mAb: Monoclonal Antibodies

MS: Multiple Sclerosis

PP-MS: primary progressive Multiple Sclerosis

RR-MS: Remitting relapsing Multiple Sclerosis

RT-PCR: Reverse transcription polymerase chain reaction

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

SP-MS: secondary progressive Multiple Sclerosis

UNIEMTG: Unitat de Neuroimmunologia i Esclerosis Múltiple Territorial de Girona

3 INTRODUCTION

3.1 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune chronic and progressive neurodegenerative disease in which we can mainly find two components, although they are not exclusive, inflammatory and degenerating. It affects specifically the Central Nervous System (CNS), and involves a loss of myelin (demyelination) and primary or secondary axonal damage. It is characterized by unpredictable and heterogeneous distribution in terms of presentations and symptomatology. (1)

Being more frequently diagnosed between the 2nd and 4th decades of life, MS is the leading cause of non-traumatic disability in adults (2). This fact has a huge implication on the quality of life, emotional burden (for those who suffer from it and their relatives) and economic impact for the health system.

3.1.1 Epidemiology

MS affects 2.8 million of people worldwide being more prevalent in European and American continents (3). This number has increased the last decade due to the improvement of epidemiological recount techniques and the greater knowledge regarding the diagnosis, as well as the correct and individualized management of MS has increased life expectance in these patients. An increase in MS incidence and prevalence also has been reported in the 3rd edition of MS Atlas (3).

MS seems to follow a latitudinal pattern, that is, its prevalence increases as we move further away from equator (greater latitude).

It is estimated that in Spain more than 55.000 of people have MS. The prevalence of MS is 120 cases per 100.000 inhabitants(4) with an annual incidence of 4.2 cases per 100.000 inhabitants. (5)

It is more frequent in women (2:1 or even 3:1 depending on the region where the ratio women/man is higher).

In Catalonia, concretely in Girona, the annual incidence of MS is 4.5 new cases per 100.000 inhabitants which means that approximately 33-34 cases are diagnosed every year (6).

The mean age of diagnosis is 32 years old but it can appear at any age (mostly between 20-40 years old).

3.1.2 Physiopathology and risk factors

MS caused by an abnormal immune response against the antigens of your own CNS in individuals who are exposed to different environmental factors and are genetically predisposed. The aetiology of this hyperactivation remains unclear and it is believed to be multifactorial.

It should be noted that MS is not a hereditary disease but having some specifical genes increases the probability to develop the disease. The gene that was clearly been linked to this disease is HLA class II, specifically the locus DRB1*15:01 (7). There is no a specifical genetic test in order to diagnose the disease.

Epstein-Barr Virus (EBV) have been described as increasing risk factors for this disease (8). Smoking is also an increased risk factor to evolve to MS progressive forms.

In the last decades, vitamin D (that acts as an Immune System modulator in CNS) had gained importance. The deficiency of vitamin D have been associated with a worse MS outcome. Levels higher than what is considered normal does not represent any benefit. (9) This is closely related to sunlight exposure and the decreased prevalence in lower latitudes.

Moreover, factors such as psychologic stress, diet, alterations in the intestinal microbiota, etc are summative risk factors of developing MS.

This immune-mediated disorder causes inflammation, demyelination and primary or secondary axonal damage, which disrupts the ability of transmission of nervous signal that causes different symptomatology. The axonal damage without regeneration is the responsible for the sequelae and the degree of disability in each MS patient.

3.1.3 Types

- (RIS) Radiologically isolated syndrome: is defined as presence of suggestive-MS lesions on MRI without clinical signs. 1/3 of these patients will develop symptoms over time (10,11).
- CIS (clinically isolated syndrome): first and single symptom that lasts more than 24h with complete or partial recovery. It is not considered MS by itself. Additional criteria have to be present. (11,12)
- RR MS (Relapsing Remitting MS): 85%. Unpredictable relapses and remissions with or without sequelae. Between remission periods usually there is no progression. Is more frequent in women.
    - Active form: MRI activity, contrast enhancing appearing of new lesions or new symptomatology.
    - Non-Active form: defined as absence of new symptomatology, acute episodes, no changes in MRI compared to the previous one.
- SP MS (Secondary Progressive MS): 50-70% of people with RRMS will progressively get worse. There is an increasing disability over time. It is needed one-year time of appearance of new symptomatology or worsening the pre-existing one, to consider the disease progresses.
- PP MS (Primary Progressive MS): 10-15%. From the onset of the first symptom, the degree of the disability increases with/without temporary plateaus without periods of frank remission. It is usually diagnosed between 40-50 years old.

3.1.4 Clinical features

MS can affect different parts of the CNS and that could explain the great variety of symptoms and their unpredictability. Visual/oculomotor, sensorial (tingling or numbness), motor (weakness, stiffness, difficulty for walking), sphincter dysfunction, decreased libido, cerebellar (unsteady gait, speech disturbances) symptoms are observed. Also, cognitive and emotional disorders can be presented. 90% of cases have different degrees of fatigue, and some patients present pain such as trigeminal neuralgia and burning pain.

Any neurological dysfunction that lasts more than 24 hours is considered as an outbreak and it must be differentiated from pseudo-outbreak or Uhthoff or Uhthoff-like

phenomenon in which pre-existing symptoms appear or worsen in a situation of increased body temperature (such as fever or physical exercise).

3.1.5 Diagnosis

- Symptoms and physical exam (13)
- Lumbar puncture: oligoclonal bands IgG in cerebrospinal fluid (CSF) are present in more than 95% of the patients with MS. This reflects intrathecal synthesis of immunoglobulins that contribute to axonal damage.
- Magnetic resonance imaging (MRI): it has to demonstrate dissemination in time and space of the inflammatory and demyelinating process. The typical lesion locations are periventricular, juxtacortical, infratentorial or spinal, in a compatible clinical context. Dissemination in time is described by McDonald criteria as an appearance of new lesions in T2 or with an enhanced gadolinium signal compared with the basal MRI; and a simultaneous presence of lesions that are enhanced and no enhanced.
- Evoked potentials test (visual, auditory, somaesthetic and/or motor)

3.1.6 Management of the disease

There is no curative treatment so far.

- **Management of acute attacks**: oral or intravenous methylprednisolone dosed 1g/day for 3-5 days followed by a descending patter of oral corticosteroids or, in critical situations, plasmapheresis.

  Corticosteroids reduce the duration of the symptomatology but do not modify disease progression. (14)
- **Disease modifying therapies (DMT)**(15,16):

The main objective is to prevent outbreaks and to reduce the disability progression. These therapies have a great impact on the disease evolution by modulating or supressing the immune system. They must be considered as chronic treatment and its early onset has a great impact on the future disability status (17). The different DMT are available in the *Figure1*.

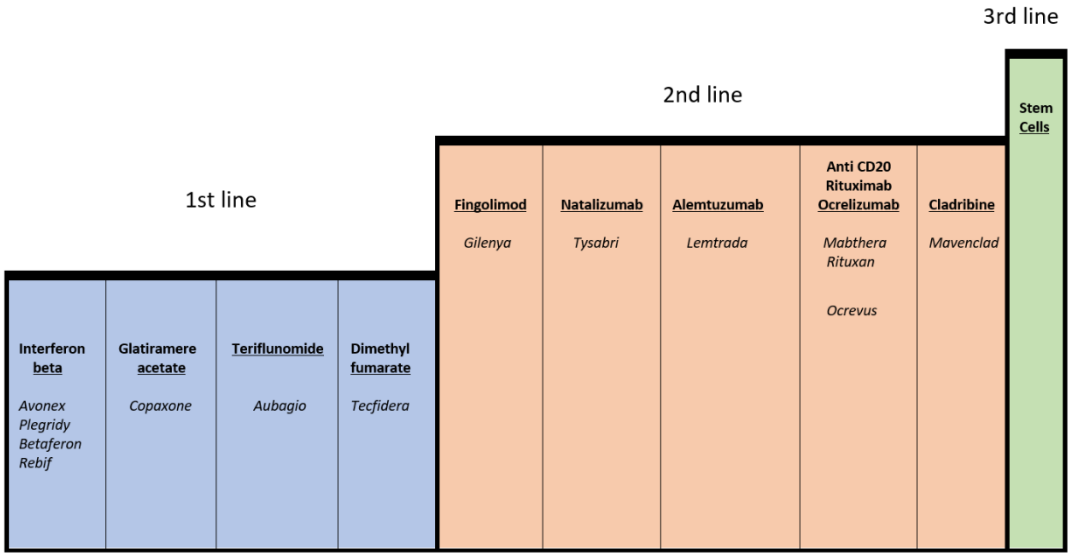


Figure 1 - Disease modifying therapies. Source: own elaboration.

- **Symptomatic treatment:**

The treatment of pain, spasticity (with botulinum toxin or cannabioids), fatigue (usually treated with amantadine), sexual dysfunction, urinary incontinence, gait impairment (usually is treated with fampiridine) etc. is conducted using drugs or psychosocial interventions in order to increase the quality of life in these patients and offer them a greater adaptation to daily life in accordance to their disabilities. In addition, they are adviced to follow the basic recommendations such as reducing cardiovascular risk factors, stop smoking, doing regular physical exercise avoiding sudden increases of body temperature, following a Mediterranean-style diet and so on. (18)

3.1.7 Prognostic

There are no clear factors to predict the course the disease will take, although certain factors have been linked to worse prognosis such as male sex, MS diagnosis >40 years old, primary progressive course from the onset, poor recovery after outbreaks or multiple lesions seen in MRI.

MS has a long-time survival prognosis but these patients tend to accumulate disability over time. At 15 years from the onset of the disease more than 80% of patients need help for walking. There is a significant reduction in quality of life.

The frequency of outbreaks, sequelae and its interference in daily life activities caused by different degrees of disability are assessed by the *Expanded Disability Status Scale (EDSS).*

In addition, E. Leray et al (2010) had described MS as two-stages disease. The disability status depends on the clinical type onset and the period until irreversible EDSS 3 (first phase). This first phase, in RR-MS disability progression, depends on the gender (females have slower disability progression rate), the age of MS onset (younger ages onset is related to slow disability progression) and history of relapses (more than 2 relapses in the first 2 years from the clinical onset and the presence of residual deficit from the first relapse contribute to faster disability progression during the first phase in RR-MS). These factors do not influence the disability progression during the second phase (a period from irreversible EDSS 3 to irreversible EDSS 6). In contrast it has neither been observed the same effect in the second phase of RR-MS nor in progressive MS onset phenotype in which the progression resulted to be much faster.

We can see Figure 2 the representation expressed as mean time of disability progression during phase 1 and phase 2, in 5 different subgroups of patients.

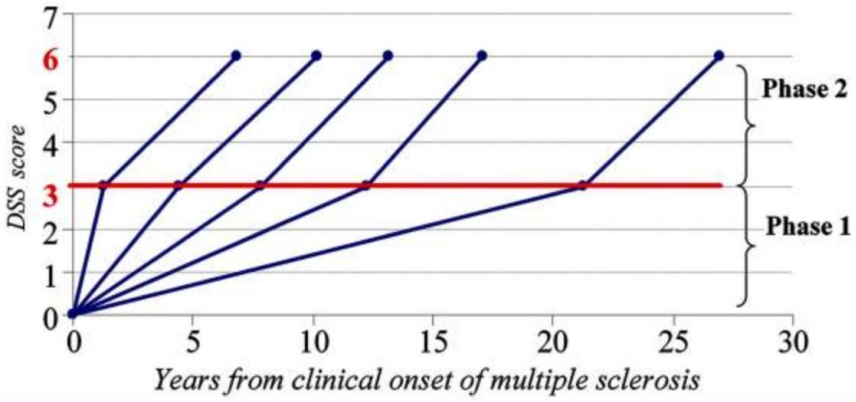


Figure 2 - Evidence for a two-stage disability progression in multiple sclerosis (19)

Here relies the importance of early therapeutic intervention that is able to delay the disability progression in short-time (first phase) (17,19).

Another issue of concern to MS patients is the vaccine administration. In general, vaccines with attenuated microorganisms are contraindicated since depending on the disease modifying therapy, the IS may not be able to fight the microorganism and cause an infection. On the other hand, inactive vaccines are usually safe.

In case of immunosuppressive therapy, vaccine administration is recommended to be done 4-6 weeks before starting a DMT or before the infusion of the next treatment cycle. (20–22)

3.2 Anti-CD20 treatments

Lymphocyte B cells are involved in the pathogenesis of Multiple Sclerosis disease. They can act as antigen-presenting cells, secrete different pro-inflammatory cytokines, co-stimulate and induce T-cell proliferation, and form ectopic lymphoid follicle-like structures in the meninges that have been related to demyelinating and axonal damage (23).

That's why one of the therapies widely used among MS patients are anti-CD20. These drugs are monoclonal antibodies whose target is CD20 ("cluster of differentiation 20" a protein antigen typical of mammals' immune system) that is present on the surface of lymphocytes pre-B and mature B cells.

The main effect of this drugs is a selective depletion of B-cells and in this way, to mitigate their activity in the pathogenesis of MS.

Rituximab and ocrelizumab are the main representatives of this group. There are more drugs within this group but its experience of use is limited since they are drugs in development, so we will focus on the other two.

Rituximab is a chimeric mouse-human IgG antibody targeting CD20. It is approved by the European Medicines Agency for the treatment of haematological oncological diseases (such as Non-Hodgkin Lymphoma or Chronic Lymphocytic Leukaemia) and autoimmune diseases such as Rheumatoid Arthritis, granulomatosis with polyangiitis and microscopic polyangiitis (24). It has completed phases I and II clinical trials for the treatment of MS but no phase III studies have been conducted for this indication. It is currently use as off-label drug for MS. It has been seen reactivation of HBV in patients treated with RTX.

On the other hand, ocrelizumab is a recombinant murine anti-CD20 antibodies molecule that has been humanized and this characteristic induces less immunogenicity and fewer infusion effects. (25)

Ocrelizumab and ofatumumab (a subcutaneous administration anti-CD20 monoclonal antibody) were created in order to improve efficacy compared to rituximab in MS disease, however, there are no studies conducted to demonstrate this increase in efficacy. Nevertheless, these two humanized drugs cause milder immunogenicity effects. They are usually well tolerated and infusion related reaction tend to reduce in intensity with the subsequent infusions.

All these anti-CD20 drugs have demonstrated benefit in radiological outcome (but also clinical benefits such as declining outbreaks and reducing disability progression) in RRMS, moreover, ocrelizumab has demonstrated to decrease the risk of disability progression in PPMS (25,26).

Repeated 6-monthly CD20 treatment can cause persistent B cell depletion and hypogammaglobulinemia, specially IgM, and then IgA and IgG. This can increase the risk of infection in these patients. This outcome would result from consequence both of immune dysregulation because of the disease process and of the immunomodulatory therapy. Therefore, these patients may be associated with higher risk of SARS-CoV-2 infection.

The Hypogammaglobulinemia caused by long-term RTX treatment has not been linked to the number of RTX cycles or duration of the treatment (years). It is transient in the majority of cases and usually recovers despite future rituximab infusions (27,28)

So, there is scientific evidence that multiple cycles of rituximab do not induce cumulative effects in lymphocytes B subpopulations. And basing on the previous evidence with other vaccines, these patients have proposed to be less likely to mount an effective antibody response.

3.3 SARS-CoV-2 and vaccine development

Severe Acute Respiratory Syndrome coronavirus 2 or, just, SARS-CoV-2 is the name of a virus that causes the COVID-19 (coronavirus disease 2019). It first appeared on December 2019 in Wuhan, China where several cases of unknown origin pneumonia were reported. The number of cases grew up quickly and this infectious disease spread all over the world, until the World Health Organization declared it a pandemic on March 11, 2020.

SARS-CoV-2 is a positive sense single-stranded RNA genome surrounded by a nucleocapsid and closed in an envelope that expresses different protein molecules on its surface conferring it a crown-like appearance under electron microscope. One of these molecules is a spike glycoprotein (S protein) that is essential for infection by interacting with the host cells (specially with Angiotensin-converting Enzyme 2 receptors located mostly in lung and small intestine tissue, kidney, vascular endothelial cells and neurological tissue (29)).

The prevalence of COVID-19 cases is updated daily in the official World Health Organization website but the number is possibly underestimated because the diagnosis depends on the availability of the tests.

About 80% of COVID-19 cases are asymptomatic or have mild-moderate symptomatology (anosmia or dysgeusia, cough, fever, sore throat, dyspnoea or gastrointestinal symptoms); 20% require hospitalisation where about 5% require critical care unit (30). Those who require hospitalisation are usually elderly people or people with comorbidities such as chronic cardiac or lung diseases, smoking, obesity, diabetes, hypertension, cancer and immunosuppression (31).

There is no evidence that MS patients are in higher risk of acquiring SARS-CoV-2 infection or get a worse COVID19 outcome but it must be pointed out that the use of some DMT can change this affirmation (32). Some drugs, especially those that cause immunosuppression or immunomodulation, confer MS patients more risk of infection and poorer prognosis in COVID 19 disease. That's the reason of why drugs such as Alemtuzumab is not recommended in this pandemic (33).

Now a days, many lines of research are being carried out trying to develop a vaccine in order to slow down the evolution of the pandemic.

"A vaccine is understood to be any preparation (a causative agent of a disease, its products, or any synthetic substitute treated to act as an antigen without inducing the disease) intended to generate immunity against a concrete disease by stimulating the production of antibodies".

Vaccines can be synthesized in different ways. The different types of SARS-Cov-2 vaccine production platforms and technologies are summarized in Table 1.

Platform	Target	Existing, Licensed Human Vaccines Using the Same Platform	Advantages	Disadvantages
RNA vaccines	S protein	No	No infectious virus needs to be handled, vaccines are typically immunogenic, rapid production possible.	Safety issues with reactogenicity have been reported.
DNA vaccines	S protein	No	No infectious virus needs to be handled, easy scale up, low production costs, high heat stability, tested in humans for SARS-CoV-1, rapid production possible.	Vaccine needs specific delivery devices to reach good immunogenicity.
Recombinant protein vaccines	S protein	Yes for baculovirus (influenza, HPV) and yeast expression (HBV, HPV)	No infectious virus needs to be handled, adjuvants can be used to increase immunogenicity.	Global production capacity might be limited. Antigen and/or epitope integrity needs to be confirmed. Yields need to be high enough.
Viral vector-based vaccines	S protein	Yes for VSV (Ervebo), but not for other viral vectored vaccines	No infectious virus needs to be handled, excellent preclinical and clinical data for many emerging viruses, including MERS-CoV.	Vector immunity might negatively affect vaccine effectiveness (depending on the vector chosen).
Live attenuated vaccines	Whole virion	Yes	Straightforward process used for several licensed human vaccines, existing infrastructure can be used.	Creating infectious clones for attenuated coronavirus vaccine seeds takes time because of large genome size. Safety testing will need to be extensive.
Inactivated vaccines	Whole virion	Yes	Straightforward process used for several licensed human vaccines, existing infrastructure can be used, has been tested in humans for SARS-CoV-1, adjuvants can be used to increase immunogenicity.	Large amounts of infectious virus need to be handled (could be mitigated by using an attenuated seed virus). Antigen and/or epitope integrity needs to be confirmed.

Table 1 - Immunity Journal. SARS-CoV-2 Vaccine: Status report(34)

Chinese scientists were the first to determine the genomic sequence of SARS-CoV-2 in January 2020. This has been a great advantage because this led to the identification of a target for the vaccine, the spike protein (it is the way through which the coronavirus can infect the host cells and therefore can replicate). It is composed by two subunits: S1, which has the receptor binding domain (RBD) and serves to bind with the ACE2 receptor of the body's cells, and S2, which is essential for the fusion of membranes between SARS-CoV-2 and the cell susceptible to being infected.

In the last 30 years, genetic engineering has gained much importance. Many of SARS-CoV-2 vaccines are being synthesized using these new techniques.

3.3.1 Understanding the time frames of a vaccine development

All clinical studies are mainly divided into two phases: pre-clinic and clinical.

- Pre-clinical phase: in which the vaccine is formulated and tested in vitro and in animal models to determine efficacy, safety and toxicity. This must always be done guaranteeing Good Laboratory Practice norms. In the same way as the

vaccine's component, their platforms should also be tested, although this step can be overlooked if there is enough evidence of their previous use.

- Clinical phase: the procedures in this phase are also subjected to Good Manufacturing Practice standards, which ensure safety and quality. Once there is enough pre-clinical data, clinical trials can be performed in humans. They are divided into 3 pre-marketing steps:
  - Phase I: evaluates the safety and immunogenicity of the vaccine in humans and it is applied to a small group of volunteers.
  - Phase II: in which the effective doses are formulated and the sample of participants is bigger. It provides more safety and adverse effects data.
  - Phase III: the efficacy and safety should be demonstrated normally compared to a control group in much larger sample.

After that, an authorization to approve and register the product must be obtained from competent regulatory agencies such as FDA.

According to FDA (Food and Drug Administration organization) in order to marketing a drug, it has to demonstrate an estimated decrease in ≥ 50% in an assessment criterion compared to placebo with a confidence interval 95% that provides a guarantee of reduction in ≥ 30%; and satisfy the criteria for assessment of clinical phases (35).

As this process normally takes years, in April 2020, ACTIV (Accelerating COVID-19 Therapeutic Interventions and Vaccines) project was created under the coordination of Foundation of National Health Institutes (FNIS) and leaders of the National Institutes of Health (NIH) with the meeting with multiple biopharmaceutical firms, FDA leaders, Advanced Biomedical Research and Development Authority (BARDA), European Medicines Agency (EMA), scientists from Centres for Disease Control and Prevention (CDC) and many other experts. So, it resulted in a public-private partnership in order to improve the development of COVID-19 therapies and vaccines.

In May 2020, another similar project called Operation Warp Speed (OWS) was launched from different US associations (Department of Health and Human Services, Department of Defense and private sectors) based on coordination of various institutions mentioned previously (CDC, BARDA, NIH) with the aim of improving the control of the COVID-19

pandemic and accelerating the development of potential candidates for SARS-CoV-2 vaccine.

All these projects have as an objective to compress the vaccine's research and development timeline from 10-15 years to 1-2 years without compromising efficacy, safety and quality of the product.

These organizations support the companies that develop vaccine candidates both, economically and technically, so that the different steps of clinical trials can be implemented in parallel. In case of FDA (Food and Drug Administration) gives them the permission to move forward in phases or gives them the license to market the vaccine, it can be administered and distributed quickly and all over the world.

To do this, they have hired several experts related to vaccine research and development in order to be able to coordinate this process. Their aim is to obtain one or more SARS-CoV-2 vaccine candidates, with proven efficacy, safety and FDA license of marketing, by the first half of 2021.

This supposes a high financial cost compared to conventional vaccine development. (36,37)

When vaccines currently in development become available, a project of COVAX Foundation (COVID-19 Vaccine Global Access) and the Coalition for Epidemics Preparedness Innovations (CEPI) and WHO will be launched in an attempt at equitable dispensing of vaccines between countries, taking into account especially the low-income countries. (38)

3.3.2 SARS-CoV-2 vaccines: a brief review

In the following paragraphs it is shown an updated summary of SARS-CoV-2 vaccine candidates that, actually, are in advanced stages of clinical trials.

- **VIRAL VECTOR BASED VACCINES**

A viral vector is a modified virus that acts as a vehicle for introducing genetic material of another pathogenic microorganism into a host cell. In consists on eliminating the genes that are related to its infectious and pathogenic capacity (so they will not be able to cause replicate) and inserting the ones that are of interest. In case of SARS-CoV-2 vaccine, as previously mentioned, the gene that codifies for Spike protein (essential for

host cells attachment and infection) is of great interest. When the vaccine is injected, this virus enters the host cells and modifies them. The infected cells begin to produce and express Spike proteins on their surface. This is what will stimulate the activation of the body's immune system and will try to produce a proper response through antibody synthesis. Vector viruses are characterized as not causing any hazard to the host body (39).

The following vaccines candidates are non-replicating combined viral vector vaccines that carry genetic sequence to codify SARS-CoV-2 Spike proteins.

- Ad5-nCov (CanSino Biologics; China)

It was the first vaccine that has been proved in humans. The vector used is a human adenovirus type 5.

It has been observed that the body begin to create specific neutralizing Ab and T-cells at 14 days post-vaccine administration, reaching its peak at 28 days post-vaccine administration.

Phase III clinical trials are currently underway, evaluating the efficacy, safety and immunogenicity of one single dose of this vaccine. The end of this clinical trial is estimated to January 2022.

Until now, it has shown that the seroconversion rate (IgG RBD and neutralizing antibodies) at 28 days is > 95%.

As Ad5 is common in humans the potency of the vaccine may be diminished by pre-existing immunity. Ab of previous contacts with type 5 adenovirus. (40)

**RBD (Receptor Binding Domain, located at S1 portion of SARS-CoV-2 Spike protein)*

- Sputnik vaccine (Gamaleya Center and RDIF; Russia)

It is similar to the previous but this one combines two different adenoviruses (two different viral vectors) in a two-dose regimen administration. The first vaccination is a human adenovirus type 26 + gene coding the spike protein. It stimulates the body's immune response. Then, after 21 days, the 2nd vaccine dose is administrated. This second one is based on human adenovirus type 5 as a viral vector (unknown to the body). They affirm that this second vaccination boosts the immune response and creates a long-lasting immunity.

Phase III clinical trials are currently underway and they are expected to finish on May 2021. (41,42)

- Janssen Vaccine (Ad26.CoV2-S)

This vaccine is also based on a non-replicating adenovirus type 26 vector that expresses the "stabilized pre-fusion spike protein of SARS-CoV-2". Ad26 is less immunogenic than Ad5 so several doses may be needed. Currently, a randomized double-blind placebo-controlled phase 3 study, ENSEMBLE, is evaluating the effectiveness of one single doses administration. It is expected to be available for emergency use authorization in 2021 but the clinical trial will not completely finish until March 2023. (39,42)

- AZD1222 (Astra Zeneca and Oxford University)

Previously known as ChAdOx1 nCoV-19; is a weakened chimpanzee adenovirus that acts as a vector + genetic sequence that encodes for the SARS-CoV-2 Spike glycoprotein.

As ChAd mainly infects chimpanzees, humans have low seroprevalence of infection with this virus. There should be no cross reaction and the vaccine shouldinduce strong immunity.

The phase I/II of the clinical trial was carried out with a single dose (+/- a booster dose) and it found out that one single dose was capable of quadrupling the levels of anti-Spike neutralizing antibodies and T-cells response in 95% of the patients a month after having been vaccinated. (43)

Currently a "Phase III Randomized Double blind, Placebo-Controlled Multicentre Study in Adults to Determine the Safety, Efficacy and Immunogenicity of AZD1222, a non-replicating ChAdOx1 vector vaccine, for the Prevention of COVID-19" evaluates two doses separated 4 week-apart. It is noteworthy that mild self-limiting adverse effects have been observed such as headache, fever or sore arm and pain at the injection site, especially after the second dose of the vaccine. The incidence of SARS-CoV-2 infection and severe COVID-19, and the reactogenicity of the vaccine is actually being tested. If in this phase, the vaccine will be able to demonstrate to prevent the previous items, the first doses will be available in the first half of 2021 event though the study will not be completed until October 2022. (39,42)

- **mRNA BASED VACCINES**

mRNA based vaccines have emerged recently and none of them has been licensed for human use. They are sequences (synthetized in vitro) that encode different genes of the pathogen the vaccine is targeting. The mRNA of SARS-CoV-2 vaccines encodes genes for the synthesis of spike proteins. The advantage is that this type of vaccine introduces a genetic sequence into the body cells and then, these cells become responsible for synthesizing what the introduced genes encode, so the entire pathogen does not entry the host cell and therefore it is unable to provoke an infection.

- BNT162 (BioNTech; Fosun Pharma; Pfizer: Germany)

There are 4 experimental vaccines that are based on modified mRNA + lipidic nanoparticles (LPN, that prevent mRNA from being degraded). All of them, encode nucleocapsid proteins. One of these four vaccines, specifically BNT162b2 in which the spike protein code is modified is actually ongoing the phase III clinical trial. I consist on administrating 2 doses, 21 days apart. Clinical trials of this vaccines are being conducted in conjunction with another similar vaccine BNT162b1, where the tolerability and safety of both are being evaluated. This study is expected to be concluded on December 2022. (39,42)

- mRNA-1273 (Moderna/NIAID: USA)

This is a mRNA-based vaccine encapsulated in lipidic nanoparticles encodes a stabilized form of S protein. Two intramuscular injections generally induce specific spike-antibody responses and T-cells in most trial participants (44). This vaccine is also in phase III of clinical trial, where two doses 29 days apart are being tested. It is expected to be concluded on October 2022. (42)

- **INACTIVATED VACCINES**

Or “killed vaccines” are particles particulates from pathogens that have been grown in a controlled culture and killed or deactivated to deprive them from their infective capacity (virulence). Because of their experience of use, structures and manufacturing methods, these types of vaccines may be quickly and easily generated in a pandemic situation. They are safe and express different types of antigens typical of the virus. (39)

- New Crown vaccine (Sinopharm; China)

It is an inactivated vaccine inactivated by beta propiolactone. Two-dose regimen 28 days apart is being tested and it has demonstrated adequate safety profile and no serious adverse reactions have been detected. The manufacturer ensures that the seroconversion rate in this regimen is 100% (42).

- CoronaVac or PiCoVacc vaccine (SinoVac China)

Is an inactivated vaccine of two-doses regimen 14 days apart. It has demonstrated to synthetize neutralizing antibodies at 14 days and the seroconversion rate is 90% at 14 days (42).

➢ **RECOMBINANT PROTEIN-BASED VACCINES**

This is purified recombinant SARS-CoV-2 S protein created by genetic engineering techniques in order to focus the immune response towards specific antigens in order to only create neutralizing Ab (don't produce non-neutralizing Ab). Normally they induce poor immunogenic response. That is because some of them need an adjuvant.

- Sanofi/GlaxoSmithKline

Actually, is in phase II clinical trial and it is scheduled to begin the Phase III study by the end of 2020. (42)

- Novavax (NVX-CoV2373 vaccine)

It is a recombinant protein subunit-based vaccine + adjuvant M-matrix that harvests antigen-presenting cells at the injection site. It is also based in two-dose regimen 29 days apart. After the 2nd dose 100% of patients developed IgG (and were correlated with neutralizing antibodies)(42).[1]

The great majority of these vaccines are conducted in age ranges between 18-65 years old (except Pfizer and AstraZeneca UK) and all of them exclude children and adolescents, immunocompromised patients, pregnant and breastfeeding women. The fact that no clinical trials have been conducted with these particular populations will have to be taken into account for the recommendations of the vaccines marketed.

[1] Underlined vaccines are expected to be administrated in the EU.

Despite all the efforts, to date, no efficacious vaccine is available. Research data results are changing every day and this introduction is aimed to be a snapshot in time in order to assist a better comprehension of this study.

4 JUSTIFICATION

Multiple sclerosis, especially progressive forms, has a great impact on the quality of life. There is a progressive accumulation of disability from the onset of symptoms. This issue is important since progressive physical disability impairs wandering within 15 years from the onset of the disease in 50% of cases (14).

Anti-CD20 treatment has demonstrated to slow down this progression and also clinical and radiological benefits. For these patients, this fact could translate to a significant improvement in their quality of life.

On the other hand, the pandemic we are currently living in, with a high rate of contagion and mortality, especially in vulnerable populations, has been a trigger for the outbreak of a new economic and social crisis. The epidemiology of this respiratory virus (SARS-CoV-2) changes rapidly and the responses need to be agile. This situation has brought the world to a standstill and led to a complete change in the public behavior at large-scale. Measures such as social distancing, mask use, frequent hand washing, and finally, confinements have had to be adopted quickly. These life changes have resulted in multiple impacts on the physical, psychological and quality of life spheres.

Another problem it brings is the collapse of hospitals, the burnout of health professionals and the restructuring of the health system in order to cope with the high demand for admissions.

For this reason, collaboration between public health scientist and the community is now the primary objective for overcoming the pandemic. There is an urgent need for research, surveillance and well-designed clinical trials to identify safe and effective therapeutic interventions and vaccines because it is widely believed the world will not return to pre-pandemic situation until vaccine become available. Multiple vaccines are being developed in parallel. But which ones will win the race?

To perform this study protocol, we have chosen AZD1222 vaccine because so far, it is the only supply contract that has been signed by the Spanish Ministry of Health and we plan to carry out this study in a Santa Caterina's Hospital in Girona, Catalonia.

In addition, from the 6 vaccine candidates that have been selected by the EU, AZD1222 and BNT162b are the ones that are making the most progress. The main differences between them is that the first one uses a more traditional mechanism based on adenovirus and the other is created based on messenger RNA of the virus (there is still no experience of use of this kind of vaccines). In addition, the German vaccine must be conserved at ultracold temperatures so that, requires special freezers. Moreover, once extracted from these freezers it should be supplied quickly (in a few hours) to the population.

Back to MS patients. Continuous treatment with anti-CD20 drugs keep pre-B-cells and B-cells in a nadir state and this fact can difficult the ability to respond immunologically to infections/vaccines by preventing antibody secretion. It has been seen with other vaccines such as influenza virus and HBV vaccines (45–48), patients treated with rituximab, had diminished humoral immune response compared to patients who were not in this treatment. It is noteworthy that the response to the vaccine was not absent.

Just to remember, activated B lymphocytes can be differentiated into memory B cells and plasma cells (which are the real ones responsible for secreting antibodies or immunoglobulins). Neither stem cells nor plasma cells do not express CD20, so they will be beyond the scope of the anti-CD20 drug effect. That's mean that although peripheral B cells have been deplored, B-cells found in other locations (e.g. spleen, bone marrow, lymph nodes, Peyer's patches and synovium) are not as susceptive to this depleting effect, and could repopulate B-cells. This process occurs before the treatment administration and takes months (27). Once these new plasma cells have been formed, they may be able to develop humoral immune response to the vaccine.

On the basis of experience with other vaccines, we have developed this study protocol to find out whether MS patients treated with anti-CD20 who are administered SARS-CoV-2 vaccine will follow the same vaccine response pattern.

The effect of the vaccines gains much importance in this public health emergency situation and, in addition, the management of patients with AI diseases such as MS who need DMT has to be performed with great caution. The main goal is to be able to provide the immune system with the ability to recover and generate an optimal vaccine

response without causing serious harm by spacing out the treatment that controls the disease.

For this reason, we have chosen anti-CD20 treatment as the independent variable and the humoral immune response to AZD1222 vaccine as a dependent one. These variables have been chosen in order to intend to offer the same SARS-CoV-2 vaccine's benefits in MS patients treated with anti-CD20 therapies as the rest of the population.

In order to see if the immune response follows the same pattern as with influenza virus vaccines, we have suggested to compare this population to the healthy one from the same range of age. To date, there is no scientific evidence of MS as a disease by itself does suppose an impairment of the ability to create antibodies as a response to the vaccines. By contrast, the increase in age has shown a decreased capacity of immune response. This phenomenon is called immunosenescense.

Anti-CD20 drugs, especially ocrelizumab, are indicated for progressive forms of MS, although they can also be used in RRMS. People with progressive forms of MS are often older, have a higher disability score (higher EDSS), and more years of illness than patients treated with other DMTs. Other DMT such as interferons, for example, do not impair the immune system's ability to produce a humoral response to different vaccines (49), but these patients tend to be younger and have less years of illness compared to anti-CD20 patients. That is why we do not compare anti-CD20 group to other DMTs.

We will evidence if both cohort groups create antibodies (IgG) and their persistence in blood within a 3-month period by analysing blood samples. Then both will be compared.

To ensure that antibody synthesis is due to the stimulation of IS by the vaccine and not by an infection, a RT-PCR will be performed in all patients before including them in the study.

The time of the serologies were chosen basing on the studies driven about Ab kinetics in SARS-CoV-2 infections, the immunoglobulins occurrence time and the probability of detecting them (50–52). We want to detect if anti-CD20 patients are capable of reproducing these results.

Finally, we will also assess the disability status in MS patients 90 days after vaccine administration. There is no data that any vaccine has influenced the deterioration of

disability status in MS patients, but it seems to be an interesting outcome as the vaccine is new and has limited experience of use. That is why early adverse events will also be collected during the follow-up period.

5 HYPOTHESIS

The proportion of patients that present seroconversion after SARS-CoV-2 vaccine administration is lower among patients with multiple sclerosis treated with anti-CD20 therapies (compared to healthy population).

This hypothesis is formulated taking in mind that duration (understood as number of infusion cycles of anti-CD20) and dose (which is constant in successive infusion cycles, except the first one at the beginning of the treatment) has no cumulative effect on the dynamics of B-cell repopulation and, therefore, on the IS's ability to mount a response to the vaccine.

6 OBJECTIVES

6.1 Main objective

To compare the proportion of patients who develop antibodies after receiving the SARS-CoV-2 vaccine.

6.2 Secondary objectives

- To determine whether the created IgG persist in blood at 60 days after vaccine administration.
- To determine whether the created IgG persist in blood at 90 days after vaccine administration.
- To collect data about symptomatology suggestive of SARS-CoV-2 infection in vaccinated patients.
- To collect data about adverse events possibly related to the vaccine.
- To objectify the absence of changes in the disability status in MS patients 90 days after vaccine administration.

7 METHODOLOGY

7.1 Study design

The study is designed as an observational, single-centre, prospective cohort study with a 5-month follow-up period (for each participant) in which we will compare the presence of immune response against a SARS-CoV-2 vaccine in two cohort groups. The whole study will last around 18 months.

7.2 Study population

7.2.1 Inclusion criteria

- Patients who express their willingness to be vaccinated against SARS-CoV-2.
- Patients who have no contraindications to the vaccine.
- Specific criteria:
  - ✓ Cohort 1: Multiple Sclerosis patients aged 18-65 years old who are being treated with anti-CD20 therapy.
  - ✓ Cohort 2: healthy people of the same range of age than the MS group.

7.2.2 Exclusion criteria

- Immunosuppressed patients or those who are receiving immunosuppressive treatment (different from anti-CD20) or are in an immunosuppressed condition because of an organic disease.
- Pregnant women
- Patients who have received corticosteroid treatment in the last 3 months.
- Patients who already have antibodies against SARS-CoV-2 at the beginning of the study (before vaccine administration).
- Patients whose RT-PCR at the onset of the study is positive.

7.3 Sample selection and sample size

7.3.1 Sample size

Since the SARS-CoV-2 vaccine is not yet available, in order to estimate the sample needed for the study, we used the results from Van Assen et al. (45) where the proportion of responders to the Influenza virus vaccine in a healthy control was 45%. Therefore, we expect this percentage of response to SARS-CoV-2 vaccine.

Taking into account this data, in order to calculate the sample size needed, we used an online software called GRANMO. So, accepting an alpha risk of 0.05 and a beta risk of less than 0.20 (in other words, a statistical power of 80% or greater) in a bilateral contrast test, 28 “exposed” and 28 “non-exposed” subjects are needed in order to recognize as statistically significant a relative risk greater than or equal to 0.11. A proportion in the group of non-exposed has been estimated to be 0.45. A rate of loss during the follow-up period of 15% has been assumed. This approximation was done using the Poisson approximation method.

7.3.2 Sample selection

We expect that sample recruitment (56 patients) will take place during a 9-month period in one single centre (“Unitat de Neuroimmunologia i Esclerosis Múltiple Territorial Girona” - UNIEMTG in Santa Caterina Hospital) using a non-probabilistic consecutive sampling-method.

For the group 1, patients who fulfil the inclusion criteria and have not any exclusion criteria will be recruited.

We will recruit group 2 (the control group) in the International Health Unit (“Unitat de Salut Internacional”) in the Santa Caterina Hospital. This group will be composed of health patients with similar characteristics (especially the age) to group 1.

All the participants will be properly informed by the doctor, given an information sheet and asked for signing the informed consent (ANNEX). They will be considered recruited only after signing this document.

7.4 Study variables

7.4.1 Independent variables

- Anti-CD20 drugs, concretely rituximab and ocrelizumab. It will be treated as a qualitative dichotomous variable. It is expressed as “yes” or “not”.

  We will compare MS patients treated vs. healthy people without anti-CD20 treatment.

7.4.2 Dependent variables

Primary outcome

- **Incidence of seroconversion**: understood as appearance of Ab against SARS-CoV-2 in blood. It will be measured as a qualitative dichotomous nominal variable. We will measure it extracting blood samples and analysing them using CMIA (see measurements and instruments section). It will show if antibodies against SARS-CoV-2 vaccine are present (+) or not (-).

  This variable will be expressed as a proportion of patients that create antibodies in response to the SARS-CoV-2 vaccine in each group.

Secondary outcomes

- **Duration of antibodies**. The presence of IgG in blood at 60 days after vaccine administration.
- **Duration of antibodies**. The presence of IgG in blood at 90 days after vaccine administration.

  Both will be considered as qualitative dichotomous variables, expressed as presence (+) or absence (-) of IgG in blood. These variables will be determined in order to speculate on the duration of the antibodies in blood.
- **Disability status**: we will assess whether there is progression in the disability status scale that could be attributed to the vaccine.

  It will be measured as a qualitative dichotomous variable. We will perform a clinic visit at 90 days after vaccine administration in order to assess the presence or absence of disability progression signs. To determine this, EDSS will be used.

This scale is a definition for a standardized neurological examination that assesses the Kurutzke's Functional Systems (visual, brainstem, pyramidal, cerebellar, sensory, bowel and bladder, cerebral and ambulation functions) and assign them a score according to the affectation. Finally, all the items are grouped in one value which is the real EDSS score. It has 10 grades where 0 is a normal neurological exam and 10 is death due to MS. That is to say, the higher the number, the greater is the dysfunction (53,54).

- **Adverse events:** any unusual symptomatology after the vaccine administration and occurred during the follow-up period will be collected.

7.4.3 Covariables

We will obtain this data directly from the clinical history, after the patients have been properly informed and signed informed consent (See ANNEX), and separate the patients into different characteristics:

- Sex: it will be considered as a qualitative dichotomous variable. Male or female.
- Age it will be considered a quantitative variable measured in years. The two cohort groups will be stratified in range of ages in order to compare them properly.
- Clinical form of MS it will be considered as a qualitative nominal variable. The patients will be distributed among RR-MS, PP-MS and SP-MS forms.
- Disease duration: it will be considered as a quantitative discrete variable measured in years.
- Disability status (EDSS): it will be considered as qualitative variable expressed in IQR.
- DMT: the group that are being treated with anti-CD20 therapy will be subdivided into two groups according to the type of anti-CD20 (qualitative nominal variables):
    - Rituximab
    - Ocrelizumab

- Comorbidities COVID-19: they will be considered as qualitative nominal variables.
    - Chronic cardio-vascular disease
    - Chronic Obstructive Pulmonary Disease (COPD)
    - Other chronic pulmonary disease
    - Diabetes mellitus
    - Obesity (BMI >30)
    - Current smoker or ex-smoker (who stopped smoking less than 15 years ago)

7.5 Measurements and instruments

In order to determine if the patients seroconvert, Chemiluminescent Microparticle Immunoassay (CMIA) will be selected as technique using the ARCHITECT machine from Abbott Laboratories. Santa Caterina Hospital (Salt, Girona) has all the facilities and instruments necessary to carry out the measures of the variables in this study.

CMIA is a laboratory device that qualitatively detects IgG (antibodies) against SARS-CoV-2 nucleoprotein in human blood or plasma samples. It is important to emphasize that CMIA does not lead to diagnose SARS-CoV-2 infection by itself but must be accompanied by characteristic symptomatology or other laboratory tests.

7.5.1 CMIA Procedure

Firstly, it must be said that one single kit contains 3 elements: a diluent, a reagent (paramagnetic microparticles + SARS-CoV-2 Antigens) and a conjugate (anti-human IgG + acridinium).

The procedure consists on the following steps:

- First of all, a venous blood sample from the patient (serum or plasma; serum or EDTA tubes respectively) has to be taken.
- Therefore, the reagent, which is composed by paramagnetic microparticles coated with SARS-CoV-2 antigens, and a diluent are added to the serum. Assuming that the plasma sample has antibodies against SARS-CoV-2 (the person has already passed the disease and has created immunoprotection), these

antibodies will bind to the antigen-coated microparticles of the new coronavirus, forming therefore antigen-antibody complexes.

- Then, the preparation is washed using a buffer solution, and the anti-human IgG + acridinium conjugate is added. In the case that the complexes antigen-antibody had been formed in the previous step, these will interact with anti-human IgG antibodies.

- After that, all this mixture has to be washed again and pre-activator and activator solutions are added.
  Acridinium acts as a tracer, so in case that the human IgG against SARS-CoV-2 are present they will react with anti-human IgG from the conjugate solution a chemiluminescent response will be seen. In other words, if there are IgG against SARS-CoV-2 in the sample, carrying out this procedure a luminescent reaction will be seen.

The chemiluminescent reaction is detected by a detector and is measured in Relative Light Units (RLU). The chemiluminescent RLU from the sample are compared to calibrator RLU and an "Index (S/C)" is calculated by the system.

The results will be interpreted as follows:

- Index S/C > or = 1.4 (established by the manufacturer) → + result or presence of SARS-CoV-2 IgG.
- Index S/C <1.4 → - result or absence of SARS-CoV-2 IgG.

Comparing to ELISA, CMIA is an automatized technique and the time of execution is shorter (30-40'). According to the Abbott CMIA description article, this technique has a positive percent agreement (PPA) > o = 14 days post-infection (probability that a positive result contains IgG in the sample) 96.77% (IC 95%: 90.86, 99.33) and a negative percent agreement (NPA) of 99.63% (IC 99.05, 99.90), (probability that a negative result does not contain IgG in the sample). PPA and NPA are not the same as sensibility and specificity terms, they are used in a comparison of two binary diagnosis tests in absence of a Referenced Gold Standard. (55)

This test is linked to a highly advanced software that automates and constantly monitors the procedures. It also provides an internal quality control of the management.

CMIA has significantly improved the profitability and productivity of the laboratory test analysis and it is helping to cope with an increase in requests for testing during the pandemic.

As the first serologic determination will be carried together with a RT-PCR test, the results should be interpreted in the following way(50,52,56):

Phase	Description	Remark
Pre-infection	RNA (-); IgM (-); IgG (-)	There may be antibodies from previous reactions that cause cross-reactions with SARS-CoV-2 and therefore IgG is detected
Infection - Incubation	RNA (+); IgM and IgG (+/-)	SARS-CoV-2 IgM appears 12-13d and IgG 13-14d post infection
- Asymptomatic resolution - Symptomatic resolution	RNA (+); IgM (+); IgG (+)	IgM begins to decline at 30 d post-infection
Healing	RNA (-); IgM (+/-); IgG (+)	PCR can give false positives because it detects the presence of remaining viral genetic material without replication capacity
Post-infection	RNA (-); IgM (-); IgG (+)	Long-term immunity (IgG) remains unknown

**RNA detected by RT-PCR (Reverse Transcription Polymerase Chain Reaction)*

Table 2 – Infection phases. Source: Own elaboration on the basis of cited references

7.6 Data collection and study procedure

7.6.1 Data collection

The information will be collected from the clinical history. We will collect the results of the serial serologies.

7.6.2 Study procedure

Each member of the study shall submit to the procedures specified in the Figure 2

In order to assure that IgG are due to the vaccine and not to the SARS-CoV-2 infection, all participants will be screened for SARS-CoV-2 infection by RT-PCR before enrolment.

Moreover, during the follow-up period the participants will be asked for:

- To record any new medications

- To record any new symptomatology such as fever, couth, dyspnea, myalgia, anosmia, ageusia and gastrointestinal symptoms.

Any of the side effects appeared during the follow up period will be registered using the standardized medical terminology that is used in the assessment of drugs for human use MedDRA (Medical Dictionary for Regulatory Activities).

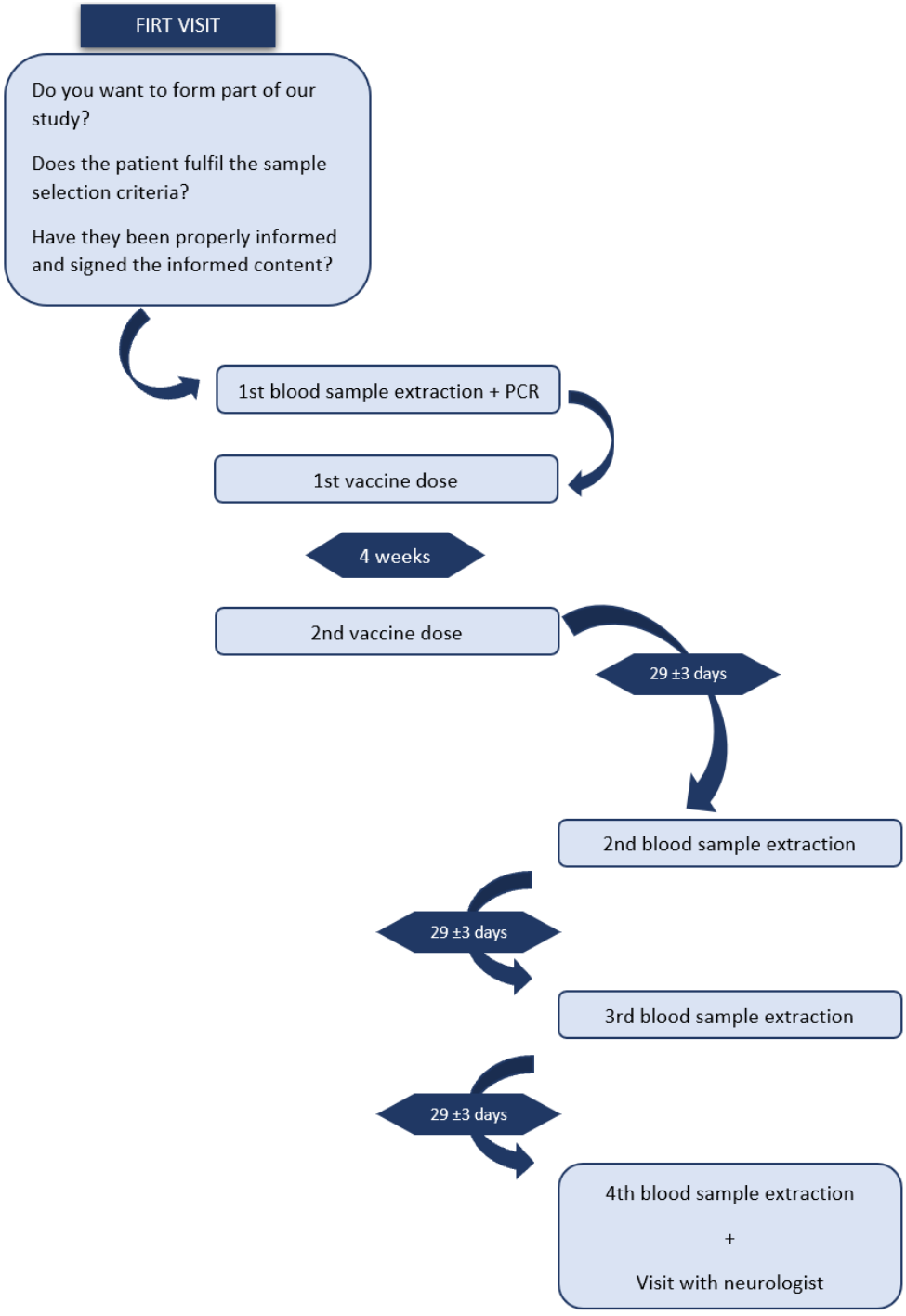


Figure 3 - Study procedure

8 WORK PLAN AND CHRONOGRAM

8.1 Work plan

All the study is organized in different steps. Taking in account the number of patients needed and the planned procedures, this study is expected to last around 18 months.

1rst step: initial process (approximate duration of 45 days)

- Activity 1: protocol elaboration
- Activity 2: Ethics Committee approval (CEIC)
- Activity 3: Team recruitment

2nd step: Patients recruitment (approximate duration of 9 months)

- 1st visit: every patient will be asked for their willingness to being vaccinated against SARS-CoV-2 and to participate in the study. In an affirmative case, they will be given an information sheet for the patient and informed consent, and they will be adequately informed of what the study consists on and about all the possible risks and benefits derived from the procedures.

  It's necessary to check the inclusion criteria in all patients and be sure they have no exclusion criteria in order to be included in the study. On that same day, a RT-PCR test and the first blood extraction will be performed (both form part of the exclusion criteria).
- We assume that the AZD1222 vaccine will be a two-dose regimen, so the patients can go to the Unitat de Salut Internacional in Santa Caterina's Hospital to receive the vaccine in the following days.
- The 2nd dose of the vaccine has to be administrated in 4 weeks from the first one. We will inform out patients that some side effects such as mild fever and fatigue can appear after the 2nd SARS-CoV-2 vaccine. It's important to say that this second dose of SARS-CoV-2 vaccine has to be administered 6 weeks before the next anti-CD20 infusion cycle.
- 2nd blood sample analysis will be performed at 29 +/-3 days after the 2nd dose of vaccine.

- 3rd blood sample analysis will be performed at 58 +/- 3 days after the 2nd dose of vaccine.
- 4th blood sample analysis will be performed at 87 +/-3 days after the 2nd dose of vaccine.

  In addition, the neurologist will visit the patient on this day. The EDSS will be assessed.

3rd step: data collection (approximate duration of 3 months)

Data collection will take place during the second stage and this activity may be extended until the las analytic results from the last patient's extraction are obtained.

4th step: Statistical analysis and data discussion (approximate duration of 45 days)

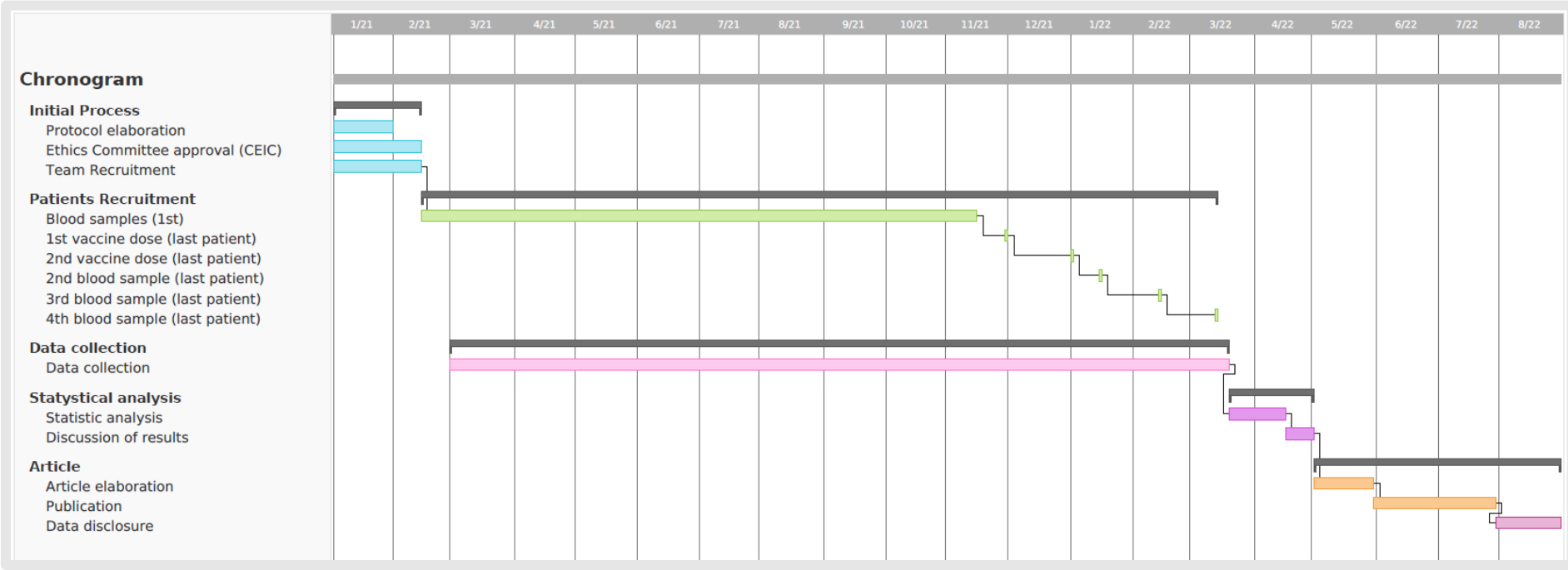
5th step: article (approximate duration of 1-2 months)

- Article elaboration
- Publication
- Data disclosure

8.2 Members of the team

- Main investigator: responsible for elaboration of the study protocol, gathering and coordinating the research team, results interpretation, writing down the discussion, article elaboration and results publication and dissemination.
- A professional from the International Health Unit (where the vaccines will be administered): responsible for administrating the vaccines and group 2 recruitment (health people who want to receive SARS-CoV-2 vaccine and fulfil the inclusion criteria and don't have any exclusion criteria).
- Nursing staff: responsible for performing blood sample extractions and data collection.
- Laboratory technician: responsible for analysing blood samples using CMIA.
- Laboratory analyst: responsible for the RT-PCR results.
- Expert in statistical analysis: responsible for the statistical analysis of the study.

8.3 CHRONOGRAM



9 STATISTICAL ANALYSES

This activity will be performed by a statistical analyst using the Statistical Package for Social Science (SPSS) version 25 software. When p-value <0.05 the result will be considered as statistically significant.

- Descriptive statistics

All the variables, dependent, independent and co-variables will be summarized.

Qualitative categorical variables will be represented as proportions and the quantitative ones will be represented as means, standard deviations, medians and interquartile range (Q1, Q3).

The relation between dependent and independent variables, being all of them qualitative, will be summarized in a crosstab.

In this analysis the variables will be stratified by categories of co-variables. Quantitative covariables will also be categorized.

- Bivariate inference

The relation between dependent and independent variables will be assessed and contrasted using Chi-quadrat test. In case that the expected number in the crosstable cells is less than 5 we will use Fisher's Exact Test.

This analysis will also be stratified by co-variable categories. The quantitative variables will also be categorized.

- Multivariate analysis

A multivariate analysis will be performed adjusting all the co-variables detected by the bivariate analysis in order to avoid possible confusion. In other words, the relation between dependent and independent variables will be adjusted using covariables-controlled logistic regressions.

10 ETHICAL AND LEGAL CONSIDERATIONS

The research protocol will be presented to the Ethical and Clinical Research Committee (CEIC) of the Hospital Universitari Dr. Josep Trueta (HUDJT), whose competences are included in the *Real Decreto 1090/2015,* for its evaluation and approval. Their recommendations will be included in the study.

This study will be conducted in accordance with the principles of Declaration of Helsinki which includes the *Ethical Principles for Medical Research Involving Human Subjects* adopted by the World Medical Association in 1964 (last reviewed in October 2013). It will also be subject to the SAS/3470/2009 Spanish State legal framework that regulates post-authorisation observational studies with medicinal products for human use; and in accordance with the law *14/2007 del Real Decreto 1716/2011* which regulates the research on biological samples.

The patients will be included in the study after being properly informed, obtained the patient information sheet and signed the informed consent. They will be informed that they are able to discontinue their participation in the study at any time if they wish so and no new data will be added to the database. Data that has already been collected will be used for the study unless the patient specifies his/her disagreement. This connotation is expressed in the informed consent document.

The exclusion criteria shall not be considered an infringement of the principle of justice. The study approach considers that patients who fulfil the exclusion criteria will not obtain benefit from the results of the study compared to the potential risks or discomfort caused.

This study does not cause physical or emotional harm to participants. The only discomfort caused will be the procedure of extracting blood samples.

The confidentiality and use of personal data will be subjected to *Regulation (EU) 2016/679 of the European Parliament and the Council of 27 April 2016,* which regulates issues of protection of personal data of natural and private legal person in terms of their use and free circulation. They will also be subjected to *Ley Orgánica 3/2018, 5 de*

diciembre, de Protección de Datos Personales y Garantía de los Derechos Digitales (LOPD-GDD).

The anonymity of any type of data that can directly identify the participants (such as first name, surname, medical history number, etc) will be assured. This data will only be handled, according to the mentioned legal framework, by the research team.

11 STUDY LIMITATIONS AND STRENGTHS

In this study the internal validity does not suppose any limitation, since the machine (CMIA) used to determine the main variable uses an automated mechanism and it is constantly subjected to internal controls.

Taking into account the external validity, that may make difficult the extrapolation of the study results, we find the following limitations:

It has to be mentioned that because of the sample number the statistical power of the results will be low. But we can't increase the sample number because it wouldn't be ethical.

The main limitation of this study is the confusion bias. The fact that the two cohort groups are different in more than one characteristic, forces us to find out confusion variables and to minimize them. First of all, as we have said before, MS is not a risk factor for having poorer immune response to vaccines, but certain DMT, such as anti-CD20 therapy, are able to impair this immune response. We have identified the age as a possible confusing variable by so-called immunosenescense. In order to eliminate the confusing variable, we could have separated the participants by age sub-groups. But apart from the age there are others candidates to be confusing variables (that are included in the co-variables section). In order to minimize the bias of confusion taking in mind all the identified confusing variables, in this case, a multivariant analysis will be performed. Through this statistical technique we can control the known confusing variables, but the confusion due to unknow variables cannot be controlled in this study.

Another limitation we should talk about is the selection bias because in this study we use a consecutive non-probabilistic sampling technique. This means that not all the patients are as likely to form part of the sample (inclusion and exclusion criteria) or may not come to the consultation during the sample selection period. In this study this doesn’t suppose a problem since the extrapolation of the results will be directed to a very specific population group (MS patients treated with anti-CD20).

Patient attrition bias in the follow-up period may affect the interpretation of the results. It will be minimized by allocating lost data with the last observation carried forward method. This statistic method consists on dragging the data from the last visit in case that the patient didn't come to the current consultation.

Coming to the hospital one a month in order to draw a blood sample for 3 consecutive months could result in a small percentage of loss in the follow-up period (loss bias), which is already minimized by assuming a small percentage of loss in the sample calculation. In our case, for example, we have assumed 15% of losses.

Finally, we will highlight that the main variable is measured using CMIA. This is an automatized procedure ruled by a machine (non-dependent on the observer) and periodical internal controls are performed. The measurement bias is minimal. However, CMIA could give false positives due to cross reactions from prior immunity to other coronaviruses.

In the same way, the EDSS scale tries to make the determination of the disability degree as objective and standardized as possible. It is also unlikely that this kind of bias will be produced.

Immunogenicity is evaluated in these patients but not the protection of the vaccine (understood as the min concentration of antibodies above which the individual is considered protected). This still remains unknown.

We will ask our patients to inform us of the presence of any type of symptomatology suggesting COVID-19. We'll only report symptomatic cases, we won't detect asymptomatic cases.

12 FEASIBILITY

In this study the main problem is its feasibility. Despite the fact that pharmaceutical companies and research centres are dedicating all their knowledge, efforts and capital in the development of a possible vaccine against the new coronavirus, the moment in which we will be able to administer it in a safety way still remains uncertain.

It's true trat the vaccine we have talked about in this study protocol, AZD1222, gives very promising insights and according to the phase 3 clinical trial (that is taking place in currently months) the primary completion of the study is expected to be scheduled for the end of the 2020. If efficacy and safety in this phase 3 study are demonstrated, the first doses will be available by the first half of 2021, although the total duration of the study will last until October 2022 (57). It is important to highlight that I/II phases of the clinical trial have demonstrated that one single dose of the vaccine induces an increase in spike specific antibodies by day 28 and a booster dose also induces or increases the production of neutralizing antibodies (43). Currently, in phase III clinical trial, the efficacy of 2 IM doses separated over a 4-week period is being evaluated.

That's the reason of why we contemplate setting up our study taking into account the administration of two doses of vaccine, hoping the primary completion of the III phase clinical trial study will take place in December 2020.

If this doesn't happen, we won't be able to carry out this study until the vaccine is commercialized.

On the contrary, there are more than a hundred of patients treated with anti-CD20 in the Santa Caterina's Hospital. So, the number of patients needed is affordable to recruit in one single hospital.

Moreover, CMIA laboratory device and all the facilities to get possible the RT-PCR tests and the serologies are available in the Hospital we want to perform the study and no additional equipment will be needed.

That's why, despite of the problem with the vaccine availability, this project can be completely feasible.

13 BUDGET

In order to calculate all the budget needed we will include into it all the expenses that don't form part of the clinical practice normally performed in the Unit's nursing consultation.

PERSONNEL EXPENSES

The recruitment of the two cohorts that fulfil the criteria of inclusion and exclusion, will be carried out by the team of neurologists of the Neuroimmunology and Multiple Sclerosis Unit and by the team of the International Health Unit of the Hospital Santa Caterina.

Recruitment, data collection and results interpretation will be not included at the calculation of costs, as this form part of their normal activities.

Additional hours will be counted for neurologist (14h from the last visits of MS patients and subsequent interpretation of the results) and nursing staff (19h that represent additional blood extractions).

In addition, the only professional who will be hired is the statistical analyst. We have estimated about 30 hours of work to perform the statistical analysis. He/she will be paid 50 €/h, so we estimate 1,200 €.

EXECUTION EXPENSES

The articles and bibliography used for the development of this protocol do not incur any additional cost.

Assuming we have 56 patients in total, and each of them will undergo 4 extractions and antigen determination tests, we estimate that we will have a total of 224 samples for which we will need the following material:

- CMIA analysis kit come at an additional cost. Counting on us to perform a total of 224 antibody determination analyses and that each reagent kit costs 500€, which is able to make about 100 determinations, we would need 3 reagent kits.

- Material such as extraction tubes and sterile needles have also been included in this budget.

PUBLICATION EXPENSES

We would like to publish this study as a journal article. Its revision, edition, formatting layout, graphic design and preparation of the digital media data will cost approximately 2.000€.

TRAVEL EXPENSES

National congress (travel and diet, meetings, conference and presentation): 1200 for 2 people

International congress (another continent): 2.500 € (this data is no included in the table).

TECHNICIAN EXPENSES			
ITEM	*PRICE*	*HOURS*	*SUBTOTAL*
Neurologist	*75 €/h*	*14h*	*1.050 €*
Nurse	*35 €/h*	*19h*	*665 €*
Statistical Analyst	*60 €/h*	*30h*	*1.800 €*

EXECUTION EXPENSES			
ITEM	*PRICE*	*UNITS*	*SUBTOTAL*
CMIA Abbot kit	*500€/u*	*3*	*1.500 €*
RT-PCR	*60€/u*	*56*	*3.360 €*
Additional material:			
Tubes (x100)	*30 €/100u*	*3*	*90 €*
Needles (x25)	*20 €/25u*	*9*	*180 €*

ARTICLE PUBLICATION	
DESCRIPTION	*SUBTOTAL*
English correction	*500 €*
Open access	*2.000 €*

TRAVEL EXPENSES	
DESCRIPTION	*SUBTOTAL*
Congress: Inscription, fee, flights, accommodation, meals (2 people)	*1.200 €*

TOTAL AMOUNT	*12.345 €*

Table 3 - Budget

14 IMPACT ON THE HEALTH SYSTEM AND FUTURE PERSPECTIVE

The main involvement of vaccines is the immunization of the population against a pathogen in order to prevent serious disease (severe COVID-19), and decrease the rate of transmission ature the progression of the pandemic.

As we have commented before, the therapies that deplete B cells in blood are related to a worse response to vaccines, in a great majority of cases. This study was raised with the aim of verifying the attenuation of the immune response to the SARS-CoV-2 vaccine in MS patients treated with anti-CD20 in order to be able to develop and to offer them specific strategies to obtain the same benefit as the rest of the population.

It must be said that there may be people who do not create antibodies when exposed to a virus, and this will not necessarily result in a worse outcome of the disease. The same can happen with the vaccine. This simply occurs in people with a strong innate immunity or/and CD8 T-cells that fight the virus before antibodies have been produced. Not creating antibodies or decreased the response doesn't predispose you to have a worse progression of the disease if you get infected.

In conclusion, we believe that this study will have a big impact on the current situation. It could offer benefits to both MS patients receiving anti-CD20 and the general population by decreasing the rate of transmission and prevention of severe disease.

There are still issues to be resolved such as the minimum titre of neutralizing antibodies considered as protective, long-term immunity and aspects related to the long-term effects of SARS-CoV-2 vaccines.

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16 ANNEXES

16.1 Patient information sheet and informed consent

PATIENT INFORMATION SHEET

Protocol number:	
Charge:	**Humoral Response to SARS-CoV-2 Vaccine in Multiple Sclerosis Patients Treated with ANTI-CD20**
Promoter:	
Principal Investigator	
Address	
Phone:	

Dear Patient,

We are pleased to contact you to inform about a research/clinical study which we would like to invite you to take part of. Because the treatment you are receiving in this centre, we are asking for your permission to use personal data about Multiple Sclerosis diagnostic and tests results.

All the details regarding the study are explained in this document.

Your participation is totally voluntary and you may withdraw from it at any time without any reason.

This study has been approved for Ethical Committee for Clinical Research of _____ hospital.

VOLUNTARY PARTICIPATION:

The main reason we are inviting you is because of you have been diagnosed in Multiple Sclerosis and you are currently treated with anti-CD20. Also, you showed your willing to receive SARS-CoV-2 vaccine.

Let us remark again, your participation is totally free and voluntary and at any time you may withdraw without any affectation in the quality of your medical attention.

STUDY PURPOSE:

The main purpose is to evaluate the immune response against SARS-CoV-2 vaccine in patients affected by Multiple Sclerosis and following anti-CD20 therapy.

STUDY PROCEDURE

In order to determine if your immune system creates antibodies against the vaccine, you will undergo 4 blood draws for further testing. Additionally, at the beginning of the study and before the vaccine is given, you will be tested for RT-PCR to make sure that the response you create is due to the vaccine and not to infection. Samples for the PCR will be collected from the nasopharynx.

The follow-up period (including administration of the two doses of vaccine) will be 5 months.

1. You will have the first blood draw along with PCR before you are given the vaccine.
2. The second extraction will be performed 4 weeks after administration of the 2nd dose.
3. The third administration will be performed at 60 days post 2nd dose of vaccine
4. The fourth and final extraction will be performed at 90 days post-vaccination.

Your samples will be analyzed in the laboratory of the Hospital Santa Caterina and a value + or – will be assigned depending on the presence or absence of antibodies.

RISKS AND DISCOMFORTS

The participation on this study lacks considerable risks and discomforts.

This study is not based on being treated by any medical drug or anything which involves alteration on the subject system or modify his actual treatment. Is based on taking blood samples during a period taking advantage of the actual patient's treatment.

PATIENT RESPONSABILITIES

The patient responsibilities are the following ones:

- Fulfilment with visits and activities.
- Inform about any untoward medical occurrence possibly related with vaccine.
- Inform about the onset of any new drug.
- Inform about suggestive symptomatology of SARS-CoV-2 occurrence during the study.

POSSIBLE BENEFITS

The conclusion of this study may be an advance in SARS-CoV-2 vaccine administration in special situations, as in patients treated with anti-CD20.

May provide information about the dose adjustment, administering the booster dose, suggest administering timings on this kind of patients.

WARNING TO PREGNANT

Clinical trials do have not included pregnant and breastfeeding women so it is not recommended to those women who are planning to get pregnant or they are pregnant already, since lack of experience with this vaccine.

PERSONAL DATA PROTECTION

The team commits the fulfilment on *"La Ley Orgánica 15/1999, de 13 de diciembre de protección de datos de carácter personal y al Real Decreto (RD 1720/2007)".*

The gathered data will be related with a code avoiding the personal identification. The access to this information will be restricted and only able to consult by the investigation team, health authorities, Ethical Committee for Clinical Research and by promotor authorized personnel but always under confidentiality. Thus, your personal data will not be revealed to external people with exemption of medical or legally requirement.

The promotor guarantees the privacy and will not allow any possibility on crossing databases to permit the identification of the patient.

You have the rights to access, modify, oppose and cancel the data. In that case, you should address to your doctor.

The results may be published or exposed in medical congresses either to Health Authorities or the Scientific Community, and in any case will contain information which could identify you directly.

CONTACT IN CASE OF DOUBTS

If during your participation in this study you have any questions or need to obtain more information, please contact _____ (responsible Dr), from the Neuroimmunology and MS Unit, located at “Parque Hospitalario Martí i Julià del Hospital Santa Caterina de Salt, Girona”.

You can also contact by phone: _____ (phone number) or write an e-mail: _____.

When you have signed the accompanying consent sheet you agree to comply with the study procedures set out above.

We appreciate your attention.

PARTICIPANT CONSENT SHEET/ INFORMED CONSENT

Study title	
Protocol number	

Me: (name and surname)
Date of birth: ID:
Phone: Patient code:

◯ I have read the information sheet that has been given to me along with this documentation

◯ I've received enough information about the study and have not any doubt

◯ I understand that my participation is voluntary

◯ I understand that I can withdraw from the study:
Whenever I want
Without affecting my medical care

◯ I consent to make use of my clinical data that is directly related to this study.

I want to be told the information derived from the research that may be relevant to my health:

◯ YES
◯ NO

I freely agree to participate in the study.

Participant's signature Researcher's signature

Date: ___/___/___ Date: ___/___/___

(Name, signature and date of handwriting by the patient)