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CLINICAL AND PLACENTAL EXPRESSION PATTERNS OF SARS COV-2 INFECTION IN PREGNANCY THROUGHOUT THE COVID-19 PANDEMIC

Autor

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UNIVERSIDAD DE ZARAGOZA
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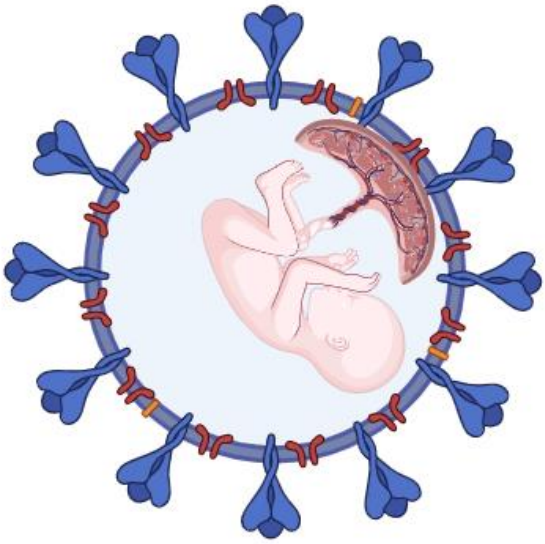

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For the degree of Doctor in Medicine

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HACE CONSTAR:

Que el trabajo de investigación titulado “CLINICAL AND PLACENTAL EXPRESSION PATTERNS OF SARS-COV-2 INFECTION IN PREGNANCY THROUGHOUT THE COVID-19 PANDEMIC” que presenta Marta Fabre Estremera, Licenciada en Ciencias Químicas para optar al GRADO DE DOCTOR, fue realizado bajo mi dirección no existiendo impedimento alguno para su defensa como compendio de publicaciones.

Y para que conste a los efectos oportunos firmo el presente en Zaragoza a 16 de diciembre de 2023.

Fdo. Dr. Daniel Orós López
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Fdo. Dr. Rafael González de Agüero Laborda
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La presente Tesis Doctoral ha sido estructurada siguiendo las directrices de la normativa para la presentación de tesis doctorales como compendio de artículos, aprobada por la el Consejo de Gobierno de la Universidad de Zaragoza el 25 de junio de 2020.

Los estudios que conforman esta Tesis Doctoral pertenecen a la misma línea de investigación. Los resultados obtenidos, gracias a la realización de estos estudios, han aportado información relevante y novedosa sobre el tema y han sido recogidos en cuatro artículos originales, publicados en diferentes revistas de amplia difusión internacional (Anexo II):

- **Fabre M,** Ruiz-Martinez S, Monserrat Cantera ME, et al. SARS-CoV-2 immunochromatographic IgM/IgG rapid test in pregnancy: A false friend?. Ann Clin Biochem. 2021;58(2):149-152. doi:10.1177/0004563220980495.
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    - Tercer cuartil.

- Abadía-Cuchí N, Ruiz-Martínez S, **Fabre M,** et al. SARS-CoV-2 congenital infection and pre-eclampsia-like syndrome in dichorionic twins: A case report and review of the literature. Int J Gynaecol Obstet. 2021;154(2):370-372. doi:10.1002/ijgo.13749.
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- **Fabre M,** Calvo P, Ruiz-Martinez S, et al. Frequent Placental SARS-CoV-2 in Patients with COVID-19-Associated Hypertensive Disorders of Pregnancy. Fetal Diagn Ther. 2021;48(11-12):801-811. doi:10.1159/000520179
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    - Tercer cuartil.

- Medel-Martinez A, Paules C, Peran M, Calvo P, Ruiz-Martinez S, Ormazabal Cundin M, Cebollada-Solanas A, Strunk M, Schoorlemmer J, Oros D, **Fabre M.** Placental Infection Associated with SARS-CoV-2 Wildtype Variant and Variants of Concern. Viruses. 2023;15(9):1918. doi:10.3390/v15091918

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TABLE OF CONTENTS

ABBREVIATIONS

1.ABBREVIATIONS

ACE2	angiotensin-converting enzyme 2
AHT	gestational arterial hypertension
cDNA	complementary deoxyribonucleic acid
CI	confidence intervals
CMIA	chemiluminescent microparticle immunoassays
COVID-19	coronavirus disease
CoVs	coronaviruses
CT	computed tomography
Ct	cycle threshold
E	envelope protein
FDA	Food and Drug Administration
GA	gestational age
HDP	hypertensive disorder of pregnancy
HIV	human immunodeficiency virus
ICA	lateral-flow immunochromatographic assay.
Ig	immunoglobulins
IL	interleukin
LFIA	Lateral flow immunoassay
M	membrane protein
MERS-CoV	Middle East respiratory syndrome coronavirus
mRNA	messenger ribonucleic acid
N	nucleocapsid protein
NGS	next generation sequencing
OR	odds ratios
PE	preeclampsia
POCT	point of care testing
R0	reproductive ratio
RBD	receptor-binding domain
RR	relative risk
RT-PCR	reverse transcription polymerase chain reaction

S	spike or surface glycoprotein
SARS-CoV-2	severe acute respiratory syndrome coronavirus-2
SARS-CoV	severe acute respiratory syndrome coronavirus
SD	standard deviation
SI	serial interval
VE	vaccine effectiveness
VOC	variant of concern
WHO	World Health Organization

INTRODUCTION

2. INTRODUCTION

2.1. SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS – 2 AND THE CORONAVIRUS DISEASE

Coronaviruses (CoVs) constitute a family of positive-sense enveloped RNA viruses known to infect a wide range of species, including avian, humans and companion animals. Six CoVs species are known to cause human disease. Four viruses — 229E, OC43, NL63, and HKU1 — are prevalent and typically cause common cold symptoms in immunocompetent individuals[1]. Until 2019, only two CoVs, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) were the only two known CoVs which caused a severe respiratory illness in humans[2].

On December 31st, 2019, the outbreak of an unknown pneumonia was first confirmed in Wuhan, China. Shortly after, the coronavirus disease (COVID-19)[3], caused by severe acute respiratory coronavirus-2 (SARS-CoV-2), was first identified and subsequently spread globally, resulting in a severe pandemic. The World Health Organization (WHO) officially declared a global pandemic in March 2020[4] (Figure 1).

The reproductive ratio (R0) denotes the transmission rate for various diseases. The R0 for COVID-19 is around 2–3 ,whereas that of influenza is 1[5]. This means that a patient who is positive for COVID-19 may spread this virus to three other people through air droplets. Each of those individuals can spread to three more people. The serial interval (SI) is the time interval between the onset of COVID-19 symptoms in the first person to the day when there is an symptoms appear in a second person. The SI for COVID-19 is 5–7.5 days while that of influenza is around 2.5 days[6]. Therefore, the extended incubation period increases the virulence of the virus as it can transmit extensively before becoming symptomatic.

In the initial months, the lethality of the COVID -19 virus remained largely unresearched. Unprepared healthcare systems in many countries, insufficient knowledge about the disease, and limited medical equipment such as respiratory machines, led to a high

death toll among the infected. This trend persisted throughout 2020 and 2021. The sole sustainable long-term solution to this global pandemic seemed to be a worldwide vaccination campaign. In May 2021, 198 countries have started vaccinating against COVID-19[7].

The WHO announced in May 2023 that it was concluding the emergency it declared for COVID-19[8]. Currently, over 771 million cases and 7 million deaths have been recorded worldwide and 14 billions vaccine doses have been administered[9].

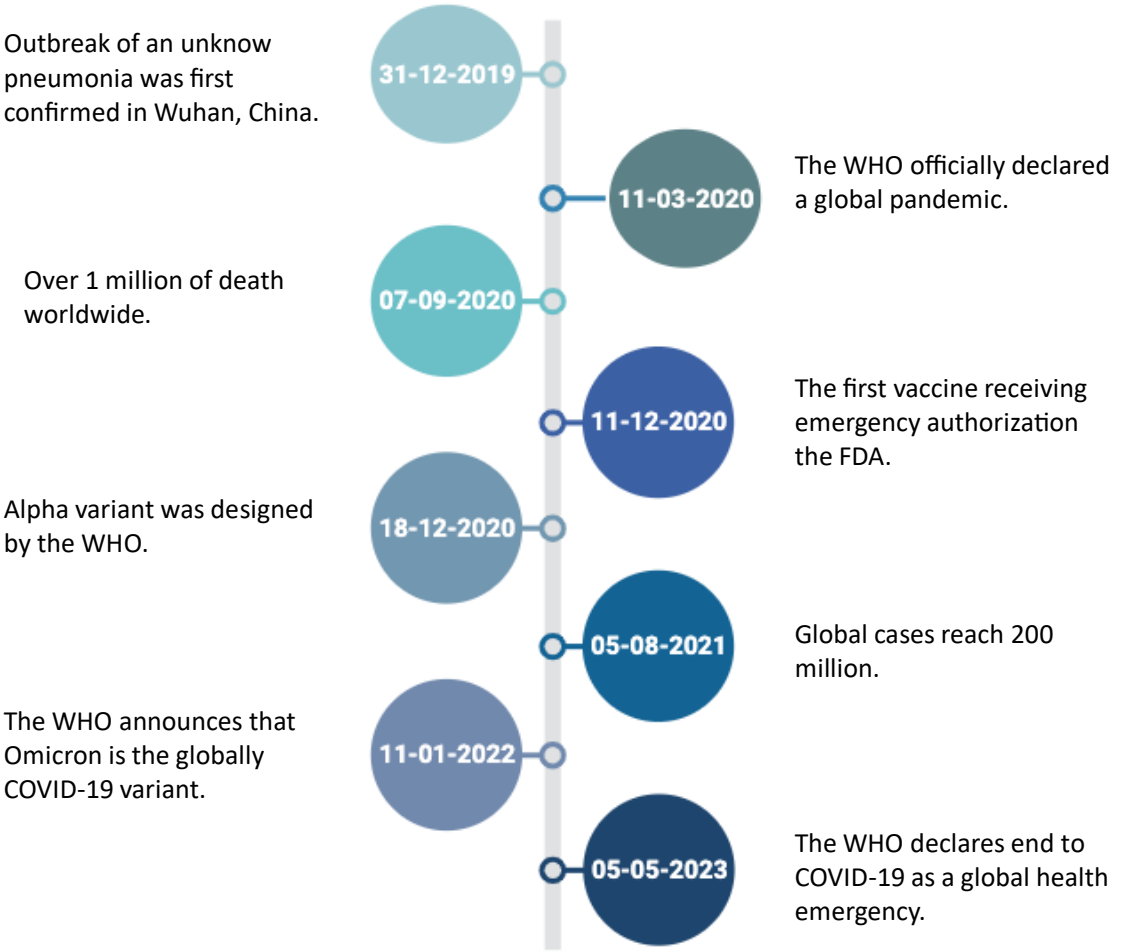

Figure 1. Timeline COVID-19 pandemic.

2.1.1 SARS-COV-2 STRUCTURE AND PATHOPHYSIOLOGY

SARS-CoV-2 encodes a non-structural polyprotein (ORF1a/b) that produces 15 proteins, including four structural proteins and five accessory proteins[10]. Structural proteins of SARS-CoV-2 are essential for assembly and consist of the spike or surface glycoprotein (S), membrane protein (M), an envelope protein (E), and nucleocapsid protein (N) (Figure 2). The surface glycoprotein is essential for attachment and entry into the host cell. Proteases can cleave into an N-terminal subunit (S1) and C-terminal (S2) membrane-bound region[11]. The spike protein contains a three-dimensional structure in the receptor-binding domain (RBD) region, loosely attached among viruses with van der Waals forces, and may lead to infection in multiple hosts[12]. SARS-CoV-2 spike links to angiotensin-converting enzyme 2 (ACE2), a membrane exopeptidase in the receptor, present in the respiratory tract of human beings and regulates both spread from human to human and cross-species[13].

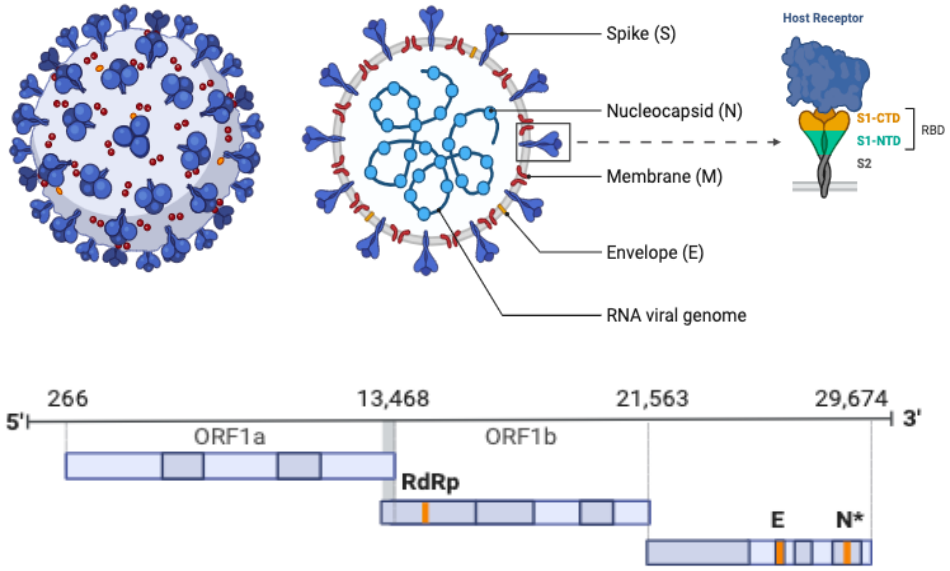

Figure 2. SARS-CoV-2 structure and the genomic organization of SARS-CoV-2. Schematic diagrams of the SARS-CoV-2 virus particle and genome. Four structural proteins of SARS-CoV-2 include Spike protein (S), Membrane protein (M), Nucleocapsid protein (N), and Envelope protein (E). The genome includes ORF1a-ORF1b-S-ORF3-E-M.

Initially, COVID-19 was classified into four types[14]: mild, moderate, severe and critical cases but, with the global outbreak of coronavirus, there was increasing evidence that many infections of COVID-19 are asymptomatic[15].

- Asymptomatic Infection: individuals with positive SARS-CoV-2 test without any clinical symptoms consistent with COVID-19 disease, but they can transmit the virus to others.

- Mild illness: individuals who have symptoms of COVID-19, such as fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, anosmia, or dysgeusia but with no abnormal radiological findings.

- Moderate illness: individuals with clinical symptoms or radiologic evidence of lower respiratory tract disease and are present with pneumonia on chest CT.

- Severe illness: individuals who have SpO2 less than 94% on room air, a ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (PaO2/FiO2) of less than 300, marked tachypnea with a respiratory frequency of greater than 30 breaths/min, or lung infiltrates that are greater than 50% of total lung volume.

- Critical illness: individuals with acute respiratory failure, septic shock, or multiple organ dysfunction. Patients with severe COVID-19 illness may become critically ill with the development of acute respiratory distress syndrome (ARDS). This tends to occur approximately one week after the onset of symptoms.

In the early phase of the infection, viral replication results in direct virus-mediated tissue damage. In the late phase, the infected host cells trigger an immune response by recruiting T lymphocytes, monocytes, and neutrophils. Cytokines such as tumor necrosis factor-α (TNF α), IL-1, IL-6, IL-1β, IL-8, IL-12 and interferon (IFN)-γ are released (Figure 3). In severe COVID-19 illness, a 'cytokine storm' is seen. This is due to the over-activation of the immune system and high levels of cytokines in circulation. This results in a local and systemic inflammatory response [16,17]. The cytokine storm is one of the possible events for the progressive and severe forms of COVID-19 and its mortality.

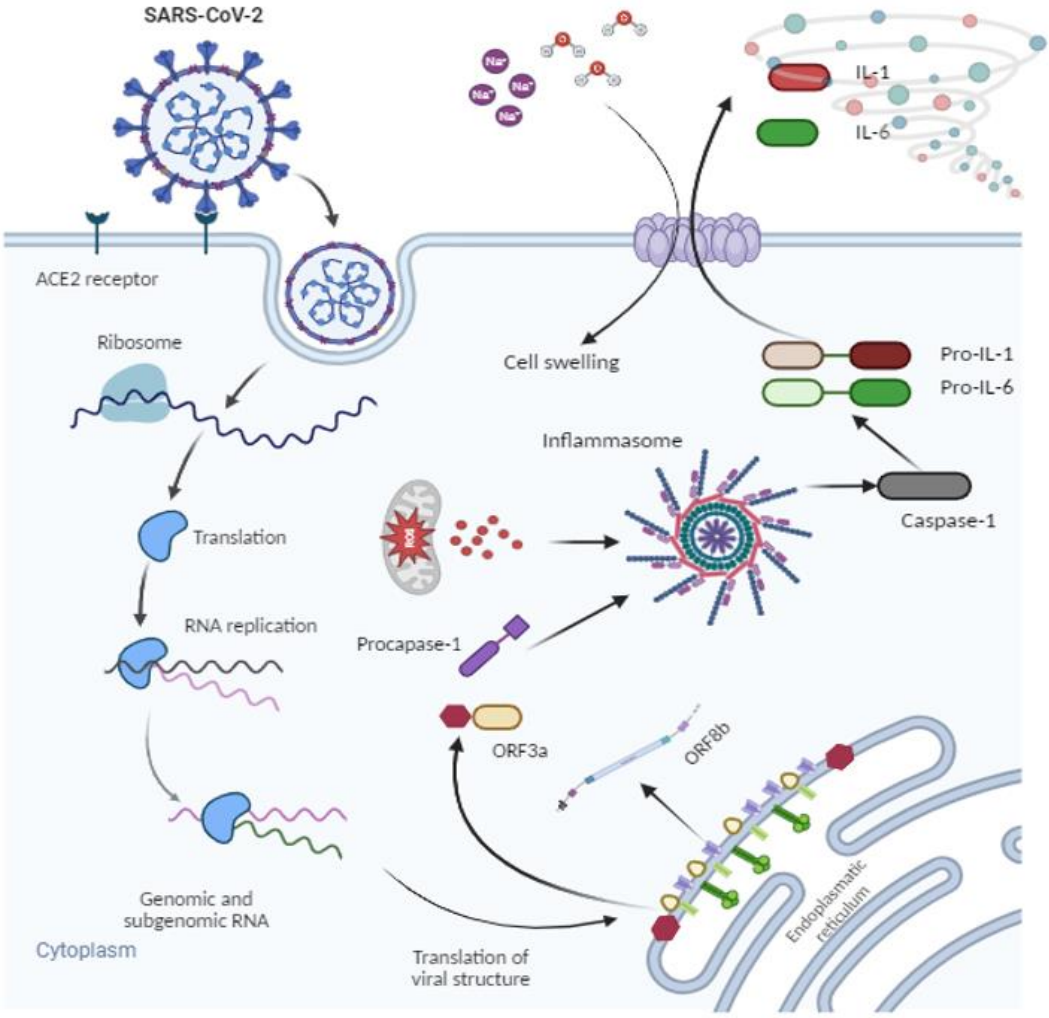

Figure 3. Cytokine storm and mechanisms of inflammasome activation in SARS-CoV-2 infection.

2.1.2 DIAGNOSTICS TEST SARS-COV-2

Specific diagnostic tests to detect SARS-CoV-2 were essential to make correct diagnosis and to control the advance of the pandemic (Figure 4). In laboratory medicine, the gold standard test is a methodology that perfectly discriminates between participants with or without the target conditions, with sensitivity and specificity equal to 100%[18]. In acute respiratory infection, reverse transcription polymerase chain reaction (RT-PCR) is the gold standard routinely used to detect causative viruses from respiratory secretions[19].

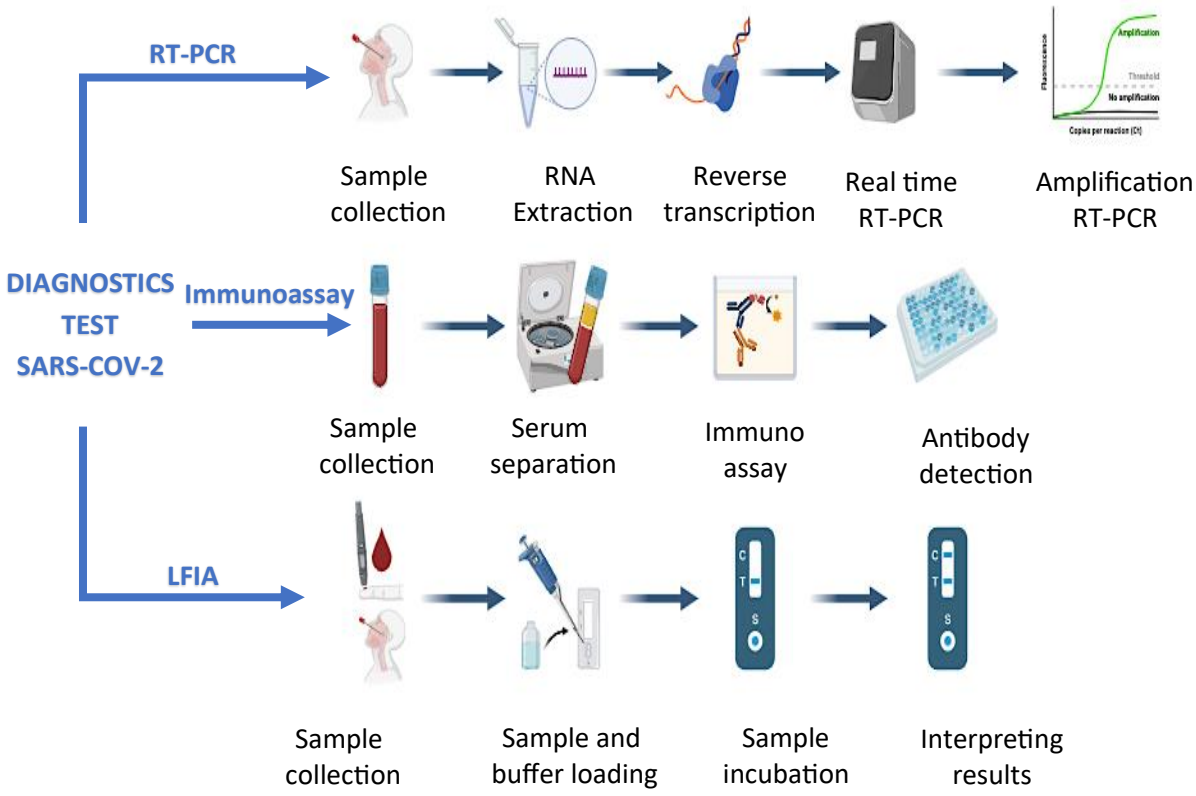

Figure 4. Screening and confirmation tests for SARS-CoV-2 infection.

The SARS-CoV-2 genome sequence was released on January 10, 2020[20]. Corman et al[21] published on 23 January 2020 a COVID-19 genetic diagnostics process that includes (viral ribonucleic acid) RNA extraction from nasopharyngeal swabs, followed by RT-PCR targeting viral genes. Generally, RT-PCR works by detecting the viral nucleotide by reversely transcribing the RNA into complementary deoxyribonucleic acid (cDNA) and amplifying the cDNA in the presence of reverse transcriptase, deoxyribonucleic acid polymerase, specific primers, and free nucleotides (Figure 4). Moreover, RT-PCR can be qualitative, semiquantitative, and quantitative. Although a qualitative RT-PCR can detect the absence or presence of the viral nucleotide sequence, a quantitative RT-PCR can also determine the copy number of the viral RNA by the inclusion of a fluorescent reporter molecule. On the other hand, a semiquantitative RT-PCR can estimate the relative copy number from the RT-PCR cycle threshold (Ct) values using standard curves of the Ct values from serial dilution samples and the estimated viral loads. This technology has been crucial to the management of the pandemic era due to its his high specificity and sensibility and its capacity to detect infection in its early phase, but also requires sophisticated instruments, expensive reagents, and laboratory expertise.

As the pandemic progressed, new diagnostic testing for SARS-CoV-2 has been included in the management of COVID-19 diagnosis. Immunoassay is a technique that utilizes the reaction between antibodies and antigens to detect the presence of a substance, typically a specific protein (Figure 4). In the context of infectious diseases and the immune response, Immunoassay can provide various data, including diagnosing microbial antigens, verifying prior exposure to the pathogen by detecting antibodies, or detecting antibodies specific to vaccine response[22]. Therefore, Serological tests play a pivotal role in the diagnosis and monitoring of SARS-CoV-2 infection, providing valuable insights into the host immune response. These tests, which detect antibodies such as IgA, IgG, and IgM, contribute to a comprehensive understanding of the progression and resolution of COVID-19 (Figure 5). The evaluation of IgM antibodies can aid in early infection detection, IgA antibodies contribute to early defense mechanisms and mucosal immunity against the virus, while IgG antibodies are associated with a more sustained immune response, potentially indicating past infection or vaccination. IgG antibodies specific to the nucleocapsid (anti-N) are often indicative of a past infection, as the

nucleocapsid protein is a conserved element of the virus[23]. On the other hand, anti-S antibodies, particularly those targeting the receptor-binding domain (RBD) of the spike protein, are instrumental in assessing vaccine-induced immunity and may contribute to protective responses[24].

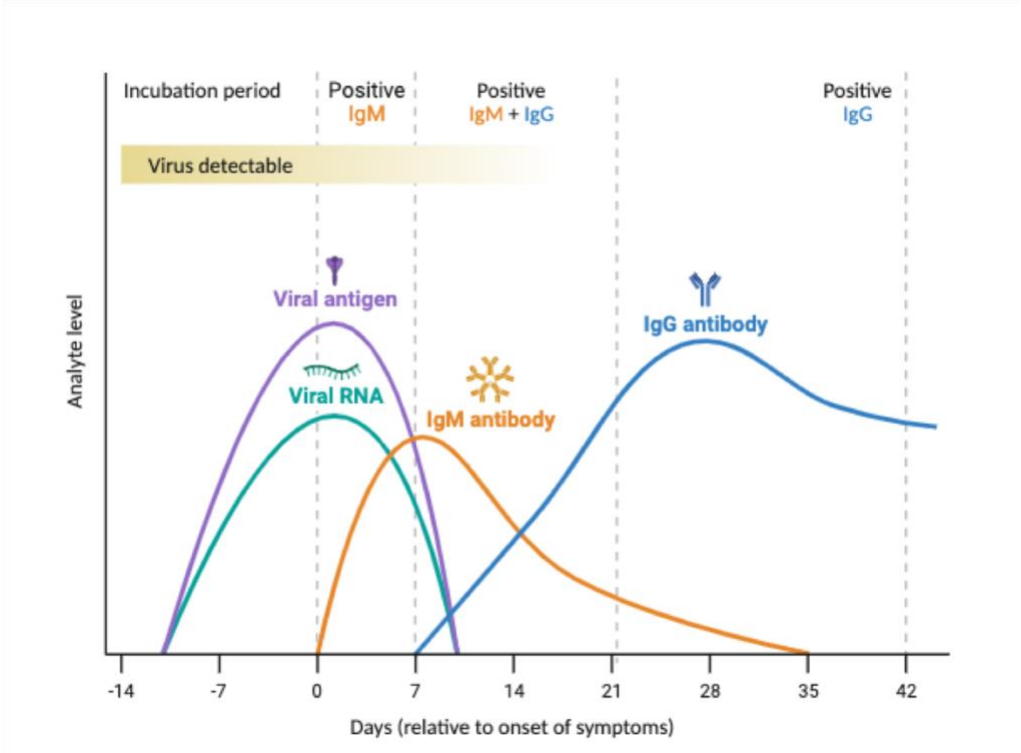

Figure 5. Antibody response against SARS-COV-2. The onset of symptoms (day 0) is usually 7 days after infection (day –7). At this early stage corresponding to the window or asymptomatic period, the viral load could be below the RT-PCR. Seroconversion may usually be detectable between 5–7 days and 14 days after the onset of symptoms.

The Lateral flow immunoassay (LFIA) is a paper-based platform for the detection of analytes in complex mixtures such as antibodies, antigens or proteins. These tests are user-friendly, rapid, and low-cost, and moreover, have acceptable sensitivity and specificity rates as a point-of-care testing (POCT) (Figure 4) . In general, the sample of interest is applied to the sample pad and then flow through the sample pad to the conjugate release pad, rehydrating the analyte-specific antibodies or the attached virus antigen. As the complex moves up the membrane, it passes the immobilized analyte-specific antibody, which recognizes and binds to it, creating a colored line. The control

antibody recognizes and captures the control well portion of the antibody detector, resulting in a colored line that eventually serves as a control. Results are interpreted in the reaction matrix as the presence or absence of lines of captured conjugate[25]. The inclusion of LFIA such as SARS-COV-2 antigens tests supposed a change in the management of the pandemic. An antigen is a molecular structure derived from the virus which elicits an immune response in the host. SARS-CoV-2 antigen can include the S protein (S1 and S2), the N protein, the RBD or a combination of these antigens that are highly immunogenic and main targets for antibody response[26]. Despite the low sensitivity and hence, the high rate of false negative during early phase of infections, LFIA played a significant role as a tool to control the SARS-CoV-2 infection due to low costs, rapid testing, and easy deployment in any setting.

2.1.3 VARIANTS OF CONCERN

In June 2020, the WHO Virus Evolution Working Group was established with a specific focus on SARS-CoV-2 variants, their phenotype and their impact on public health. These variants are the results of spontaneous mutations in the viral RNA derivate from errors in its replication within the host cell [27]. Over the years, some variants have increased the risk to global public and prompted WHO to characterize some as variant of interest (VOI), variant of concern (VOC), and variant under monitoring (VUM)[28]. According to the WHO definition:

- VOI: variant with genetic changes that are predicted or known to affect virus characteristics such as transmissibility, disease severity, immune escape, diagnostic or therapeutic escape; and identified to cause significant community transmission or multiple clusters of infected persons, in multiple countries with increasing relative prevalence as well as increasing number of cases over time, or other apparent epidemiological impacts to suggest an emerging risk to global public health.

- VOC: variant that meets the definition of a VOI and has been demonstrated to be associated with one or more of the following changes at a degree of global public health significance: transmissibility, the potential for more severe disease, reduced effectiveness of diagnostics, vaccines, and therapeutics and challenges in public health interventions. As of December 2023, the WHO has designated five VOCs (Alpha, Beta, Gamma, Delta and Omicron) (Figure 6).

- VUM: variant with genetic changes that are suspected to affect virus characteristics and early signals of growth advantage relative to other circulating variants, but for which evidence of phenotypic or epidemiological impact remains unclear, requiring enhanced monitoring and reassessment pending new evidence.

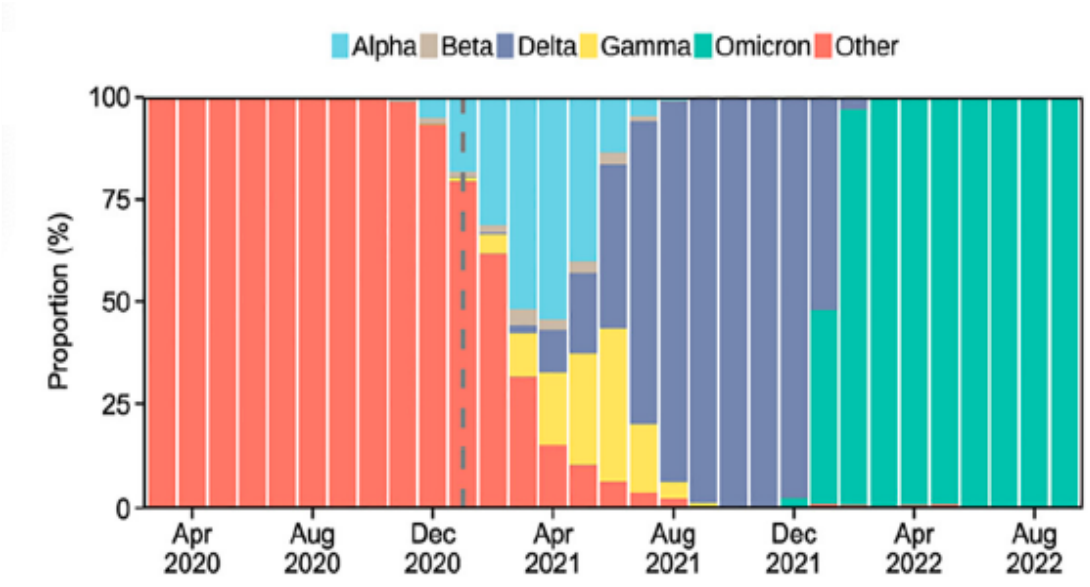

Figure 6. Global relative abundance of VOCs based on subsampled dataset for 63 countries. Extracted from: Yang L, Wang Z, Wang L, et al. Association of vaccination, international travel, public health and social measures with lineage dynamics of SARS-CoV-2. Proc Natl Acad Sci U S A. 2023;120(33):e2305403120**.**

2.1.3.1 ALPHA VARIANT

The B.1.1.7 strain (Alpha variant) was first detected in England in September 2020 and designated on December 18, 2020. In a few months was the dominant lineage around the world[29]. The alpha variant includes 17 mutations (14 nonsynonymous point mutations and three deletions) in the viral genome. Additionally, N501Y within the RBD enhances virus binding affinity to ACE2 receptor of host cells and P681H is adjacent to the furin cleavage site in spike, which is a key determinant for transmission[30,31]. Furthermore, the deletion H69/V70 in the S protein is linked to immune escape and could cause testing kit failures[32]. Studies have reported that the B.1.1.7 variant has 43–82% more transmissible, higher hospitalization rate [OR 1.40 (95% CI: 1.02–1.93)] and a higher mortality rate (from 2.5 to 4.1 death per 1000 cases) compared to preexisting variants[33,34]. However, B.1.1.7 variants showed a minimal impact to neutralization by convalescent and/or post-vaccination sera[35]. This indicates that a prior infection or vaccination with wild-type SARS-CoV-2 may still protect against the B.1.1.7 variants.

2.1.3.2 BETA VARIANT

In May 2020, researchers from South Africa reported SARS-CoV-2 lineage B.1.351 (Beta variant) and was designated as of December 18, 2020. In January 2021, the variant had spread to others African countries. The Beta variant includes 17 mutations (7 in the S protein and three deletions), notably, the E484K mutation, which is associated with immune escape[36]. This mutation explains why beta variant is more resistant to neutralization by sera from vaccinated individuals or convalescent plasma and its high risk of reinfection[37,38]. Compared with the progenitor strain, the B.1.351 has higher transmissibility, 50% more than previously circulating variants in South Africa[34]. The European Centre for Disease Prevention and Control [39] showed that cases with the Beta variant had significantly higher adjusted odds ratio for hospitalization [OR:3.6 (95% CI: 2.1–6.2)], however the risks for death was not significantly higher compared to non-variant cases [OR for death was 1.1 (95% CI: 0.4–3.4)].

2.1.3.3 GAMMA VARIANT

The Gamma variant, designated as P.1, was detected in November 2020 in Brazil and designated on January 11 2021. It rapidly spread rapidly in other South American countries[40]. The P.1 variant contains multiple spike protein mutations, but the N501Y, K417N and E484K mutations, which are also found in the alpha and beta variants, are the biologically important and have been associated with increased transmissibility[41]. A study using a model-based approach estimated that the transmissibility of P.1 is about 2.5 times higher than previous variants in Brazil, and it has a probability of reinfection of 6.4%[42]. However, it has decreased the risk of hospitalization and death compared variant Alpha and Beta variants[43].

2.1.3.4 DELTA VARIANT

B.1.617.2 (Delta variant) is a variant of lineage B.1.617 of SARS-CoV-2[44]. The B.1.617.2 variant was first detected in India in October 2020 and designated on May 11, 2021. In a short period, the delta variant become the most dominant strain worldwide due to its high transmissibility[45]. Several studies have estimated that the Delta variant could be up to 50% more transmissible than the Alpha variant[46,47]. The Delta is characterized by several notable mutations, with a particular emphasis on L452R and P681R on spike protein[48]. It has been suggested that these mutations contribute to increased transmissibility and may have potential impacts on viral replications[49]. Hence, B.1.617.2 has the potential to infect vaccinated and unvaccinated individuals. However, the progression of illness seems to be prevented by vaccination[50]. Twohig et al[51] in a cohort study on 43.338 patients, reported a higher hospital admission risk for patients with COVID-19 infected with the Delta variant compared with the alpha variant, especially unvaccinated patients.

2.1.3.5 OMICRON VARIANT

The Omicron variant (B.1.1.529) was first detected in Botswana and South Africa in November 2021[52]. Subsequently, the Omicron variant was identified from different

parts of the world, rapidly became the dominant strain. It was designated as a VOC on November 26, 2021. In January 2022, the U.S and many European countries reported a record of COVID-19 infections, almost 5 times higher than the peak points of all previous waves[53]. A crucial feature of Omicron is that it comprises distinct sub-lineages (BA.1, BA.2, BA.3, BA.4 and BA.5)[54]. Initially, BA.1 was the most prolific sub-lineage detected worldwide; however, BA.2 was overtaking BA.1 as the dominant variant globally[55]. The Omicron variant is the highly mutated variant, containing up to 59 mutations in its genome, including H655Y, N679K, and P681H and the 69-70del. These mutations are responsible for increased transmissibility and reduced neutralization by vaccine-induced immunity[56;57,58,59]. Lyngse et al[60] in a large-study found that among vaccinated people, Omicron was 2.6–3.7 times more infectious than the Delta variant. However, they found no significant difference in transmissibility between the 2 variants in unvaccinated people. On the other hand, the literature showed a significantly reduced severity of Omicron infections in people, including hospital admission, intensive care unit, shorter median stay at the hospital, and lower mortality rates[61,62]. The current prevalence of severe disease may be associated with large-scale vaccination. In addition, it has been reported that booster/third doses are more effective than one/two dose in protecting against symptomatic COVID-19 infections by Omicron variant[63].

2.1.3 VACCINES

Among the most effective strategies for preventing viral infections, vaccination represents the best tool for helping the immune system to activate protective responses. The advent of COVID-19 vaccines marked a pivotal moment in the global response to the pandemic.

The first authorized COVID-19 vaccine, BNT162b2 developed by Pfizer-BioNTech, was approved by The United States Food and Drug Administration (FDA) on December 11, 2020. It was administered on December 14, 2020, in the United Kingdom (UK)[64]. The mRNA-1273 vaccine, developed by Moderna, received Emergency Use Authorization from the FDA on December 18, 2020[65]. Nowadays, more than 100 vaccines have been

developed and over twenty vaccines were approved in different parts of the world. These include inactivated virus vaccines, virus-like particle vaccines, protein subunit vaccines, virus-vectored vaccine, mRNA and DNA vaccines and vaccines based on live-attenuated SARS-CoV-2[66] (Figure 7).

Phase III clinical trials first demonstrated a high vaccine effectiveness (VE) for several vaccines against symptomatic COVID-19 from the original strain, such as 95% VE for the BNT162b2 mRNA vaccine (Pfizer-BioNTech)[67], 94.1% VE for the mRNA-1273 vaccine (Moderna)[65], 70.4% VE for the ChAdOx1nCoV-19 vaccine (AZD1222 Oxford AstraZeneca)[68], and 50.7% VE for the inactivated CoronaVac[69]. Results of a metanalysis showed a global 97% VE for the prevention of hospitalization and severe disease and a 99.0% VE for the prevention of COVID-19 related mortality[69].

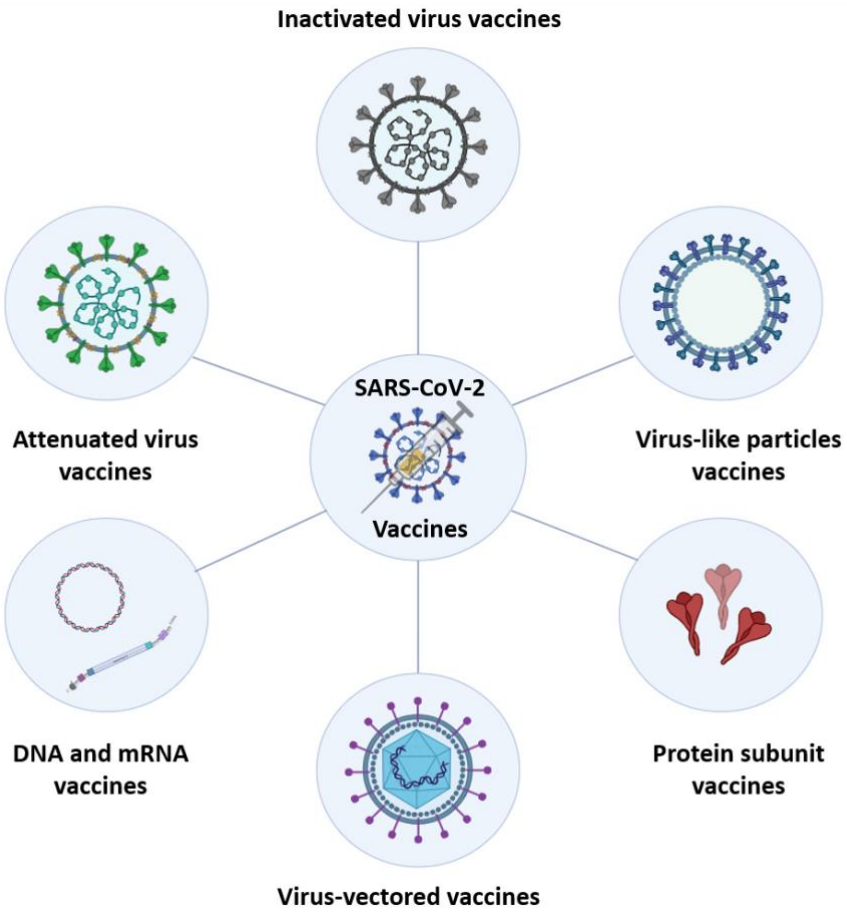

Figure 7. COVID-19 vaccines and their different platforms.

Due to the mutation nature of the spike protein, the new variants of concern may increase the risk of immunological escape, decreasing the effectiveness of a vaccine[70]. The emergence of Delta and Omicron variants partially decreased the efficacy of two-dose vaccine regimens, which was specifically contrasted via administration of third and booster doses to enhance protection versus symptomatic and severe COVID-19[71].

2.2. PREGNANCY AND COVID-19

Pregnant women develop a special immunological adaptation, which is necessary for maintaining tolerance of the fetal semi-allograft. This state of transient suppressed immunity is modulated by suppressing T cell activity, predisposes pregnant women to viral infections. Hence, pregnant women and their fetuses are particularly susceptible to the effects of viruses during outbreaks[72,73]. Hence, compared with the general population, pregnant women are particularly susceptible to specific viral infections, including the cytomegalovirus, herpes simplex virus, and Zika virus, and exhibit greater complications and mortality rates associated with varicella, rubeola, and H1N1 infections. Particularly, SARS-CoV and MERS-CoV have been associated with higher case fatality rates and more severe complications during pregnancy[74].

At the beginning of the COVID-19 pandemic there was considerable controversy about the impact of SARS-CoV-2 infection on pregnant women and their fetus. The first studies suggested that pregnant women did not have more risk or distinct symptoms from non-pregnant[75, 76]. However, subsequent studies have shown an increased risk of developing severe COVID-19 if they are infected, compared with non-pregnant women of a similar age or conditions [77,78]. The INTERCOVID study[79] demonstrated for first time, that women with COVID-19 diagnosis, compared with those without COVID-19 diagnosis, were at substantially increased risk of severe pregnancy complications such as preeclampsia, ICU admission, preterm birth or low birth weight. A large number of evidence on the impact of SARS-CoV-2 on perinatal outcomes have been published on the same line[80,81,82].

The clinical manifestations are similar to those in nonpregnant women[83]. The risk factors for severe disease include black race, age >35 years, overweight or obese and some comorbidities, such as diabetes or hypertension[81,84]. Furthermore, pregnant women with COVID-19 attending to the hospitals are more likely to be admitted to the intensive care unit or needing invasive ventilation than non-pregnant women of reproductive age[82]. The INTERCOVID study[79] described a risk of mortality 22 times higher in the group of women with COVID-19 diagnosis vs non COVID-19 diagnosis.

2.2.1 TRIMESTER OF INFECTION ON COVID-19 DISEASE

Studies on other viral infections in pregnancy have shown that the effect of infection on outcomes depends on the timing of exposure to the virus. For instance, in cytomegalovirus infection the severity of sequelae decreases with advancing gestation and the risk of transmission increases from approximately 40% in the first two trimesters to 60% or more in the third trimester of gestation[85]. In the case of Rubella, the highest risk of fetal infection occurs in the first trimester, especially prior to 10 weeks of gestation[86]. In contrast, exposure to the Influenza during pregnancy is associated with increased neonatal mortality during the first trimester, decreased birthweight during the second trimester, and increased preterm birth and decreased birthweight during the third trimester[87]. Due to the similarities regarding transmission, clinical features, and related immune responses between SARS-CoV-2 and Influenza, at the beginning of the pandemic it expected different outcomes based on the timing of infection with the SARS-CoV-2 virus.

Several studies have focused on the impact of gestational age at infection with the SARS-CoV-2 virus on maternal, obstetrical, and neonatal outcome. Seif et al[88] concluded that patients infected early in pregnancy seem to have a less severe disease course than women infected in third trimester; however, they have an increased risk for preterm delivery. Similar results are found by other researchers[89,90,91]. On the other hand, there is still controversy about the association between spontaneous miscarriage and SARS-CoV-2 infection during the first trimester of pregnancy. While authors such as Cosma et al[92] consider that SARS-CoV-2 infection during the first trimester of pregnancy does not seem to predispose to early pregnancy loss. Others authors such as Balachandren et al[93] and Kazemi et al[94] suggest than pregnant women diagnosis of SARS-CoV-2 in the first trimester had a higher risk of early miscarriage. Peikos et al[95] propose that increased levels of angiotensin converting enzyme 2 (ACE2) in the placenta earlier in gestation and the interaction between SARS-CoV-2 spike protein and ACE2 for entry into human cells could explain for worse outcomes with SARS-CoV-2 infections earlier in gestation.

2.2.2 VERTICAL TRANSMISSION

There is scientific evidence of transplacental transmission of emerging diseases such as HIV, Zika and Ebola[96] [97]. Viruses can be vertically transmitted from mother to infant through three different mechanisms (Figure 8):

- Intrauterine viral transmission: It can occur via 2 major mechanisms, the hematogenous route and the ascending route. The hematogenous route is characteristic of most viral agents such as rubella or cytomegalovirus [98,99]. In this process, the virus circulates within the maternal bloodstream during pregnancy, gaining access to the placenta a through the uterine arterioles and crosses the maternal-placental interface to reach the fetal vessels and be transmitted to the fetus. The ascending route of intrauterine, more common in bacterial infection, fetal infection occurs when microorganisms present in the lower genital tract ascend the cervicovaginal tract to reach the pregnant uterine cavity, from where they breach the placental membranes and infect the amniotic fluid.

- Intrapartum transmission: it occurs around the time of labor and delivery when the fetus passes through an infected birth canal during vaginal delivery. This mechanism can occur with herpes simplex virus or HIV[100].

- Postpartum vertical transmission: it develops following delivery. It can occur through contaminative transmission of a virus from an infected mother via respiratory secretions and fomites, skin-to-skin contact, and breast milk. Respiratory viruses or agents present in breast milk may be transmitted by this mechanism.

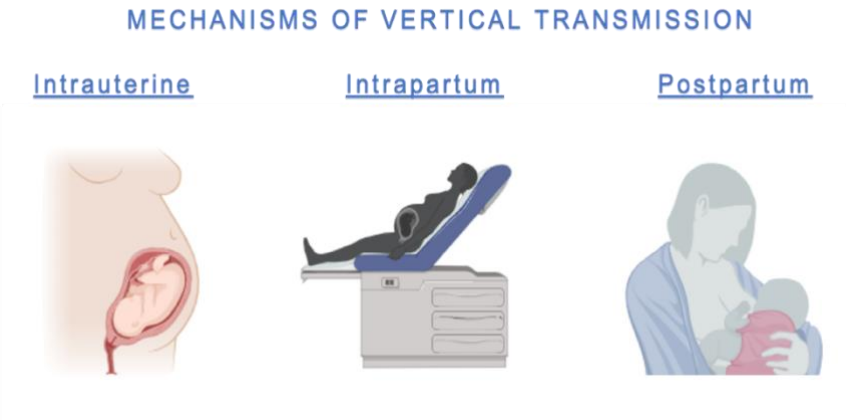

Figure 8. Mechanisms of vertical transmission of infections in pregnancy.

At the begging of the pandemic, the literature was scarce and diverse concerning vertical transmission. Some studies reported that was not vertical transmission[101,102,103] and others published that the vertical transmission could occur[104,105,106]. Kotlyaret al[107] conducted a systematic review of 68 cases or cohort published from 2020, in which they pooled information from 936 newborn whose mother were infected with SARS-CoV-2 and the 3.2% of the population tested positive. Another systematic review by Robaina-Castellanos et al[108] contained 87 studies that reported congenital, intrapartum and postpartum infection of SARS-CoV-2 during pregnancy. The investigators identified 53 reported neonates who tested positive for SARS-CoV-2 in the first 48 hours of life either showing presence of the virus by RT-PCR or IgM tests. The investigators estimated congenital or intrapartum infections occur in only 1.8% − 8.0% of newborns born to individuals with COVID-19 during the pregnancy. However, the vast majority of studies describing a vertical transmission did not meet the criteria of a correct diagnosis. The confirmation of intrauterine infection transplacental transmission of COVID-19 need to demonstrate the presence of the virus in amniotic fluid, cord blood, and neonatal throat swab samples[109,110]. For example, Almazara et al[111] reported a case with RT-PCR of neonatal nasopharyngeal swab after delivery but they did not evaluated amniotic fluid, cord blood, or placental tissue. Zeng et al[112] showed 6 infants negatives by RT-PCR but antibodies (IgM and IgG) were detected in their blood sera samples. Therefore, in the large available literature about a possible vertical transmission, there are limited cases

with sufficient evidence of vertical transmission confirmed [113,114]. Consequently, the vertical transmission seems to be possible but rare.

2.2.3 PLACENTAL INFECTION

There is evidence of placental infection in absence of vertical transmission. Cribiù et al[115] reported a rate of 47% SARS-CoV-2-positive placenta tissue in 21 women diagnosed (using RT-PCR) with COVID-19, suggesting that the placenta may be an effective maternal-neonatal barrier against the virus even in the presence of severe infection. Baud et al[116] described a pregnant with symptomatic coronavirus disease who experienced a second-trimester miscarriage in association with documented placental SARS-CoV-2 infection but negative cord blood and fetus RT-PCR. Moreover, some authors reported changes in the placental pathology of COVID-19 women compared with non-COVID-19 women. Shanes et al[117] studied 16 placentas from pregnant women with COVID-19 and concluded that the placentas have higher rates of decidual arteriopathy and other maternal vascular mal perfusion features than non-COVID-19 women. Smithall et al[118] compared placentas from 51 COVID-19 pregnant women with 25 non-COVID-19 pregnant women. They reported nonspecific microscopic changes related to SARS-CoV-2 infection but a variety of pathological finding were identified in placentas of women with COVID-19. Dagelic et al[119] suggest that time of the COVID-19 infection may have the influence on trophoblast damage and thus morphological appearance of the placenta. However, is still unknown the consequences of these placental infection or histological changes. Some studies reported that SARS-CoV-2 placental infection could be relation with adverse maternal and perinatal outcomes[120]. Hosier et al.[121] described for the first time an inflammatory infiltrate and a high viral load in the placenta of a single woman with severe PE and SARS-CoV-2 infection during the late second trimester of pregnancy. Hamilton et al[122] suggest that the placental lesion could result in chronic fetal hypoxia, insufficient nutrient transfer and fetal growth restriction.

2.2.4 MATERNAL ANTIBODIES TRANSFER

Maternally derived pathogen specific antibodies represent a tool to protect the vulnerable infants until their immune system can adequately respond to infections. In fact, maternal antibodies are passively transferred throughout the placenta and later in breast milk (Figure 9). IgG is the only antibody class that significantly crosses the human placenta[123]. Instead, maternal secretory IgA and, to a lesser extent IgG and IgM, are secreted in the colostrum and breastmilk[124]. Maternal passive immunity has been observed in viruses including HIV, cytomegalovirus and Zika[125].

Transplacental passage of maternal-derived SARS-CoV-2 antibodies has been documented in seropositive women infected with COVID-19. Studies have shown that cord blood IgG antibody concentrations are directly associated with maternal antibody concentrations and with time elapsed from maternal infection to delivery[112,113]. A systematic review has evaluated IgG and IgM levels in pregnant women and their neonates. They found that 72.73% of infected mothers had positive IgG against SARS-CoV-2 and 67.16% of neonates born to SARS-COV-2-positive mothers had elevated IgG levels in their serology tests; however, the prevalence of IgM rise in the neonates was lower (20.6%)[126].

The presence of SARS-CoV-2 RNA and its antibodies in human breast milk is reported in several studies. In fact, it is not definitely clear whether the virus in breastmilk has the potential to infect infants or not. Some studies have found SARS-CoV-2 RNA in breast milk, while the infants' nasopharyngeal RT-PCR tests were negative and no symptoms related to SARS-CoV-2 were detected[127,128]. Others, have shown positive SARS-CoV-2 PCR test of breast milk in addition to the reported infection in infants[129130]. However, antibodies against SARS-CoV-2 in breast milk samples could provide long-term passive immunity for newborns. Low et al[131] reported systematic review of women with COVID-19 infection and human milk tested for anti-SARS-CoV-2. The included 161 women from 14 studies and concluded that human milk of lactating individuals after COVID-19 infection contains anti-SARS-CoV-2-specific IgG, IgM and/or IgA in 133 cases (82.6%),

even after mild or asymptomatic infection. Out of the evaluated samples, 78.5% were positive for IgA antibody.

Figure 9. Mechanisms of vertical transmission of infections in pregnancy. A) Antibody production and transplacental transfer after maternal immunization during pregnancy. B) Breastfeeding enables the transfer of maternal antibodies, mainly IgA.

2.2.5 COVID-19 AND PREECLAMPSIA

Throughout the pandemic, and especially during the first year, a higher incidence of preeclampsia has been observed in COVID-19 cases compared to non-infected women. The INTERCOVID study[132], conducted in 18 countries during 2020 with 1430 women enrolled, reported 123 women who had preeclampsia of which 59 of 725 (8.1%) were in the COVID-19 diagnosed group and 64 of 1459 (4.4%) were in the not-diagnosed group (risk ratio, 1.86; 95% confidence interval, 1.32–2.61). Wei et al[133] conduced a meta-analysis of observational studies with SARS-CoV-2 infection and prenatal outcomes, including 42 studies involving 438.548 pregnant. They concluded that COVID-19 disease was associated with preeclampsia [OR 1.33 (95% CI: 1.03–1.83)], preterm birth [OR

1.82(95% CI: 1.38–2.39)], and stillbirth [OR 2.11(95% CI: 1.14–3.90)]. Other meta-analysis with 28 studies comprising 15,524 women who were diagnosed with SARS-CoV-2 infection, showed that the odds of developing preeclampsia were significantly higher among pregnant women with SARS-CoV-2 infection than among those without SARS-CoV-2 infection [7.0% vs 4.8%; OR: 1.62 (95% CI: 1.45–1.82); p<.00001]. Even after adjusting for other risk factor, the association between COVID-19 and PE remained significant[134]. Moreover, some data showed that women with a history of a positive risk screening of preeclampsia in the first trimester of pregnancy have a significantly high risk of developing COVID-19[135].

Nowadays, the pathogenesis linking SARS-CoV-2 infection and preeclampsia remains uncertain. Extensive endothelial dysfunction (ED) is considered the common and overlapping pathophysiological mechanism behind both preeclampsia and COVID-19 disease. Both pathologies exert symptoms related to vasoconstriction and organ ischemia such as acute kidney lesions, liver injuries, thrombocytopenia, coagulation abnormalities, and hypertension [136,137] (Figure 10). Mendoza et al[138] described a PE-like syndrome as PE induced by severe COVID-19 in women with low angiogenic factors values. They observed that the women were completely recovered after severe pneumonia and suggested that it might not be a placental complication. Palomo et al[139] demonstrated differences and similarities in the endothelial and angiogenic profiles of pregnant women with preeclampsia and COVID-19, suggesting both conditions activate inflammatory signaling pathways. Another theory focuses on the placenta. It is critical organ that protects the fetus from infections and has a fundamental role in the development of preeclampsia. Verma et al[140] concluded that the presence of virus in placental villi and fetal membranes suggests that the virus can access the placenta and could affect fetal and maternal development. Lu-Culligan et al[141] hypothesized that maternal SARS-CoV-2 infection may activate the maternal endothelium, leading to endotheliitis, impaired fibrinolysis, and excess fibrin deposition, similar to observations in preeclampsia. Bachnas et al[142] studied placentas from women with preeclampsia, COVID-19 and preeclampsia and COVID-19. Placental expressions of caspase-3, caspase-1, and TNF-alpha were significantly high in pregnancies complicated by both COVID-19

and preeclampsia, suggesting highly morbid placental damage with unfavorable perinatal outcomes.

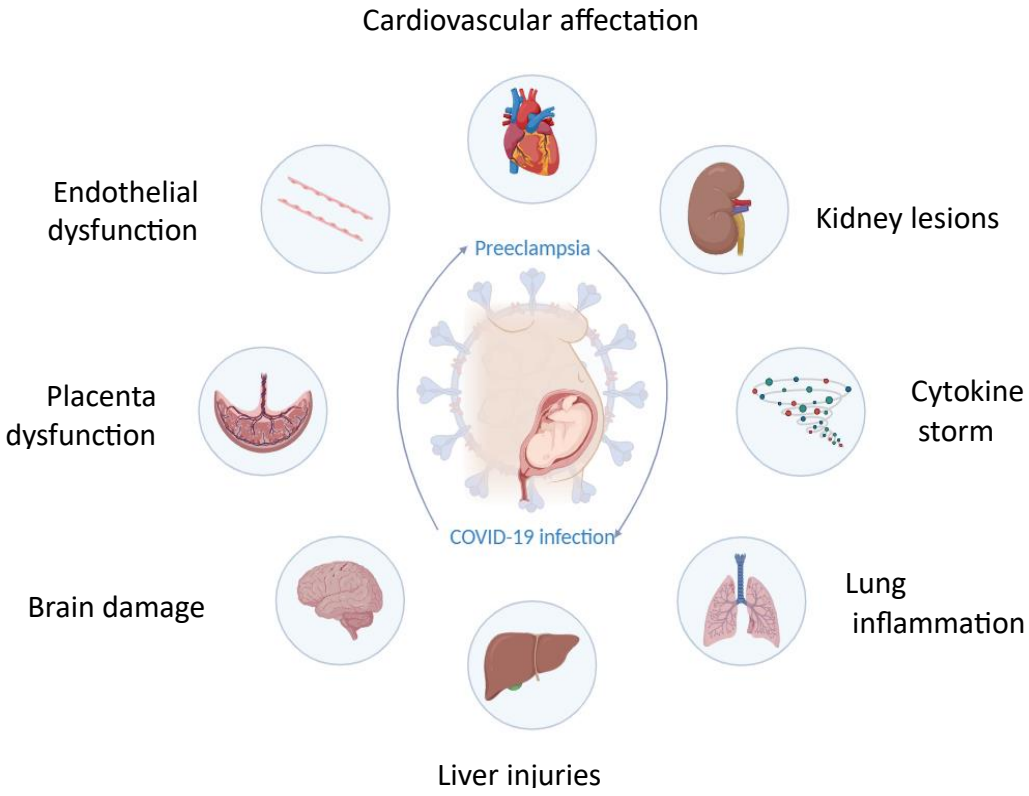

Figure 10. Schematic symptoms of several effects of COVID-19 in pregnant women and preeclampsia.

2.2.6 VACCINATION IN PREGNANCY

Vaccination in pregnancy to prevent maternal morbidity and mortality, or to confer passive immunity to the infant, has a long and successful history. Notably, The WHO has recommended Influenza vaccination of pregnant women irrespective of trimester since 2005[143] . Similarly, the United Kingdom has advised a single dose of the Pertussis vaccine between 28 and 32 weeks of gestation since 2012[144]. Vaccinating pregnant women offers dual benefits. Firstly, it shields women from infections that pose heightened risks during pregnancy, thereby protecting the fetus from congenital infection and related maternal

infection effects. Secondly, maternal vaccination aims to safeguard the developing fetus and newborn by transferring neutralizing IgG and/or secretory IgA antibodies via the placenta and breast milk.

Pregnant individuals were not included in the phase III trials of the COVID-19 vaccines approved in the EU and the US. However, due to the urgent need to protect this group, vaccines were rolled out to them in advance of the completion of clinical trials. On 16 April 2021, the United Kingdom's Joint Committee on Vaccination and Immunisation advised that pregnant women should be offered the COVID-19 vaccine at the same time as the rest of the population, based on their age and clinical risk group[145]. Currently, four types of COVID-19 vaccines (mRNA, viral vector, inactivated, and protein subunit) are available worldwide. Vaccines using mRNA technology are by far the most used type in pregnant women and have the largest accumulated safety data[146]. The primary vaccination schedule is considered complete after receiving two doses, administered 3–8 weeks apart. Booster shots, that are given to reverse the decline in the humoral response, are recommended at least 5 months after the primary vaccination series[147].

To date, accumulating evidence indicates that COVID-19 vaccines are safe for the mother and fetus. A prospective cohort study involving pregnant women immunized with COVID-19 mRNA vaccines revealed that the incidence of COVID-19 vaccine side effects was relatively similar between pregnant and nonpregnant women, and no serious complications occurred in either group[148]. In a meta-analysis conducted by Ma et al[149] at the end of 2021 concluded that no adverse effects of COVID-19 vaccination were found on pregnant, fetal, or neonatal outcome, confirming the effectiveness and safety of COVID-19 vaccines for pregnant women. Similarity Dick et al[150] studied 2305 women vaccinated during pregnancy and reported no increase in maternal and neonatal adverse events compared to unvaccinated women.

The INTERCOVID-2022, which included 4618 women with a COVID-19 diagnosis and 3017 women without diagnosis from November 2021 to June 2022, highlighted the substantial benefits of COVID-19 vaccination during pregnancy. The vaccine effectiveness (all vaccines combined) for preventing severe complications of COVID-19

in women with a complete regimen was 48% (95% CI 22–65) and 76% (47–89) after a booster dose. For women with a COVID-19 diagnosis, vaccine effectiveness of all vaccines combined for women with a complete regimen was 74% (95% CI 48–87) and 91% (65–98) after a booster dose. Notably, mRNA vaccines showed exceptional effectiveness in preventing severe symptoms and complications. Moreover, unvaccinated women with a COVID-19 diagnosis had a greater risk of maternal morbidity and mortality index (RR: 1.36 [95% CI 1.12–1.65]). The researchers concluded that COVID-19 in pregnancy, during the first 6 months of omicron as the variant of concern, was associated with increased risk of severe maternal morbidity and mortality, especially among symptomatic and unvaccinated women. These significant decreases in maternal and perinatal morbidity and deaths were reported in several studies during the years 2022 and 2023[151,152,153].

Additionally, specific antibodies generated by vaccinated mothers during pregnancy can transfer across the placenta barrier. Nir et al[154] demonstrated efficient transfer of SARS-CoV-2 IgG across the placenta in women vaccinated with mRNA vaccines during pregnancy, establishing a positive correlation between maternal serum and cord blood antibody concentrations. Moreover, Yang et al[155] assessed the transplacental transmission of SARS-CoV-2 IgG antibodies to infants from maternal COVID-19 vaccine immunization before pregnancy. The investigators concluded that the maternal SARS-CoV-2 IgG antibodies produced from inactivated COVID-19 vaccine before pregnancy can be transferred to newborns by the placenta ant the levels of antibodies was positively correlated with the number of COVID-19 vaccine dose.

2.3 REVELANCE AND JUSTIFICACION OF THE RESEARCH STUDY

The onset of the pandemic marked an unprecedented and challenging landscape, redefining the dynamics of work and collaboration for healthcare professionals. The research groups were compelled to provide evidence for the consequences of SARS-CoV-2 infection. In our project, we investigate the clinical and placental expression patterns of SARS-CoV-2 Infection in pregnancy to improved management of COVID-19-positive women during gestation throughout the pandemic (Figure 11).

Our group has demonstrated the impact of SARS-CoV-2 infection during the pregnancy. We contribute to the understanding how SARS-CoV-2 virus affects pregnancy, exploring the efficacy of point of care diagnostics tests in pregnant women, investigating the potential of vertical transmission and examining placenta infection by different variants of SARS-CoV-2 and their association with the hypertension disorder of pregnancy. In fact, we were the first in suggested that SARS-CoV-2 infection during pregnancy does trigger gestational hypertensive disorders through placenta-related mechanisms. The release of each of these projects has had a dual purpose, customizing clinical protocols employed throughout the pandemic for the management of SARS-CoV-2 positive pregnant women. Concurrently, it has substantially contributed to the scientific evidence base concerning the impact of COVID-19 during pregnancy.

Finally, it is important to note that, despite the conclusion of the COVID-19 pandemic as a public health emergency of international concern by the WHO officially in May 2023, the exhaustive research conducted throughout the pandemic to understand the impact of COVID-19 infection have provided essential scientific evidence. This research serves as a reservoir of knowledge, offering valuable insights and pathways to navigate potential future pandemics.

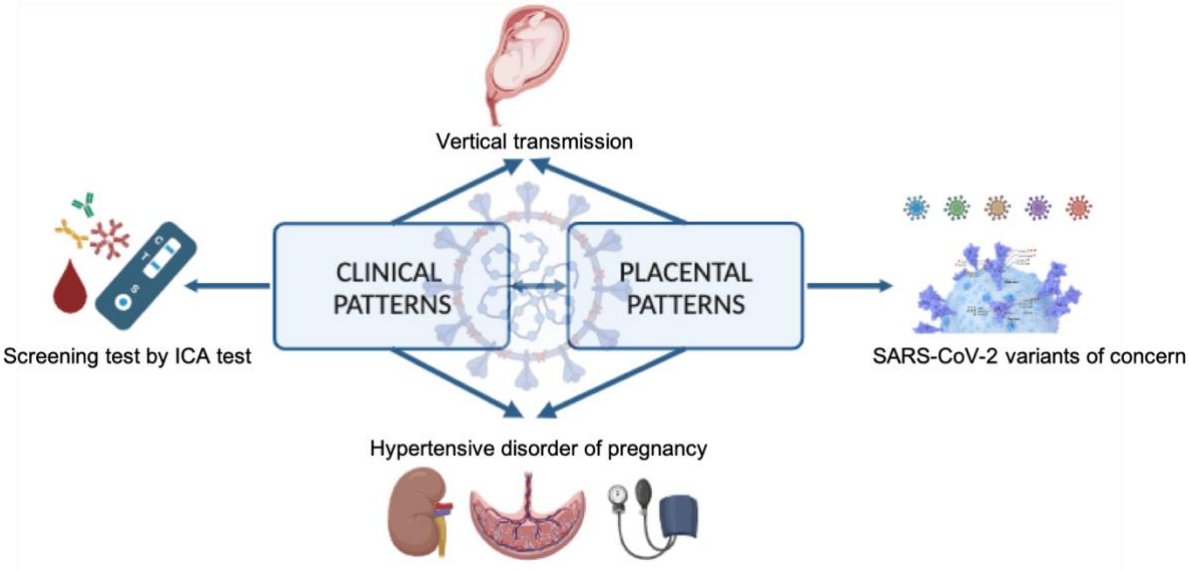

Figure 11. Project summary.

HYPOTHESIS

3. HYPHOTESIS

3.1 MAIN HYPHOTESIS

- The SARS-CoV-2 infection during pregnancy has the potential to influence maternal-fetal outcomes by modulating clinical and placental expression profiles.

3.2 SECONDARY HYPHOTESIS

- The immunochromatographic assay rapid test for qualitative detection of SARS-CoV-2 IgG/IgM antibodies has not diagnostic efficacy in pregnancy population.

- The SARS-CoV-2 vertical transmission may occur, but it is rare phenomenon.

- The SARS-CoV-2 infection during pregnancy may potentially instigate gestational hypertensive disorders via sustained placental infection and subsequent placental damage.

- The presence of SARS-CoV-2 in placenta tissue may vary depending on SARS-CoV-2 variants.

OBJECTIVES

4. OBJETIVES

4.1 MAIN OBJETIVE

- To examine the potential impact of SARS-CoV-2 infection during pregnancy on maternal-fetal outcomes through the analysis of clinical and placental expression patterns.

4.2 SECONDARY OBJETIVE

- To examine the utility of immunochromatographic assay rapid test for qualitative detection of SARS-CoV-2 IgG/IgM antibodies in the pregnant population.

- To investigate the possibility of SARS-CoV-2 vertical transmission and to assess studies reporting infected neonates born to infected mothers.

- To determinate whether SARS-CoV-2 infection during pregnancy has the potential to initiate gestational hypertensive disorders, exploring the mechanisms involving persistent placental infection and resultant placental damage.

- To analyze the frequency of placental infection by SARS-CoV-2 in SARS-CoV-2 positive women during pregnancy throughout the successive pandemic waves.

STUDY DESIGN

5. STUDY DESIGN

To address the hypothesis and objectives described previously, we conducted a biobank and database of pregnant women diagnosed of COVID-19 disease at Lozano Blesa Hospital during the course of the pandemic (Figure 12). The project was approved by the Research Ethics Committee of the Community of Aragon (C.I. PI21/155 and COL21/000) (Annex I). Written informed consent was obtained from participants.

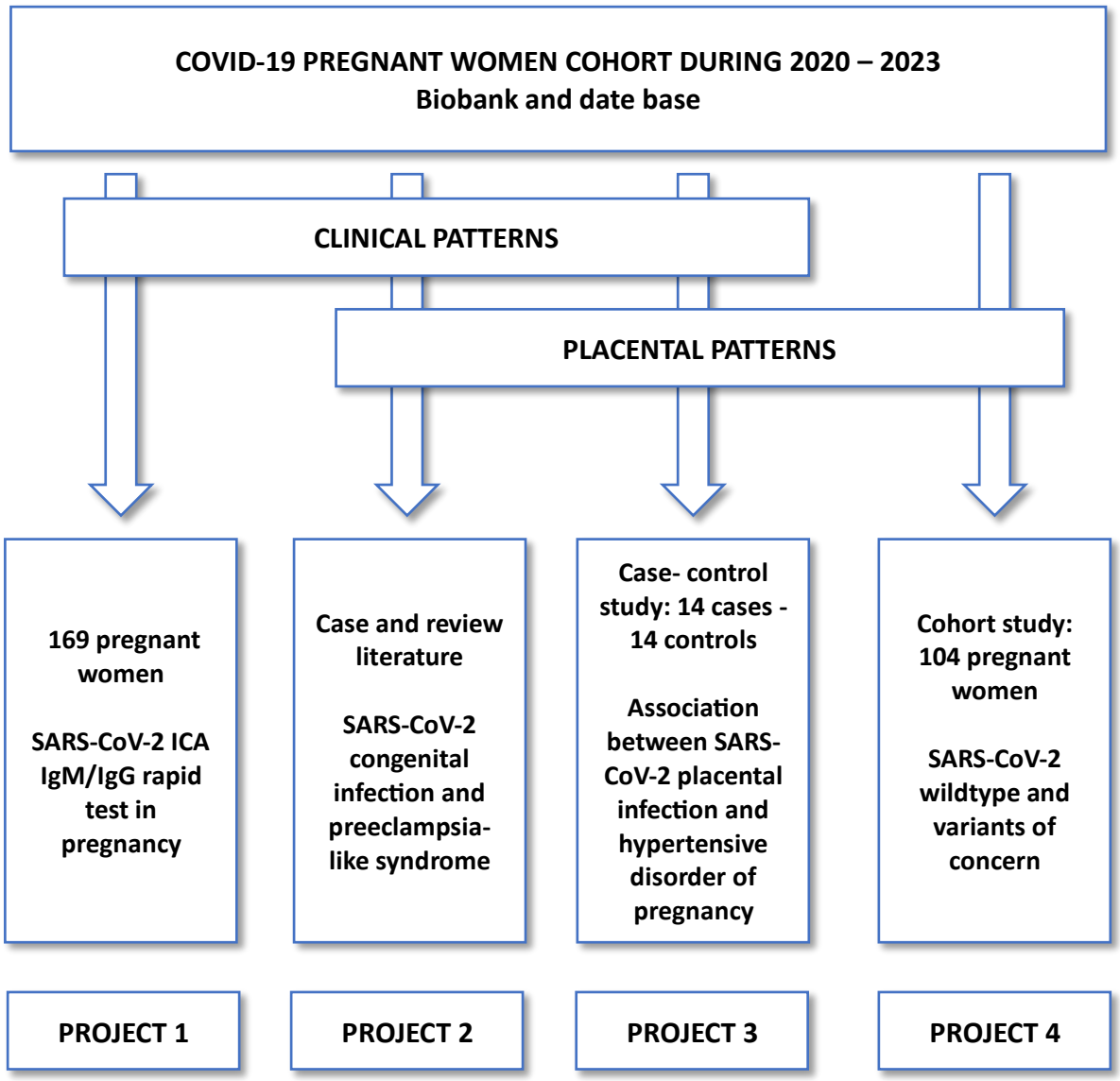

Figure 12. Study design.

For this purpose, the first consisted of adapting protocols previously applied in others projects to the COVID-19 studies. Our circuits consisted of collecting sample in the

laboratory of remnants of samples collected for clinical purposes (maternal blood and urine samples), the delivery room (umbilical cord blood and placenta samples) and the newborn room (urine, stool and milk samples). Subsequently, these samples are received in the emergence laboratory for centrifugation and preservation (Figure 13).

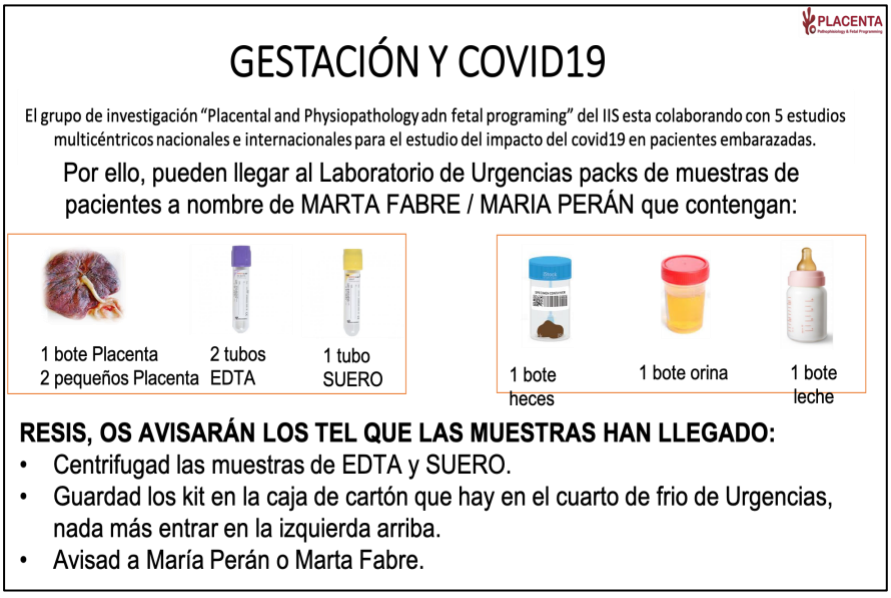

Figure 13. Posters included in the Obstetrics and Gynecology service and Clinical Biochemistry service to ensure the successful execution of the samples collection.

The projects and consequential papers included in this thesis were internally conducted within our center. On the other hand, since the beginning of the pandemic, several research groups offered us to collaborate in different multicentric projects on the influence of SARS-CoV-2 infection during pregnancy. Some of the projects that we had collaboration on May 2020:

- OxTREC Ref: 526-20: Oxford University. INTERCOVID: A prospective cohort study of the effects of COVID-19 in pregnancy and the neonatal period. Funded by Bill and Melinda Gates Foundation.

- COV20/310: Instituto de investigaciones Biomédicas August Pi i Sunyer. Seguimiento ecográfico en gestantes con infección por COVID-19. Riesgos asociados a la infección severa y al trimestre del embarazo. Funded by Instituto de Salud Carlos III (27.375 euros).

- COV20/188: Fundación Instituto de Investigación Valle De Hebrón. Gestación y COVID-19: estudio clínico y microbiológico (GESTA-COVID19). Funded by Instituto de Salud Carlos III (136.478 euros)

- RedSEGO: Spanish database created by the Spanish Group of Obstetric Emergencies. A prospective observational study in 78 centers.

These collaborations have led to the publication of the following articles in which the doctoral candidate is author or collaborator:

- Villar J, Ariff S, Gunier RB, et al. Maternal and Neonatal Morbidity and Mortality Among Pregnant Women With and Without COVID-19 Infection: The INTERCOVID Multinational Cohort Study. JAMA Pediatr. 2021;175(8):817-826.

- Papageorghiou AT, Deruelle P, Gunier RB, et al. Preeclampsia and COVID-19: results from the INTERCOVID prospective longitudinal study. Am J Obstet Gynecol. 2021;225(3):289.e1-289.e17.

- Bäuerl C, Randazzo W, Sánchez G, et al. SARS-CoV-2 RNA and antibody detection in breast milk from a prospective multicentre study in Spain. Arch Dis Child Fetal Neonatal Ed. 2022;107(2):216-221.

- Eskenazi B, Rauch S, Iurlaro E, et al. Diabetes mellitus, maternal adiposity, and insulin-dependent gestational diabetes are associated with COVID-19 in pregnancy: the INTERCOVID study. Am J Obstet Gynecol. 2022;227(1):74.e1-74.e16.

- Villar J, Soto Conti CP, Gunier RB, et al. Pregnancy outcomes and vaccine effectiveness during the period of omicron as the variant of concern, INTERCOVID-2022: a multinational, observational study. Lancet. 2023;401(10375):44.

5.1. PROJECT 1: SARS-COV-2 IMMUNOCHROMATOGRAPHIC IGM/IGG RAPID TEST IN PREGNANCY

In the initial months of the pandemic when diagnostics tools were limited, we designed an evaluation of the diagnostic efficacy of a lateral-flow immunochromatographic assay (ICA) for qualitative detection of SARS-CoV-2 IgG/IgM antibodies between April 27 and May 29 2020. Qualitative rapid tests had the advantage of being rapid, easy to perform and low cost but they did not have high diagnostic efficacy rates.

To study ICA test, we conducted an universal screening including 169 pregnant women who were either at 36 weeks of gestation or had COVID-19 symptoms in the third trimester. In positive ICA test cases, the result were confirmed by RT-PCR for ruled out active infection and by chemiluminescent microparticle immunoassays (CMIA) for quantitative detection of SARS-CoV-2 IgG and IgM+IgA antibodies, as a gold standard (Figure 14).

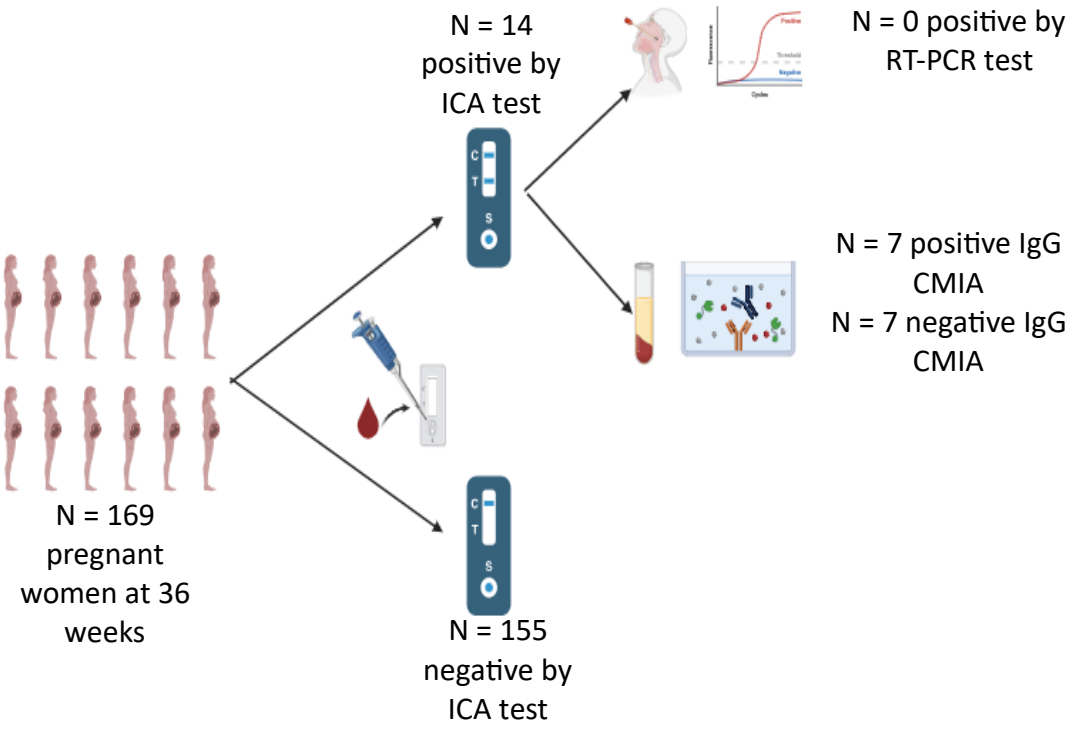

Figure 14. SARS-CoV-2 immunochromatographic IgM/IgG rapid test in pregnancy study design.

Publications generated by this project:

- Fabre M, Ruiz-Martinez S, Monserrat Cantera ME, et al. SARS-CoV-2 immunochromatographic IgM/IgG rapid test in pregnancy: A false friend?. Ann Clin Biochem. 2021;58(2):149-152. doi:10.1177/0004563220980495.

5.2 PROJECT 2: SARS-COV-2 CONGENITAL INFECTION AND PREECLAMPSIA-LIKE SYNDROME

The second project aimed to investigate the possibility of SARS-CoV-2 vertical transmission and to assessed studies reporting infected neonates born to infected mothers.

In January 2021, vertical transmission of SARS-CoV-2 was still a controversial issue and studies on transplacental transmission were limited (Figure 15). We found 41 studies reporting infected neonates born to infected mothers.

We reported a case of a probable transplacental transmission of SARS-CoV- 2 to both twins in which the mother also developed a preeclampsia-like syndrome. The develop of both clinical picture was the case unique.

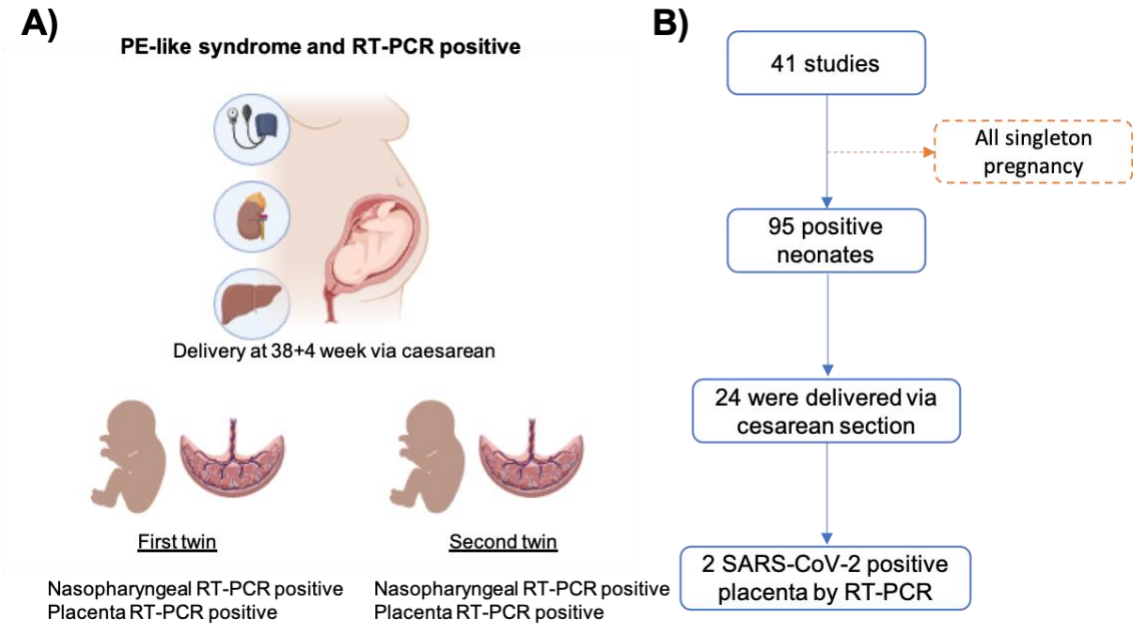

Figure 15. SARS-CoV-2 vertical transmission study design. A) Description of case. B) Assessed studies reporting infected neonates born to infected mothers.

Publications generated by this project:

- Abadía-Cuchí N, Ruiz-Martínez S, Fabre M, et al. SARS-CoV-2 congenital infection and pre-eclampsia-like syndrome in dichorionic twins: A case report and review of the literature. Int J Gynaecol Obstet. 2021;154(2):370-372. doi:10.1002/ijgo.13749.

5.3 PROJECT 3: ASSOCIATION BETWEEN SARS-COV-2 PLACENTAL INFECTION AND HYPERTENSIVE DISORDER OF PREGNANCY

Several studies conducted towards the end of the 2020 and at the beginning of 2021 reported an increase in hypertensive disorders of pregnancy associated with SARS-CoV-2 infection during pregnancy. The probable pathophysiological mechanism in many complications related to COVID-19 and pregnancy may be endothelial dysfunction. Concurrently, there was no robust evidence regarding the incidence of SARS-CoV-2 invading the placenta.

In light of the increased incidence of hypertensive disorders in COVID-19 pregnant women, the third project aimed to explore the relationship between the SARS-CoV-2 viral load in placental tissue at birth and the development of hypertensive disorder of pregnancy after COVID-19 across different gestational periods. We conducted a case-control study to analyze SARS-CoV-2 RNA levels in placental tissue and assess SARS-CoV-2 antibodies in the umbilical cord, comparing women diagnosed with COVID-19 during pregnancy with and without gestational hypertensive disorders (Figure 16).

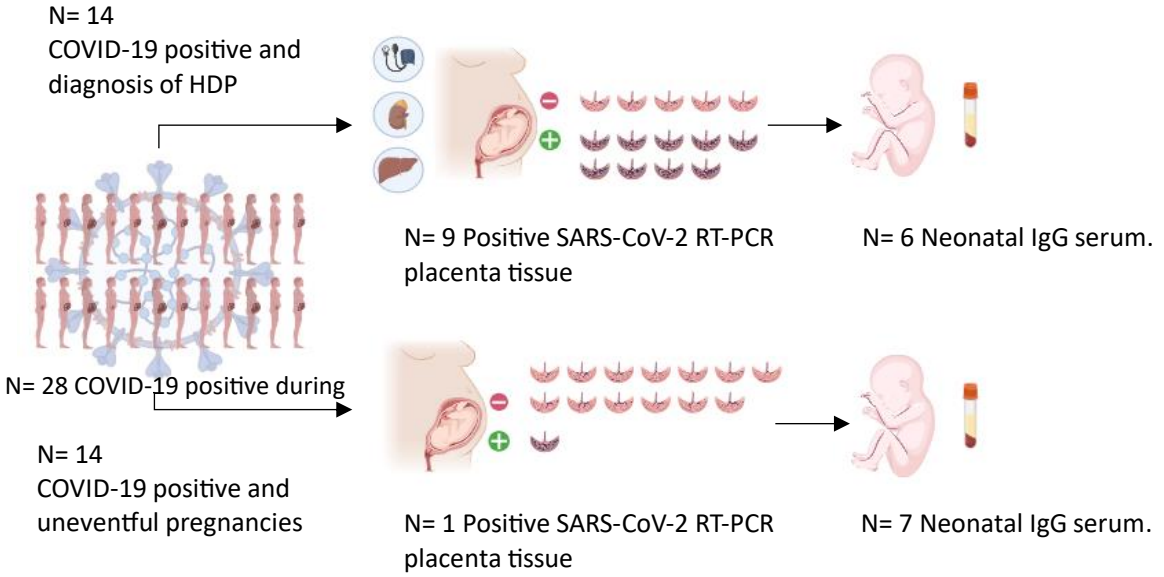

Figure 16. Association between SARS-CoV-2 placental infection and hypertensive disorder of pregnancy study design.

Publications generated by this project:

- Fabre M, Calvo P, Ruiz-Martinez S, et al. Frequent Placental SARS-CoV-2 in Patients with COVID-19-Associated Hypertensive Disorders of Pregnancy. Fetal Diagn Ther. 2021;48(11-12):801-811. doi:10.1159/000520179

- Peran M. 2021. [Master Final Project. Universidad de Zaragoza] "Elevada carga viral de SARS-CoV-2 en la placenta de pacientes con trastornos hipertensivos tras el diagnóstico de COVID-19 durante el embarazo".

- Peran M, Fabre M, Aparicio D, Medrano A. 2022. Elevada carga viral de SARS-CoV-2 en la placenta de pacientes con trastornos hipertensivos tras el diagnóstico de COVID-19 durante el embarazo. Congreso LabClin. Málaga. Spain

- Luna Álvarez C, Fabre Estremera M, Calvo Cardo MP, Abadía Cuchi N. 2021. Elevada carga viral de SARS-CoV-2 en la placenta de pacientes con trastornos hipertensivos tras el diagnóstico de COVID-19 durante el embarazo. 36º Congreso SEGO. Murcia. Spain

5.4 PROJECT 4: SARS-COV-2 WILDTYPE AND VARIANTS OF CONCERN

Finally, the advent of vaccines, herd immunity, and the diverse SARS-CoV-2 variants have prompted modifications in both clinical protocols and public health control measures for COVID-19 pandemic.

In the last project, we examined a cohort of 104 placentas to assess the frequency of placental infection by SARS-CoV-2 in pregnant women with COVID-19 disease throughout the successive pandemic waves. We analyzed the placenta tissue by RT-PCR, and the viral variant was determined by whole genome sequencing (Figure 17).

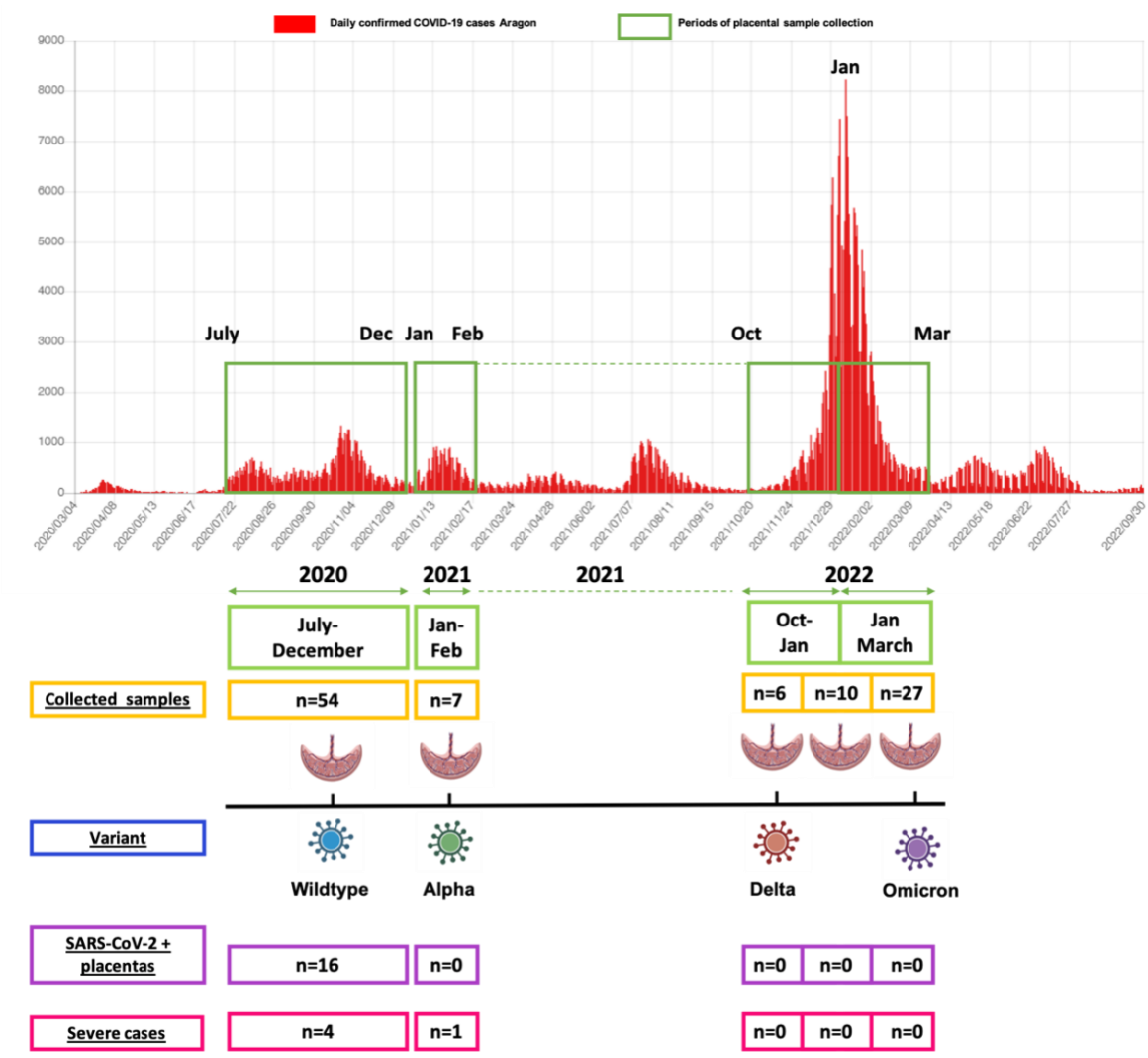

Figure 17. SARS-CoV-2 wildtype and variants of concern study design.

Publications generated by this project:

- Medel-Martinez A, Paules C, Peran M, Calvo P, Ruiz-Martinez S, Ormazabal Cundin M, Cebollada-Solanas A, Strunk M, Schoorlemmer J, Oros D, Fabre M. Placental Infection Associated with SARS-CoV-2 Wildtype Variant and Variants of Concern. Viruses. 2023;15(9):1918. doi:10.3390/v15091918

- Pardina G. 15-16 diciembre 2022. Evolución de la tasa de infección placentaria según la variante de SARS-COV-2. 10º Curso de formación continuada de la Asociación de Ginecología y Obstetricia de Aragón. Zaragoza. Spain.

DISCUSSION

6. DISCUSSION

In our project, we examine the clinical and placental patterns linked to SARS-CoV-2 infection in pregnancy, aiming to improve the care of pregnant women diagnosed with COVID-19 throughout the pandemic. Our research indicates that SARS-CoV-2 infection during pregnancy may have consequences throughout gestation, involving both clinical and placental aspects.

In the early stages of the pandemic, there were not enough diagnostic tests for the entire population. It was therefore imperative to ensure accurate interpretation in order to make accurate diagnoses. Our first project investigated the utility of rapid antibody tests (RATs) to detect SARS-CoV-2 IgG/IgM antibodies in pregnant women, which have been published to have moderate sensitivity but high specificity in non-pregnant patients. However, in our study, a low positive predictive value and a high false-positive rate were observed in pregnant women. We also reviewed potential interferences such as rheumatoid factors and cross-reactivity with other viruses. Rapid antibody tests may be less accurate, whereas chemiluminescent microparticle immunoassay tests may mitigate these interferences. Despite their potential limitations as rapid and cost-effective screening tools, the importance of confirming ICA test results with other methods in the pregnant population is emphasized.

As the months progressed, concerns regarding the potential vertical transmission of the SARS-CoV-2 virus from mothers to infants escalated . Numerous studies intensified the focus on understanding the mechanisms and implications of maternal-fetal transmission. In this context, we investigate the possibility of SARS-CoV-2 vertical transmission and to assessed studies reporting infected neonates born to infected mothers. In January 2021, We found 41 studies reporting infected neonates born to infected mothers. Moreover, we described the first case suggesting likely vertical transmission of SARS-CoV-2 to both twins, with the added uniqueness of a pre-eclampsia-like syndrome. Therefore, we concluded that the SARS-CoV-2 vertical transmission may occur but it is rare.

The INTERCOVID study, coordinated by the University of Oxford, concluded in April 2021 that COVID-19 disease increases the risk of complications during pregnancy for mothers and babies, a greater risk than had been found at the beginning of the pandemic. Our third project investigated the association between the presence of SARS-CoV-2 in placental tissue and the development of hypertensive disorders of pregnancy (HDP) according to COVID-19 disease. Out of 28 samples, 10 placenta samples tested positive for SARS-CoV-2, with a higher frequency observed in the HDP group. In particular, severe cases of pre-eclampsia had higher placental viral loads, and mothers with persistent placental infection before 27 weeks were more likely to develop HDP. The omission of maternal tissue during sampling implies that positive placental RT-PCR results reflect viral load in the villous placenta. Despite limitations such as small sample size and uneven placental distribution, our study highlights an increased viral presence in HDP placentas.

As the pandemic progressed, the SARS-CoV-2 virus underwent mutations, leading to the emergence of variants of interest (VOIs) of COVID-19. These variants have prompted adjustments in public health protocols, clinical management strategies, and vaccination campaigns worldwide. The genetic changes observed in these variants can impact various aspects of the virus, including transmissibility, virulence, and immune evasion. Consequently, the identification and characterization of VOIs have become integral to understanding the evolving nature of the pandemic and implementing effective control measures. Our study examined the prevalence of positive placental tests for SARS-CoV-2 across different variants of the virus. We found a higher frequency of positive placental RT-PCR SARS-CoV-2 in placentas from women infected with the wildtype SARS-CoV-2 compared to variants of concern (VOCs), even after adjusting for maternal vaccination status. Placental infection correlated with the predominant virus lineage at the time of infection. While detecting the viral genome in placental tissue doesn't necessarily indicate active infection, our data suggested potential active replication. Limited published data exist on the connection between SARS-CoV-2 variants and placental health. Our hypothesis suggests that increased vascular permeability may facilitate greater infiltration of infected cells into the placenta, potentially leading to placental

infection. Our findings also indicate that the occurrence of placental SARS-CoV-2 may vary depending on the timing of infection and the virus variant.

One significant limitation we faced in our investigation was the inherent challenge of studying COVID-19, a disease characterized by a constantly evolving and largely unknown pathophysiology. The dynamic nature of the virus, combined with the rapid accumulation of scientific knowledge and clinical data, presented considerable challenges to our research efforts. It is crucial to consider these investigations within the context of the understanding and perspectives available at the time they were conducted. Given the unprecedented nature of the COVID-19 pandemic and the rapid pace at which scientific knowledge was developing, our research was influenced by the prevailing understanding of the virus, its transmission dynamics, and clinical manifestations during the study period. As our understanding of COVID-19 continued to evolve, subsequent findings and interpretations may provide nuanced insights into the disease's complex nature.

In summary, our work has contributed throughout the pandemic to provide evidence of the implications of SARS-CoV-2 infection during the pregnancy and to improve clinics protocols for managing these pregnancies. Our findings suggest that SARS-CoV-2 infection during pregnancy can affect clinical and placental aspects, necessitating thorough monitoring of these pregnancies.

6.1 CLINICAL PATTERNS

6.1.1 SARS-COV-2 IMMUNOCHROMATOGRAPHIC IGM/IGG RAPID TEST IN PREGNANCY

While RT-PCR has been the primary method for confirming SARS-CoV-2 diagnoses since the onset of the pandemic, serological assays can play a pivotal role in managing virus infections. Given that both RT-PCR and serologic assays require determination by a clinical laboratory, exploring point-of-care testing, such as the ICA rapid test, is worth considering.

Recently, lateral-flow immunochromatographic assay in non-pregnant patients have demonstrated a moderate sensitivity (84.4%) and high specificity (98.6%)[156]. In contrast, within our series of cases, we noted a positive predictive value of 50% with a corresponding false positive rate of 50% in pregnant women. Regrettably, our study did not allow for an assessment of the false-negative rate. Nonetheless, it's crucial to acknowledge that false-positive outcomes may adversely impact pregnant patients, potentially leading to a false sense of security, increased infection risk, unnecessary treatment, as well as heightened anxiety or depression. False-positive ICA results in pregnant women may be due to factors like rheumatoid factors, antinuclear antibodies, and cross-reactivity with other viruses[157]. The CMIA can minimise these interferences by careful choices of reagents, dilution, depletion or blocking, while rapid antibody tests cannot.

This study is limited by a small participant number and the absence of confirmation for all ICA tests using CMIA. Moreover, we evaluate the test using different sample. Recent evidence shows higher sensitivity with serum (80%) than whole blood (57%) in ICA tests[156].

Our study aimed to be a practical tool for other professionals in clinical practice. This was the first population-based study to evaluate a SARS-CoV-2 rapid antibody test in pregnant women. Our findings indicated that while ICA test for detecting SARS-CoV-2

IgG/IgM antibodies may offer a rapid and cost-effective screening tool, they exhibit a high false positive rate in pregnant women. It is imperative to verify ICA antibody SARS-CoV-2 tests with other methods, in pregnant population.

6.2. CLNICAL AND PLACENTAL PATTERNS

6.2.1 SARS-COV-2 CONGENITAL INFECTION AND PREECLAMPSIA-LIKE SYNDROME

We described a woman with a dichorionic diamniotic twin pregnancy, admitted at 38+4 weeks of gestation due to severe preeclampsia and a SARS-CoV-2 infection. The maternal clinical features in this case suggest the possibility of a preeclampsia-like syndrome caused by SARS-CoV-2[138]. Moreover, an emergency caesarean section was performed. The babies were promptly separated and admitted to the neonatal intensive care unit. RT-PCR for SARS-CoV-2 was conducted in two swabs deep in the thickness of both placentas, yielding positive results (cycle threshold 16.7/19.1 and 16.7/19.1). Both babies also tested positive for SARS-CoV-2 RT-PCR 8 days after delivery.

Up to March 2021, we found 41 studies reporting infected newborns of infected mother. Among 95 cases identified, only 24 were delivered by caesarean section to mothers who tested positive for SARS-CoV-2 and were immediately separated at birth. Notably, none of these cases involved multiple pregnancies. Two of these cases reported placentas with a positive test for SARS-CoV-2, showing inflammatory histologic changes not found in noninfected controls[104,158]. Other study other studies have noted the presence of intervillositis in placentas of infected mothers, correlating with adverse perinatal outcomes such as severe pre-eclampsia, fetal growth restriction, and miscarriage[121].

This was the first case reported that suggest a probable vertical transmission of SARS-CoV-2 to both twins, with the added uniqueness of a preeclampsia-like syndrome.

6.2.2 ASSOCIATION BETWEEN SARS-COV-2 PLACENTAL INFECTION AND HYPERTENSIVE DISORDER OF PREGNANCY

The presence of SARS-CoV-2 in the placenta has been studied in the context of horizontal transmission of the virus[107]. Nevertheless, only a limited number of studies offer association between the presence of SARS-CoV-2 in the placenta and hypertensive disorders of pregnancy [121,159,160].

We investigated the relationship between SARS-CoV-2 viral load in placental tissue at birth and the development of PE or gestational hypertension after COVID-19 throughout different periods of gestation in a cohort of 14 hypertensive disorder of pregnancy cases and their matched controls, all diagnosed with COVID-19 disease. Out of 28 samples examined in our study, we identified 10 placental tissue samples that tested positive for SARS-CoV-2 (35.7% of total). Notably, nine of them belonging to the hypertension disorders during pregnancy group. Our results revealed a significantly higher frequency of positive placental SARS-CoV-2 RT-PCR test among women with SARS-CoV-2 infection during pregnancy in HDP group compared to the non-HDP group. Additionally, we noted a trend in the percentage of cases with a positive placental SARS-CoV-2 RT-PCR test between PE and AHT ($p = 0.065$). Indeed, the three most severe cases of preeclampsia exhibited a higher placental viral load than all the others. Furthermore, each of the three tested positive for SARS-CoV-2 in a different trimester of gestation. This data indicates that placental infection may persist even in the absence of maternal infection.

Furthermore, all six mothers who tested positive for SARS-CoV-2 before week 27 of gestation and exhibited persistent placental infection at birth subsequently developed HDP. These findings may indicate that chronic COVID-19 disease might increase the risk of preeclampsia. Consequently, we propose that healthcare professionals should intensify monitoring of pregnant women following SARS-CoV-2 infections, given that potential placental infection cannot be detected until delivery.

In light of the deliberate omission of maternal tissue during sample collection and for the sake of simplicity, we interpret positive placental RT-PCR results as indicative of viral

load in villous placenta. This suggests that in women susceptible to HDP, the existence of placental SARS-CoV-2 may play a role in exacerbating the severity of the disorder. We propose that the correlation between an elevated viral load in placental tissue and the severity of the hypertensive disorder warrants careful consideration.

The main limitation of our study is the small sample size. In addition, the distribution of SARS-CoV-2 within the placenta may not be uniform, and our testing was conducted on a single sample. The categorization of samples as positive or negative should be understood within this context. On the other hand, the primary strength of the study is that we emphasize the inclusion of women who tested positive for SARS-CoV-2 in each of the three trimesters of pregnancy. Our findings reveal an elevated viral load in placental tissue within the case group compared to the control group, accompanied by an increased prevalence of infected placentas.

In summary, our study revealed an increased presence of SARS-CoV-2 in placentas of the HDP group compared to those without HDP. Further investigation in a larger cohort is necessary to understand the frequency and potential role of SARS-CoV-2 in triggering gestational hypertensive disorders via placental mechanisms.

6.3 PLACENTAL PATTERNS

6.3.1 SARS-COV-2 WILDTYPE AND VARIANTS OF CONCERN

Our findings indicated that the prevalence of positive placental tests for SARS-CoV-2 may vary depending on the SARS-CoV-2 variants. The prospective collection of 106 placentas throughout the pandemic establishes a reliable foundation for evaluating the risk of placental infection over time, taking into account different VOCs and clinical scenarios.

We observed a significantly higher frequency of positive placental RT-PCR SARS-CoV-2 in the placentas from women infected with SARS-CoV-2 wildtype compared with women infected with variants of concern of SARS-CoV-2, even adjusting for the vaccination

status of the mother. In fact, we only detected cases in 2020, when SARS-CoV-2 wildtype predominated. Furthermore, we could affirm that the lineage infecting the placenta matches with the predominant lineage at the time of SARS-CoV-2 infection, and not with rare variants exhibiting a tropism for the placenta. However, while detecting the viral genome in placental tissue does not automatically imply the presence of active infectious particles, our observed genome-wide coverage in most positive samples suggested that the replication occurred and there was a potential of active infection.

There are very limited published describing the connection between SARS-CoV-2 variants of concern and placental health[117,161,162]. However, the potential differences in the frequency of placental infection, replication, and/or symptoms in placenta between pre-VOC and VOC, are yet to be determined. Our hypothesis propose that the increased permeability of blood vessels may facilitate greater infiltration of SARS-CoV-2 infected cells into the placenta, leading to subsequent placental infection. A recent study supports this theory, indicating reduced disease severity in pregnant women infected with newer variants, such as the Omicron strain, compared to those infected with the wild-type SARS-CoV-2 at the onset of the pandemic[163]. Our data show that the presence of placental SARS-CoV-2 is less frequent in mothers infected with VOC compared to SARS-CoV-2 wildtype. Therefore, if placental infection correlates with disease severity, the observed reduced frequency post-2020 could be attributed to the limited vascular damage associated with less severe illness.

Severity in several viral diseases often vary depend on the trimester of infection[164]. In our analysis, we examined the frequency of placental SARS-CoV-2 in relation to the trimester of infection. Unlike many studies concentrating on women with a positive SARS-CoV-2 diagnosis at the time of labor or hospital admission[115,118] , our study involved women infected with SARS-CoV-2 throughout the trimesters of pregnancy. The majority of SARS-CoV-2 positive placentas (12 out of 16) were from women diagnosed with COVID-19 over 10 days before delivery. Notably, all seven cases corresponding to women diagnosed with COVID-19 before the third trimester belong to the Pre-VOC group. While we cannot rule out the possibility of reinfection later in pregnancy, an equally plausible explanation lies in persistent infection. This timeframe categorizes these cases as post-

COVID-19 syndrome, according to the definitions in that moment[165]. As the persistence of SARS-CoV-2 infection post-early pregnancy exposure may lead to distinct placental lesions, further studies are essential to mechanistically elucidate the impact on both placental and fetal health. Additionally, more investigations are required to assess potential implications for newborns.

It is crucial to highlight that the collection of placentas from all SARS-CoV-2 positive pregnancies during the study period supposed a real challenge. Identification of women with a history of COVID-19 disease during pregnancy at the time of delivery, along with the availability of research staff for sample collection and preservation, were essential requisites. Due to this difficulty, a much more limited number of sample was collected. This may have caused selection bias.

To sum up, placentas positive for SARS-CoV-2 were exclusively identified in pregnancies affected by the wildtype variant. The occurrence of placental SARS-CoV-2 may be influenced by the SARS-CoV-2 variant, timing of infection, or the mother's vaccination status. Based on our findings, the current assessment of the risk of SARS-CoV-2 placental infection following maternal COVID-19 disease during pregnancy should be updated.

CONCLUSION

7. CONCLUSION

The conclusions of this thesis are as follows:

1. SARS-CoV-2 infection during pregnancy may have consequences throughout gestation, involving clinical and placental aspects.

2. Immunochromatographic assay rapid tests for qualitative detection of SARS-CoV-2 IgG/IgM antibodies may have a high false positive rate and low positive predictive value in pregnant women. Therefore, immunochromatographic assay for qualitative detection of SARS-CoV-2 IgG/IgM antibodies must be verified by other test in pregnant patients.

3. SARS-CoV-2 vertical transmission may occur but it is rare.

4. The SARS-CoV-2 infection during pregnancy could trigger gestational hypertensive disorders through persistent placental infection and resulting placental damage. Furthermore, there appears to be a correlation between high viral load in the placenta and the development of hypertensive disorders.

5. Placental SARS-CoV-2 presence could be influenced by SARS-CoV-2 variants, infection timing, or vaccination status. In our series, Positive SARS-CoV-2 placentas were only detected in pregnancies infected with the SARS-CoV-2 wildtype.

SPANISH SUMMARY

8. SPANISH SUMMARY

8.1 INTRODUCCIÓN

El 31 de diciembre de 2019, se confirmó un brote de neumonía desconocida en Wuhan, China, que posteriormente definiría la enfermedad por coronavirus (COVID-19), desencadenada por el virus SARS-CoV-2. Este virus se propagó globalmente, llevando a la Organización Mundial de la Salud (OMS) a declarar oficialmente una pandemia en marzo de 2020.

En los primeros meses, la incertidumbre sobre la causa de la enfermedad, su origen y transmisión, llevaron a un alto número infectados y de muertes. La implementación de pruebas diagnósticas específicas para detectar el SARS-CoV-2 fue esencial para realizar diagnósticos precisos y controlar el avance de la pandemia. En junio de 2020, se fundó el Grupo de Trabajo de Evolución de Virus de la OMS con un enfoque específico en las variantes del SARS-CoV-2. Estas variantes resultan de mutaciones espontáneas en el ARN viral derivado de errores en su replicación dentro de la célula huésped. A lo largo de estos años, algunas variantes han aumentado el riesgo para la salud pública global, llevando a la OMS a categorizar algunas como variantes de interés (VOI), variantes de preocupación (VOC) y variantes bajo vigilancia (VUM).

La tendencia de infección y letalidad persistió a lo largo de 2020 y 2021. La única solución a largo plazo para esta pandemia global fue una campaña de vacunación a nivel mundial. En mayo de 2021, 198 países habían comenzado la vacunación contra el COVID-19.

En mayo de 2023, la OMS anunció la conclusión de la emergencia declarada para el COVID-19. Actualmente, se han administrado 14,000 millones de dosis de vacunas y se han registrado más de 771 millones de casos y 7 millones de muertes en todo el mundo.

Las mujeres embarazadas desarrollan una adaptación inmunológica especial necesaria para mantener la tolerancia al feto. Este estado de inmunidad transitoriamente suprimida, predispone a las mujeres embarazadas a infecciones virales. Por lo tanto, las

embarazadas y sus fetos son particularmente susceptibles a los efectos de los virus durante brotes, en comparación con la población general.

Al comienzo de la pandemia del COVID-19, hubo controversia sobre el impacto de la infección por SARS-CoV-2 en mujeres embarazadas y sus fetos. Los primeros estudios sugirieron que las mujeres embarazadas no tenían un riesgo mayor ni síntomas distintos a las no embarazadas. Sin embargo, investigaciones posteriores demostraron un aumento en el riesgo de desarrollar COVID-19 grave en mujeres embarazadas en comparación con mujeres no embarazadas de edad y condiciones similares. El estudio INTERCOVID[79] demostró por primera vez que las mujeres con diagnóstico de COVID-19 tenían un riesgo sustancialmente mayor de complicaciones graves durante el embarazo, como preeclampsia, admisión en UCI, parto prematuro o bajo peso al nacer.

Respecto a la transmisión vertical del virus, la literatura a lo largo del 2020 y 2021 era escasa y diversa. Algunos estudios informaron que no había transmisión vertical, mientras que otros publicaron que la transmisión vertical podría ocurrir. Sin embargo, la mayoría de los estudios que describieron una transmisión vertical no cumplían con los criterios de un diagnóstico correcto. Actualmente, en la bibliografía disponible sobre la posible transmisión vertical, hay casos limitados con evidencia suficiente para confirmar tal hecho.

Por otro lado, conforme avanzaba la pandemia surgía cierta evidencia sobre que era posible una infección placentaria con ausencia de transmisión vertical. Cribiù et al[115] informaron una tasa del 47% de tejido placentario positivo para SARS-CoV-2 en 21 mujeres diagnosticadas con COVID-19, sugiriendo que la placenta podría ser una barrera eficaz entre la madre y el neonato. Shanes et al[116] estudiaron 16 placentas de mujeres embarazadas con COVID-19 y concluyeron que estas presentan tasas más altas de características de mala perfusión vascular materna en comparación con mujeres sin COVID-19. Sin embargo, aún se desconocen las consecuencias de estas infecciones placentarias o cambios histológicos.

8.2 HIPÓTESIS PRINCIPAL

- La infección por SARS-CoV-2 durante el embarazo puede afectar los resultados materno-fetales mediante cambios en los perfiles de expresión clínicos y placentarios.

8.3 HIPÓTESIS SECUNDARIAS

- Los test rápidos de inmunocromatografía para la detección cualitativa de anticuerpos IgG/IgM SARS-CoV-2 no tienen buena eficacia diagnóstica en la población gestante

- La transmisión vertical de SARS-CoV-2 puede ocurrir, pero es poco frecuente.

- La infección por SARS-CoV-2 durante el embarazo tiene el potencial de inducir trastornos hipertensivos gestacionales a través de la infección de la placenta y el consiguiente deterioro placentario.

- La presencia de SARS-CoV-2 en el tejido placentario puede variar dependiendo de las variantes de SARS-CoV-2.

8.4 OBJETIVO PRINCIPAL

- Examinar el impacto de la infección por SARS-CoV-2 durante el embarazo en los resultados materno-fetales a través del análisis de patrones clínicos y de expresión placentaria.

8.5 OBJETIVOS SECUNDARIOS

- Evaluar la utilidad de la prueba rápida de ensayo inmunocromatográfico para la detección cualitativa de anticuerpos IgG/IgM SARS-CoV-2 en la población gestante.

- Investigar la posibilidad de la transmisión vertical de SARS-CoV-2.

- Determinar si la infección por SARS-CoV-2 durante el embarazo tiene el potencial de iniciar trastornos hipertensivos gestacionales, mediante mecanismos que implican la infección placentaria persistente y el consecuente daño placentario.

- Analizar la frecuencia de la infección placentaria por SARS-CoV-2 en mujeres embarazadas positivas para SARS-CoV-2 a lo largo de las sucesivas olas de la pandemia.

8.6 DISEÑO DEL ESTUDIO

El inicio de la pandemia marcó un panorama sin precedentes, obligando a redefinir las dinámicas de trabajo y la colaboración entre los profesionales de la salud. Existía una imperiosa necesidad de proporcionar evidencia sobre las consecuencias de la infección por SARS-CoV-2. En nuestro proyecto de tesis, investigamos los patrones de expresión clínica y placentaria de la infección por SARS-CoV-2 durante el embarazo con el objetivo de examinar el posible impacto de la infección por SARS-CoV-2 durante el embarazo en los resultados materno-fetales.

Con este propósito, lo primero que hicimos fue adaptar protocolos previamente implementados para otros proyectos a los estudios de COVID-19. Nuestros circuitos consisten en la recolección de muestras, tanto en paritorio como en el laboratorio a partir de remanentes de muestras clínicas, para su conservación y posterior investigación. Además, desde el inicio de la pandemia, varios grupos de investigación nos ofrecieron colaborar en diferentes proyectos multicéntricos sobre la influencia de la infección por SARS-CoV-2 durante el embarazo. Esto supuso la publicación de varios artículos en revistas de alto impacto.

Los proyectos y los consiguientes artículos incluidos en esta tesis se llevaron a cabo exclusivamente en nuestro centro.

8.6.1. PROYECTO 1: SARS-COV-2 IMMUNOCHROMATOGRAPHIC IGM/IGG RAPID TEST IN PREGNANCY

En los primeros meses de la pandemia, cuando las herramientas de diagnóstico eran limitadas, diseñamos una evaluación de la eficacia diagnóstica de una prueba de inmunocromatografía lateral (ICA) para la detección cualitativa de anticuerpos IgG/IgM contra el SARS-CoV-2. Se realizó un cribado universal en 169 mujeres embarazadas en la semana 36 de gestación o con síntomas de COVID-19 en el tercer trimestre.

Las pruebas ICA, a pesar de su rapidez y bajo costo, no demostraron una alta eficacia diagnóstica. Los resultados positivos se confirmaron mediante RT-PCR y CMIA para IgG e IgM+IgA.

8.6.2 PROYECTO 2: SARS-COV-2 CONGENITAL INFECTION AND PREECLAMPSIA-LIKE SYNDROME

El segundo proyecto tuvo como objetivo investigar la posibilidad de transmisión vertical del SARS-CoV-2 y realizar una revisión bibliográfica sobre los estudios publicados sobre neonatos infectados de madres positivas a SARS-CoV-2.

En enero de 2021, la transmisión vertical de SARS-CoV-2 seguía siendo un tema controvertido y los estudios sobre la transmisión transplacentaria aún eran limitados. Encontramos 41 estudios que informaban sobre neonatos infectados nacidos de madres infectadas.

Reportamos un caso de probable transmisión transplacentaria de SARS-CoV-2 a ambos gemelos, en el cual la madre también desarrolló un síndrome preeclampsia-like. El desarrollo de ambos cuadros clínicos durante la gestación hace el caso prácticamente único.

8.6.3 PROYECTO 3: ASSOCIATION BETWEEN SARS-COV-2 PLACENTAL INFECTION AND HYPERTENSIVE DISORDER OF PREGNANCY

Ante el aumento de los trastornos hipertensivos en mujeres embarazadas con COVID-19, el tercer proyecto tuvo como objetivo explorar la relación entre la carga viral del SARS-CoV-2 en el tejido placentario al nacer y el desarrollo de trastornos hipertensivos del embarazo después de la enfermedad COVID-19 en diferentes períodos gestacionales.

Realizamos un estudio de casos y controles para analizar los niveles de ARN de SARS-CoV-2 en el tejido placentario y evaluar los anticuerpos contra el SARS-CoV-2 en el

cordón umbilical, comparando mujeres diagnosticadas con COVID-19 durante el embarazo con y sin trastornos hipertensivos gestacionales.

8.6.4 PROYECTO 4: SARS-COV-2 WILDTYPE AND VARIANTS OF CONCERN

Finalmente, el surgimiento de vacunas, la inmunidad colectiva y las diversas variantes de SARS-CoV-2 han motivado modificaciones tanto en los protocolos clínicos como en las medidas de control de salud pública para la pandemia de COVID-19.

En el último proyecto, examinamos una cohorte de 104 placentas para evaluar la frecuencia de la infección placentaria por SARS-CoV-2 en mujeres embarazadas con la enfermedad COVID-19 a lo largo de las sucesivas olas de la pandemia. Analizamos el tejido placentario mediante RT-PCR, y la variante viral se determinó mediante secuenciación completa del genoma.

8.7 DISCUSIÓN

En nuestro proyecto, examinamos patrones clínicos y placentarios vinculados a la infección por SARS-CoV-2 en el embarazo, con el objetivo de mejorar la atención de las mujeres embarazadas diagnosticadas con COVID-19 durante toda la pandemia. Nuestra investigación indica que la infección por SARS-CoV-2 durante el embarazo puede tener consecuencias a lo largo de la gestación, involucrando aspectos clínicos y placentarios.

En las etapas iniciales de la pandemia, no había suficientes pruebas de diagnóstico para toda la población. Por lo tanto, era necesario garantizar una interpretación precisa para realizar diagnósticos certeros. Nuestro primer proyecto investigó la utilidad de las pruebas rápidas de anticuerpos (ICA) para detectar anticuerpos IgG/IgM contra el SARS-CoV-2 en mujeres embarazadas. Estas pruebas rápidas es conocido que tienen una sensibilidad moderada pero una alta especificidad en pacientes no embarazadas. Sin embargo, en nuestro estudio, se observó un bajo valor predictivo positivo y una alta tasa de falsos positivos en mujeres embarazadas. Revisamos posibles interferencias como factores reumatoides y reactividad cruzada con otros virus. A pesar de sus posibles ventajas como herramientas de cribado rápidas y económicas, concluimos la importancia de confirmar los resultados de las pruebas ICA con otros métodos, como quimioluminiscencia, en la población embarazada.

A medida que avanzaban los meses, aumentaron las preocupaciones sobre la posible transmisión vertical del virus SARS-CoV-2 de madres a bebés. Numerosos estudios intensificaron el enfoque en comprender los mecanismos e implicaciones de la transmisión materno-fetal. En este contexto, investigamos la posibilidad de transmisión vertical de SARS-CoV-2 y realizando una revisión de los trabajos publicados sobre neonatos infectados nacidos de madres infectadas. En enero de 2021, encontramos 41 estudios que informaron sobre neonatos infectados nacidos de madres positivas a SARS-CoV-2. Además, describimos el primer caso que muestra una probable transmisión vertical probable de SARS-CoV-2 a ambos gemelos, con la singularidad adicional de un

síndrome preeclampsia-like. Por lo tanto, concluimos que la transmisión vertical de SARS-CoV-2 puede ocurrir, pero es rara.

El estudio INTERCOVID, coordinado por la Universidad de Oxford, concluyó en abril de 2021 que la enfermedad COVID-19 aumenta el riesgo de complicaciones durante el embarazo para las madres y los bebés, describiendo un riesgo mayor al que se había encontrado al principio de la pandemia. Nuestro tercer proyecto investigó la asociación entre la presencia de SARS-CoV-2 en el tejido placentario y el desarrollo de trastornos hipertensivos del embarazo (HDP) según la enfermedad COVID-19. De 28 muestras, 10 muestras de placenta dieron positivo para SARS-CoV-2, con una frecuencia más alta observada en el grupo de HDP. En particular, los casos graves de preeclampsia tenían cargas virales placentarias más altas, y las madres con infección placentaria persistente antes de las 27 semanas tenían más probabilidades de desarrollar estados hipertensivos del embarazado. La ausencia del tejido materno durante el muestreo implica que los resultados positivos de RT-PCR placentaria reflejan la carga viral en la placenta villosa. A pesar de limitaciones como el tamaño muestral pequeño y la distribución placentaria desigual, nuestro estudio destaca una presencia viral aumentada en placentas de gestantes que desarrollaron estados hipertensivos durante la gestación.

A medida que avanzaba la pandemia, el virus SARS-CoV-2 sufrió mutaciones, lo que llevó a la aparición de variantes de interés de SARS-CoV-2. Estas variantes han provocado ajustes en los protocolos, las estrategias de manejo clínico y las campañas de vacunación en todo el mundo. Los cambios genéticos observados en estas variantes pueden afectar varios aspectos del virus, incluida la transmisibilidad, la virulencia y la evasión inmunitaria. En consecuencia, la identificación y caracterización de las VOIs se han vuelto fundamentales para comprender la naturaleza evolutiva de la pandemia y para implementar medidas de control efectivas. Nuestro estudio examinó la prevalencia de placentas positivas para SARS-CoV-2 durante epócas de diferentes variantes de SARS-CoV-2. Encontramos una frecuencia más alta de RT-PCR positivas en placentas para SARS-CoV-2 en mujeres infectadas con el SARS-CoV-2 de tipo salvaje en comparación con las variantes de interés (VOCs), incluso después de ajustar por el estado de vacunación materna. La infección placentaria se correlacionó con la linaje viral predominante en el

momento de la infección. Si bien detectar el genoma viral en el tejido placentario no necesariamente indica una infección activa, nuestros datos sugirieron una posible replicación activa. Existen datos limitados publicados sobre la conexión entre las variantes de SARS-CoV-2 y la salud placentaria. Nuestra hipótesis sugiere que una mayor permeabilidad vascular puede facilitar una mayor infiltración de células infectadas en la placenta, lo que potencialmente conduce a una infección placentaria. En definitiva, placentas positivas para el SARS-CoV-2 se identificaron exclusivamente en embarazos afectados por la variante de tipo salvaje.

Una limitación significativa que enfrentamos en nuestra investigación fue el desafío inherente de estudiar COVID-19, una enfermedad caracterizada por una fisiopatología en constante evolución y en gran parte desconocida. La naturaleza dinámica del virus, combinada con la acumulación rápida de conocimientos científicos y datos clínicos, presentó desafíos para nuestros esfuerzos de investigación. Es crucial considerar estas investigaciones dentro del contexto del entendimiento y las perspectivas disponibles en el momento en que se llevaron a cabo. Dada la naturaleza sin precedentes de la pandemia de COVID-19 y el ritmo rápido al que se desarrollaba el conocimiento científico, nuestra investigación fue influenciada por la comprensión prevaleciente del virus, sus dinámicas de transmisión y manifestaciones clínicas durante el período de estudio.

En resumen, nuestro trabajo ha contribuido a lo largo de la pandemia para proporcionar evidencia de las implicaciones de la infección por SARS-CoV-2 durante el embarazo y para mejorar los protocolos clínicos para el manejo de estos embarazos. Nuestros hallazgos sugieren que la infección por SARS-CoV-2 durante el embarazo puede afectar aspectos clínicos y placentarios, lo que hace necesario un monitoreo exhaustivo de estos embarazos.

8.8 CONCLUSIÓN

Las conclusiones de la tesis doctoral son las siguientes:

1. La infección por SARS-CoV-2 durante el embarazo puede tener consecuencias a lo largo de la gestación, implicando aspectos clínicos y placentarios.

2. Las pruebas rápidas de ensayo inmunocromatográfico para la detección cualitativa de anticuerpos IgG/IgM SARS-CoV-2 pueden tener una alta tasa de falsos positivos y un bajo valor predictivo positivo en mujeres embarazadas. Por lo tanto, la prueba de ensayo inmunocromatográfico para la detección cualitativa de anticuerpos IgG/IgM contra SARS-CoV-2 debe ser confirmada por otras pruebas en pacientes embarazadas.

3. La transmisión vertical de SARS-CoV-2 puede ocurrir, pero es rara.

4. La infección por SARS-CoV-2 durante el embarazo podría desencadenar trastornos hipertensivos gestacionales mediante la infección placentaria persistente y el consecuente daño placentario. Además, parece haber una correlación entre la carga viral elevada en la placenta y el desarrollo de trastornos hipertensivos.

5. La presencia de SARS-CoV-2 en la placenta podría estar influenciada por variantes de SARS-CoV-2, el momento de la infección o el estado de vacunación. En nuestra serie, las placentas positivas para SARS-CoV-2 solo se detectaron en embarazos infectados con el SARS-CoV-2 tipo wildtype.

JUSTIFICACIÓN DE AUTORÍA DEL DOCTORANDO

9. JUSTIFICACIÓN DE AUTORIA DEL DOCTORANDO

Mi contribución abarcó desde la planificación inicial hasta la presentación final de los resultados en los cuatro proyectos incluidos en esta tesis doctoral. En concreto:

- Creación de una base de datos clínicos: desarrollé y gestioné una base de datos para el manejo y control de las pacientes incluidas en los estudios.

- Recogida y conservación de muestras: desempeñé un papel esencial en la recogida de muestras, garantizando la representatividad y calidad la calidad de las mismas, para los análisis posteriores.

- Realización de trabajo de campo: fui parte del equipo investigador que realizó los experimentos científicos, entre otras, pruebas de RT-PCR o NGS.

- Elaboración de análisis estadísticos: contribuí de manera significativa en la realización de los análisis estadísticos de los datos obtenidos.

- Escritura de los artículos: asumí un papel principal en la redacción de los artículos científicos.

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ANNEX 1

Informe Dictamen Favorable

C.P. - C.I. PI21/155 y COL21/000

7 de abril de 2021

Dña. María González Hinjos, Secretaria del CEIC Aragón (CEICA)

CERTIFICA

1º. Que el CEIC Aragón (CEICA) en su reunión del día 07/04/2021, Acta Nº 07/2021 ha evaluado la propuesta del investigador referida al **estudio** y a la creación de la **colección de muestras biomédicas**:

Título: Función endotelial y angiogénesis tras infección por SARS-CoV2 durante el embarazo (ANGIO-COVID)

Investigador Principal: Daniel Orós López, HCU Lozano Blesa

Versión protocolo: Versión 1. 16/03/2021

Nombre de la colección: ANGIO-COVID
Responsable clínico: Daniel Orós López
Responsable científico: Jon Schoorlermmer
Versión documento de información y consentimiento de la colección: Versión 2.0 08/04/2021

2º. Considera que

- El proyecto se plantea siguiendo los requisitos de la Ley 14/2007, de 3 de julio, de Investigación Biomédica y su realización es pertinente.
- La colección de muestras cumple los requisitos establecidos en el RD1716/2011.
- Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y están justificados los riesgos y molestias previsibles para el sujeto.
- Es adecuada la utilización de los datos y los documentos elaborados para la obtención del consentimiento.
- El alcance de las compensaciones económicas previstas no interfiere con el respeto a los postulados éticos.
- La capacidad de los Investigadores y los medios disponibles son apropiados para llevar a cabo el estudio.

3º. Por lo que este CEIC emite **DICTAMEN FAVORABLE a la realización del estudio y a la creación de la colección de muestras biológicas**.

Lo que firmo en Zaragoza

GONZALEZ HINJOS MARIA - DNI 03857456B

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ANNEX 2

Short Report

The Association for Clinical Biochemistry & Laboratory Medicine
Better Science, Better Testing, Better Care

Annals of Clinical Biochemistry
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SARS-CoV-2 immunochromatographic IgM/IgG rapid test in pregnancy: A false friend?

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Abstract

Background: An increasing body of evidence has revealed that SARS-CoV-2 infection in pregnant women could increase the risk of adverse maternal and fetal outcomes. Careful monitoring of pregnancies with COVID-19 and measures to prevent neonatal infection are warranted. Therefore, rapid antibody tests have been suggested as an efficient screening tool during pregnancy.

Cases: We analysed the clinical performance during pregnancy of a rapid, lateral-flow immunochromatographic assay for qualitative detection of SARS-CoV-2 IgG/IgM antibodies. We performed a universal screening including 169 patients during their last trimester of pregnancy. We present a series of 14 patients with positive SARS-CoV-2 immunochromatographic assay rapid test result. Immunochromatographic assay results were always confirmed by chemiluminescent microparticle immunoassays for quantitative detection of SARS-CoV-2 IgG and IgM+IgA antibodies as the gold standard. We observed a positive predictive value of 50% and a false positive rate of 50% in pregnant women, involving a significantly lower diagnostic performance than reported in non-pregnant patients.

Discussion: Our data suggest that although immunochromatographic assay rapid tests may be a fast and profitable screening tool for SARS-CoV-2 infection, they may have a high false positive rate and low positive predictive value in pregnant women. Therefore, immunochromatographic assay for qualitative detection of SARS-CoV-2 IgG/IgM antibodies must be verified by other test in pregnant patients.

Keywords

Clinical studies, immunoassay, laboratory methods, pregnancy

Accepted: 18th November 2020

Introduction

Pregnant women and their fetuses represent a high-risk population during infectious disease outbreaks. Immunological changes during pregnancy may affect the risk of developing severe complications in COVID-19 patients.[1] Although the majority of mothers do not develop any major complications, an increasing body of evidence revealed that SARS-CoV-2 infection in pregnant women can cause miscarriage, severe maternal morbidity and preterm delivery.

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Nevertheless, there is still considerable controversy about the impact of SARS-CoV-2 infection on pregnant women and the fetus. Recent studies report SARS-CoV-2 viral particles in breast milk, placental infection with the associated inflammatory changes demonstrated by immunohistochemistry and high viral load, and following by neonatal viremia.[2]

Careful monitoring of pregnancies with COVID-19 and measures to prevent neonatal infection are warranted. Some authors recommend universal screening for SARS-CoV-2 infection in pregnant women at the time of delivery or during pregnancy. Currently, the reverse-transcription polymerase chain reaction (RT-PCR) on respiratory samples are the gold standard for the diagnosis of active SARS-CoV-2 infection. Unfortunately, RT-PCR is time-consuming and requires specialized operators. However, easy to perform, fast and low-cost immunochromatographic IgM/IgG rapid antibody tests have been suggested as an efficient screening tool during pregnancy.

Materials and methods

We analysed the clinical performance during pregnancy of a lateral-flow immunochromatographic assay (ICA) for qualitative detection of SARS-CoV-2 IgG/IgM antibodies (Wondfo SARS-CoV-2 Antibody Test, Guangzhou Wondfo Biotech Co., Guangzhou, China). Between April 27 and May 29 2020, we performed a universal screening including 169 pregnant women who were either at 36 weeks of gestation or had COVID-19 symptoms in the third trimester. ICA was performed according to manufacturer's instruction, was carried out by a healthy care professional, the fingerstick whole blood specimen has been applied to the test by hanging drops and any band intensity was considered as a positive result. Fourteen patients had positive ICA results. Almost half of them (57.1%) reported having presented in the previous weeks mild-symptoms (Table 1). Active infection was ruled out by negative RT-PCR results (Abbott RealTime SARS-CoV-2 Assay, Abbott Molecular, Abbott Park, IL, USA) from nasopharyngeal swabs. ICA results were also confirmed by chemiluminescent microparticle immunoassays (CMIA) for quantitative detection of SARS-CoV-2 IgG (IgG-CMIA; SARS-CoV-2 IgG Assay, Abbott Laboratories Ireland, Dublin, Ireland) and IgM+IgA antibodies (IgM+IgA-CLIA; COVID-19 VIRCLIA IgM+IgA, Vircell Microbiologists, Granada, Spain) as the gold standard.[3] The maternal blood was taken with vacutainer in a tube without anticoagulant and has been allowed to clot. The samples were centrifuged and were determined on the same day. A signal of-cut-off (S/CO) index >1.4 were considered positive for IgG. IgM+IgA samples with a S/CO index less than 0.4 were considered negative samples, those with a S/CO index more than 0.6 were considered positive and samples with a S/CO ranging from 0.4 to 0.6 were interpreted as grey zone.

Results

According to the gold standard results, a total of 7 out of 14 (50%) patients were considered true positive ICA tests. Six patients were directly positive for IgG-CMIA. Additionally, one case (number 10) initially presented an uncertain result for IgM+IgA-CLIA with negative IgG-CLIA, hence she was considered as recent infection, as two weeks later she finally had SARS-CoV-2 positive IgG-CLIA (Table 1). The remaining seven patients tested negative for both IgG-CMIA and IgM+IgA-CLIA, and therefore were considered ICA false-positive results. It is important to note that, in contrast with the previously described case number 10, cases number 6 and number 12 were considered as false-positive ICA results, as after being initially positive for IgM+IgA-CLIA (S/Co index 1.1 and 1.09, respectively) but negative for IgG-CMIA, both IgM+IgA-CLIA and IgG-CMIA assays were negative two weeks later (Table 1). Recent studies suggest that humoral immunity against SARS-CoV-2 may not be long lasting in persons with mild illness. We evaluated this possibility in cases number 6 and number 12, as in both patients levels of IgM+IgA and IgG decreased between test. However, they never presented positive results for IgG-CMIA or any symptoms or signs of SARS-CoV-2 infection.

Discussion

Although since the beginning of this pandemic RT-PCR has been used to confirm diagnosis of SARS-CoV-2, serological assays can play an important role in the management of virus infection. Serologic tests detect waning or past SARS-CoV-2 virus infection indirectly by measuring the host humoral immune response to the virus. A meta-analysis of diagnostic performance of serological test in general population yielded a sensitivity of 82% (95%CI: 75–88%) for IgM, and 85% for IgG (95%CI: 73–93%).[4] The fact that both RT-PCR and serologic assays, need to be determinate by a clinical laboratory, make interesting the evaluation of the point of care testing as the ICA rapid test.

It should be noted that as pandemic progresses, the value of diagnostic testing for SARS-CoV-2 has enhanced. We considered our available technology as gold standard but virus neutralization remains the gold standard for determining antibody efficacy. Although, this is currently not available. For future studies, it

Table 1. Demographic, obstetrical characteristics and chronological description of laboratory testing of 14 positive cases.

Cases	Date ICA test	Age (years)	Gestational age (weeks)	Symptoms	Time from symptoms to test (days)	RT-PCR SARS-CoV-2 Result	First SARS-CoV-2 antibodies					Second SARS-CoV-2 antibodies					Diagnosis SARS-CoV-2 by ICA
							Date	IgM+IgA (S/Co)		IgG (S/Co)		Date	IgM+IgA (S/Co)		IgG (S/Co)		
1	28/04/20	41	35+6	Cough	30	Negative	26/05/20	0.97	Positive	3.10	Positive						TRUE POSITIVE
2	29/04/20	33	30+1	Cough	30	Negative	28/05/20	1.71	Positive	4.86	Positive						TRUE POSITIVE
3	29/04/20	36	30+3	Anosmia, headache, arthralgia	35	–	25/05/20	0.17	Negative	1.73	Positive						TRUE POSITIVE
4	05/05/20	34	37+1	Cough, asthenia, anosmia	90	Negative	14/05/20	0.31	Negative	3.48	Positive						TRUE POSITIVE
5	08/05/20	33	36+3	None	–	Negative	29/05/20	0.09	Negative	0.02	Negative						FALSE POSITIVE
6	11/05/20	36	36+0	None	–	Negative	27/05/20	1.10	Positive	0.05	Negative	12/06/20	0.10	Negative	0.04	Negative	FALSE POSITIVE
7	12/05/20	22	35+5	None	–	Negative	01/06/20	0.34	Negative	0.03	Negative						FALSE POSITIVE
8	13/05/20	33	36+1	Dyspnoea, fever, cough	40	Negative	27/05/20	1.37	Positive	9.15	Positive						TRUE POSITIVE
9	15/05/20	38	35+0	None	–	Negative	26/05/20	0.29	Negative	0.01	Negative						FALSE POSITIVE
10	19/05/20	37	37+1	Diarrhoea, arthalgia	20	Negative	19/05/20	0.50	Grey zone	0.19	Negative	09/06/20	0.20	Negative	2.14	Positive	TRUE POSITIVE
11	20/05/20	40	36+5	Cough, arthalgia	60	Negative	20/06/20	0.25	Negative	6.25	Positive						TRUE POSITIVE
12	22/05/20	30	36+2	None	–	Negative	22/05/20	1.09	Positive	0.26	Negative	15/06/20	0.56	Grey zone	0.26	Negative	FALSE POSITIVE
13	26/05/20	35	37+3	Asthenia	7	Negative	26/05/20	0.33	Negative	0.15	Negative						FALSE POSITIVE
14	26/05/20	31	36+1	None	–	Negative	26/05/20	0.28	Negative	0.06	Negative						FALSE POSITIVE

would be helpful to compare different immunoassay for SARS-CoV-2 antibodies in pregnant population.

Newly, lateral-flow immunochromatographic assay in non-pregnant patients has shown a moderate sensitivity (84.4%) and high specificity (98.6%).[5] In contrast, we observed in our series of cases a positive predictive value of 50% with a false positive rate of 50% in pregnant women. Unfortunately, we could not evaluate the false-negative rate in our study. However, false-positive results may have a detrimental effect on pregnant patients, such as false security awareness increasing the risk of infection, unnecessary treatment, anxiety or depression.

A false-positive result by ICA test in pregnant women may be explained by several factors. Rheumatoid factors, antinuclear and heterophile antibodies are known to interfere with rapid antibody tests. Moreover, cross-react with various agents such as influenza-A, respiratory syncytial virus or other coronaviruses have been reported.[6]

The complexity of the immunology of pregnancy makes it hard to interpret the test results. If there is any clinical suspicion of discordance between the clinical and the laboratory data, an attempt should be made to resolve the difference. The CMIA can minimize these interferences by careful choices of reagents, dilution, depletion or blocking, while rapid antibody tests cannot.

It is important to emphasize that the false positive rate depends on the prevalence of disease in the population. Instead, the sensitivity and specificity of the diagnostics test are independent of the prevalence. Thus, decisions made in low prevalence settings must not be automatically extrapolated to settings with high disease prevalence.

This study has several limitations. The first limitation is the small number of participants and the inability to confirm all ICA test by CMIA. It would have been interesting to perform ICA test and CMIA test on the same sample or at least on the same type of sample. In fact, recently it has been demonstrated significantly superior sensitivity with serum (80%) than with whole blood (57%) in ICA test.[5] However, while serum show better results than whole blood, serum samples have the disadvantage of requiring additional laboratory material. Despite these constrains, our study is designed to be a tool for others professionals in clinical practice.

To our knowledge, this is the first population-based study to evaluate a SARS-CoV-2 rapid antibody test in pregnant women. Our data suggest that although immunochromatographic assay for qualitative detection of SARS-CoV-2 IgG/IgM antibodies may be a fast and profitable screening tool, they have a high false positive rate in pregnant women. Thus, ICA antibodies SARS-CoV-2 tests must be verified by other test methods such as chemiluminescent microparticle immunoassays in pregnant patients.

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Ethical approval

This study was approved by the Hospital Clinico Lozano Blesa.

Guarantor

SRM.

Contributorship

MF and MP have analysed the diagnostic test, SRM, PM, CP and DO have collected and analysed the clinical and demographic data of each patient, MF, SRM, RB and DO have written the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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Obstetrics

SARS-CoV-2 congenital infection and pre-eclampsia-like syndrome in dichorionic twins: A case report and review of the literature

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Keywords: COVID-19, multiple pregnancy, pre-eclampsia, SARS-CoV-2, twin pregnancy, vertical transmission

Although the route of transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is mainly respiratory, vertical transmission seems possible.[1] We report the case of a woman with a dichorionic diamniotic twin pregnancy admitted to Hospital Clínico Universitario Lozano Blesa at 38^{+4} weeks of gestation due to severe pre-eclampsia in the context of a SARS-CoV-2 infection (positive nasopharyngeal PCR; Viasure, CerTest Biotec., Zaragoza, Spain) with a probable transplacental transmission of the virus to both twins.

The patient presented with fever and high blood pressure (160/90 mmHg). Evolution of laboratory parameters is shown in Table 1. Given the diagnosis of severe pre-eclampsia, the patient underwent an emergency cesarean section due to breech presentation of both babies, who were immediately handed to pediatricians in a separate room and admitted to the neonatal intensive care unit. Apgar scores were 6/4/8 and 2/3/8 at 1, 5 and 10 min, cord blood pH was 7.18 and 7.22, and birthweight was 2820 and 2845 g. Nasopharyngeal PCR for SARS-CoV-2 was performed in each twin, and both tested positive. Chest X-ray of each twin was normal, and they remained asymptomatic. PCR for SARS-CoV-2 was also performed in two swabs taken deep in the thickness of both placentas, both resulting in positive results (cycle threshold 16.7/19.1 and 16.7/19.1).

Following delivery, the mother required treatment with oxygen and dexamethasone due to mild dyspnea. Chest X-ray showed bilateral interstitial infiltrates. On the following day, blood parameters worsened and were compatible with HELLP syndrome (hemolysis, elevated liver, low platelets) (Table 1). The mother and babies were discharged 9 days after delivery.

Although this is a single case report and the amniotic fluid was not tested, the mother had no contact with the newborns after the cesarean section, making horizonal transmission unlikely.

We found 41 studies reporting infected neonates born to infected mothers. Among 95 cases identified, only 24 were delivered via cesarean section to mothers who tested positive for SARS-CoV-2 and were immediately separated at birth. Two of these reported placentas with a positive test result for SARS-CoV-2 with inflammatory histologic changes that were not found in noninfected controls.[2,3] Moreover, further studies have described the presence of intervillositis in the placentas of infected mothers, which is associated with adverse perinatal

TABLE 1 Analytical and microbiological parameters in the mother and twins

Parameter	Delivery	Day 1	Day 2	Day 5	Day 8
Analytical findings (mother)					
Blood pressure, mmHg	160/90	106/75	111/74	125/76	120/88
Temperature, °C	38.0	36.3	36.0	36.0	36.4
Creatinine, mg/dl	1.19	1.34	0.85	0.77	0.67
AST, U/L	43	80	67	30	33
ALT, U/L	12	24	30	17	22
LDH, U/L	443	670	578	339	398
CRP, mg/L		70.7	37.4	3.0	27.9
Hemoglobin, g/dl	12.0	10.6	10.7	7.1	8.9
Hematocrit, %	34.8	31.5	32.0	20.9	26.1
Leukocytes, per mm^3	5400	19 200	32 400	14 800	8600
Neutrophils, per mm^3 (%)	4400 (80.7)	15 900 (82.6)	27 200 (83.9)	11 400 (76.2)	6200 (72.7)
Lymphocytes, per mm^3 (%)	700 (12.4)	2400 (12.5)	3200 (10.0)	2400 (15.9)	1600 (19.1)
Platelets, per mm^3	59 000	79 000	172 000	184 000	270 000
Ferritin, ng/ml		992	1585	692	547
D-dimer, µg/L		3964	1072	1283	1639
Proteins in urine, g/L	>2		0.16		

Parameter	Delivery	Day 2	Day 3	Day 8	Day 19
Microbiological findings (mother)					
Nasopharyngeal PCR (CT)	Positive (21.3/18.7)			Positive (33.5/34.3)	
Anti-SARS-CoV–2 IgM (index)				Positive (64.7)	Positive
Anti-SARS-CoV–2 IgM (index)		Negative (0.01)		Negative (1.1)	Positive (9.0)
Microbiological findings (first twin/second twin)					
Nasopharyngeal PCR (CT)	Negative/ negative	Negative/positive (27.3/30.6)	Positive (30.2/30.7)	Positive (19.5/19.2)/ positive (17.7/17.4)	
Anti-SARS-CoV–2 IgM (index)		Negative (0.02)/ negative (0.02)			
Anti-SARS-CoV–2 IgG (index)		Negative (0.01)/ negative (0.01)		Negative (0.01)/ negative (0.01)	Positive (6.0)/ positive (6.0)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; CT, cycle threshold; IgG, immunoglobulin G; IgM, immunoglobulin M; LDH, lactate dehydrogenase; PCR, polymerase chain reaction.

outcomes such as severe pre-eclampsia, fetal growth restriction, and miscarriage.[4]

It is important to highlight the maternal clinical features in the present case. All data led to a diagnosis of severe pre-eclampsia. However, there is the possibility of a pre-eclampsia-like syndrome caused by SARS-CoV-2, which has been previously described.[5]

To our knowledge, this is the first case of probable vertical transmission of SARS-CoV-2 to both twins. Moreover, the possibility that the mother could have developed a pre-eclampsia-like syndrome makes this case unique.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

NAC, SRM, and CP wrote the article. MRS and PMA critically reviewed and corrected the article. MFE critically reviewed and corrected the analytical findings. RBR and JBS provided and reviewed

the microbiological parameters. PVF provided, reviewed, and corrected the article.

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Obstetrics

Successful extracorporeal cardiopulmonary resuscitation for a puerpera with amniotic fluid embolism

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Keywords: amniotic fluid embolism, disseminated intravascular coagulation, extracorporeal cardiopulmonary resuscitation, puerpera, thromboelastography

Amniotic fluid embolism (AFE) is one of many high-mortality maternal complications, with approximately 50% of patients with AFE suffering cardiac arrest.[1] Additionally, 89% of deaths in cases of AFE are caused by cardiac arrest, and there are no statistical data for refractory cardiac and respiratory arrest.[2]

A 35-year-old woman with autoimmune diseases presented to our hospital for an elective cesarean delivery. The patient was treated with hydroxychloroquine, methylprednisolone, and clexane before her pregnancy. She had regular prenatal care and her routine prenatal screening tests were normal. Written informed consent was obtained from the patient for publication of this study.

The patient was diagnosed with placenta previa and underwent a cesarean delivery. Ventricular fibrillation was detected during cesarean delivery, which rapidly progressed into pulseless electrical activity. Cardiopulmonary resuscitation (CPR) was immediately started. As this was unsuccessful, we opted for open chest CPR (OC-CPR) due to severe obesity (body mass index 35.2 kg/m^2).

There was no detectable heart rate within 15 min of OC-CPR; therefore, extracorporeal cardiopulmonary resuscitation (ECPR) (BioConsole™ 560; Medtronic) was initiated with an initial flow rate of 3.1 L/min. Due to surgical bleeding and disseminated intravascular coagulation caused by AFE, we did not administer any anticoagulation therapy

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Frequent Placental SARS-CoV-2 in Patients with COVID-19-Associated Hypertensive Disorders of Pregnancy

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Mini Summary

- What does this study add to current knowledge? (80 words max.)–Our study shows that among women diagnosed with COVID-19 during pregnancy, the presence of SARS-CoV-2 in the placenta was more frequent among women suffering from preeclampsia or gestational hypertension. Moreover, we find that more severe hypertensive disorders were associated with higher viral load in placenta. We identify several cases of placental SARS-CoV-2 positivity at delivery (RT-PCR) in women who have tested negative for SARS-CoV-2 after an initial COVID-19 episode.
- What are the main clinical implications? (80 words max.)–SARS-CoV-2 infection could trigger pathophysiological pathways that could favor the development of hypertensive disorders. The presence of viral load in the placenta weeks after the infection, accompanied by hypertensive disorders, could be considered as a form of chronic COVID-19. We should take this information into account when controlling pregnant women after SARS-CoV-2 infection.

Keywords
SARS-CoV-2 · Placenta · Hypertensive disorders of pregnancy · COVID-19 · Viral load

Abstract
Introduction: Studies described an increased frequency of hypertensive disorders of pregnancy (HDP) after a COVID-19 episode. There is limited evidence about SARS-CoV-2 viral load in placenta. This study aimed to investigate the relationship between SARS-CoV-2 viral load in the placenta and clin-

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ical development of HDP after COVID-19 throughout different periods of gestation. ***Methods:*** This is a case-control study in women with and without gestational hypertensive disorders after SARS-CoV-2 infection diagnosed by RT-PCR during pregnancy. Patients were matched by gestational age at the moment of COVID-19 diagnosis. We performed an analysis of SARS-CoV-2 RNA levels in placenta. ***Results:*** A total of 28 women were enrolled. Sixteen patients were diagnosed with COVID-19 during the third trimester and the remaining 12 patients in the other trimesters. Ten placentas (35.7%) were positive for SARS-CoV-2, 9 of them (9/14, 64.3%) belonged to the HDP group versus 1 (1/14, 7.2%) in the control group ($p = 0.009$). Those cases with the highest loads of viral RNA developed severe preeclampsia (PE). ***Conclusion:*** Among women diagnosed with COVID-19 during pregnancy, the presence of SARS-CoV-2 in the placenta was more frequent among women suffering from PE or gestational hypertension. Furthermore, the most severe cases of HDP were associated with high placental viral load, not necessarily associated with a positive nasopharyngeal RT-PCR at delivery. Our data suggest that SARS-CoV-2 infection during pregnancy could trigger gestational hypertensive disorders through persistent placental infection and resulting placental damage.

Introduction

Several international studies have recently suggested an increase in preeclampsia (PE) and other hypertensive disorders of pregnancy (HDP) associated with SARS-CoV-2 infection during pregnancy [1–4] with classic signs and symptoms related to PE, such as hypertension, proteinuria, thrombocytopenia, and elevated liver enzymes [5, 6]. PE is a pregnancy-specific multisystem disorder characterized by endothelial dysfunction that leads to the release of soluble factors into de maternal circulation responsible for the multi-organ injury [7]. These so-called PE-like syndromes are in some way clinical manifestations of maternal endothelial dysfunction.

The clinical manifestations of the SARS-CoV-2 infection support a key role for endothelial dysfunction in the pathobiology of this condition. SARS-CoV-2 infection induces an endotheliitis in different organs as a direct consequence of viral infection and the host inflammatory response [8, 9]. Angiotensin-converting enzyme 2 is the major cellular-entry receptor for SARS-CoV-2 virus [10]. Angiotensin-converting enzyme 2 is present prominently in the alveoli, but also in endothelial cells, which can be directly infected by the virus [11]. Besides, cytokine release syndrome can also drive endothelial damage independently [12]. Consequently, a distinctive feature of SARS-CoV-2 infection is vascular harm, with severe endothelial injury, widespread thrombosis, and microangiopathy, in response to endothelial damage. Therefore, endothelial dysfunction seems to be the pathophysiological substrate for severe COVID-19 complications.

It is at present unknown how COVID-19 is associated with increased frequency of HDP (and especially PE) and whether similar mechanisms operate. COVID-19-associated PE-like disease may be an indirect result of maternal disease or more directly related to the presence of SARS-CoV-2 in the placenta. While initial studies had suggested that SARS-CoV-2 invades the placenta in some cases [13–15], posterior data suggest that it is not very common [16–19]. Consistent with a low frequent presence of SARS-CoV-2 in the placenta [20], vertical transmission is rather infrequent [21]; a systematic review reported a rate of 3.2% vertical transmission in mothers who tested positive for SARS-CoV-2 (diagnosed by RT-PCR) during the third trimester of pregnancy [22]. A recent study of placentas taken from COVID-19-positive mothers [23] describes a low-level presence of RNA in about half of placentas. No substantial histopathological, maternal, or neonatal outcome features were detected associated with this low level of viral RNA. However, a high SARS-CoV-2 RNA level was detected in only one patient, who exhibited severe placental injury in the form of extensive fibrin deposition, necrosis of the syncytiotrophoblast layer of the villi and apoptosis. This observation fits the hypothesis that only limited placental pathology is associated with COVID-19, except for cases with very high SARS-CoV-2 RNA levels, the latter indicative of high viral load.

Considering the increase in hypertensive disorders in COVID-19 patients, this study aimed to investigate the relationship between SARS-CoV-2 viral load in placental tissue at birth and the development of PE or gestational hypertension after COVID-19 throughout different periods of gestation. The clinical history of 3 cases of PE, with positive placental RT-PCR tests for SARS-CoV-2 during each of the 3 semesters of pregnancy, is described in detail as an example.

Materials and Methods

We conducted a case-control study in women diagnosed with COVID-19 during pregnancy with and without gestational hypertensive disorders. We performed an analysis of SARS-CoV-2 RNA levels in placental tissue and SARS-CoV-2 antibodies in the um-

bilical cord. Patients were recruited at the moment of delivery between May 2020 and February 2021 at a tertiary university center. Cases were defined as women who tested positive for SARS-CoV-2 during pregnancy and were diagnosed with gestational hypertensive disorders. Controls, defined as SARS-CoV-2-positive women without HDP during pregnancy, were paired by gestational age at the moment of SARS-CoV-2 diagnosis.

SARS-CoV-2 infection was diagnosed based on the positive RT-PCR test for SARS-CoV-2 from nasopharyngeal swabs. RT-PCR test kits from different companies were used: Viasure (CerTest Biotec, Zaragoza, Spain), M2000 SARS-CoV-2 Assay (Abbott RealTime SARS-CoV-2 Assay, Abbott Molecular, Abbott Park, IL, USA), TaqPath COVID-19 (Thermo Fisher Scientific, USA-FDA) and Alinity SARS-CoV-2 (Abbott Alinity, Abbott Molecular, Abbott Park, IL, USA). Information on these test kits are listed in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000520179). As suggested by the manufacturer for nasopharyngeal specimens, cycle threshold (CT) values below 37 were taken as positive. Besides, all women who were hospitalized for delivery were screened for SARS-CoV-2 infection using RT-PCR on nasopharyngeal swabs shortly before giving birth. However, in the case of pregnant women who had already overcome the infection, RT-PCR was only performed around delivery if the latest negative RT-PCR test was more than 3 months ago. As a result, 4 patients (3, 4, 15, and 16) were not tested for SARS-CoV-2 infection at the time of delivery. Prophylactic treatment with low molecular weight heparin for 14 days was applied to COVID-19 women diagnosed by RT-PCR [24]. COVID-19 has been divided into 3 types [25]: asymptomatic infection refers no clinical symptoms or signs; mild infection refers to symptoms such as fever, cough, headache, anosmia and asthenia; and severe infection refers dyspnea, hypoxemia accompanied by chest imaging compatible with pneumonia and respiratory infection.

Placental tissue samples were taken at the moment of delivery, taking villous tissue while carefully trying to avoid the overlying maternal tissue. Placental samples, approximately 1 cm^3 in size, were placed into tubes containing 1 mL of preservative solution (RNA*later* Fisher Scientific) at 4°C. After 24 h, excess RNA*later* was removed, and the samples were stored at −80°C. Tissue was homogenized; RNA was extracted, treated with DNase I, repurified and resuspended as previously described [26]. Concentrations were measured using Nanodrop, 100 ng of RNA was used in downstream RT-PCR analysis. RT-PCR for SARS-CoV-2 in placenta tissue samples was performed using the TaqPath COVID-19 CE-IVD RT-PCR kit (Catalog number A48067; Life Technologies Europe, Bleiswijk, The Netherlands) on a QuantStudio5 (Applied Biosystems; Thermo Fisher Scientific) apparatus. The limit of detection of this kit is listed as 10 genomic copy equivalents (GCEs). As this assay targets N, ORF1aboratory and S genes, only samples with 3 positive targets were considered as SARS-CoV-2 positive. SARS-CoV-2 tissue load in the placenta is referred to in the manuscript as viral load and is expressed as CT values of RT-PCR [27].

We collected umbilical cord plasma in EDTA and samples were centrifuged. Serological assays were determined on the same day by chemiluminescent microparticle immunoassays for quantitative detection of SARS-CoV-2 IgG. The SARS-CoV-2 IgG assay is designed to detect IgG antibodies to the nucleocapsid protein of SARS-CoV-2 (IgG; SARS-CoV-2 IgG Assay, Abbott Laboratories Ireland, Dublin, Ireland) and IgM + IgA antibodies (IgM + IgA; COVID-19 VIRCLIA IgM + IgA, Vircell Microbiologists, Granada, Spain).

Gestational hypertensive disorders were divided into 4 categories, defined according to the criteria proposed by American College of Obstetrics and Gynecologists [28]: PE, chronic hypertension, chronic hypertension with superimposed PE and gestational hypertension. First trimester risk of early-onset PE was retrospectively calculated according to maternal characteristics, obstetric history, maternal blood pressure, maternal serum pregnancy-associated plasma protein-A, and uterine artery pulsatility index, using SsdwLab6 version 6.1 package (SBP Soft 2007 S.L.) [29].

Clinical characteristics, laboratory results, and maternal and neonatal outcomes were collected from medical records. All patients provided written informed consent. The study was approved by the Research Ethics Committee of the Community of Aragon (C.I. PI21/155 and COL21/000) and all patients provided written informed consent.

Statistical analysis was performed using SPSS 22.0. Categorical variables were presented as frequencies or percentages. Continuous variables were presented using mean ± standard deviation (SD), median, or range. For continuous variables, Shapiro-Wilk tests of normality were used to evaluate the distributions. Data were analyzed using the Student *t* test or Mann-Whitney U test. Statistical significance was considered $p < 0.05$.

Results

Fourteen women in the case group (COVID-19 positive and diagnosis of HDP) and 14 women in the control group, matched for gestational age at the time of COVID-19 diagnosis, were included. Out of those 14 cases, 9 cases (64.3%) had PE and 5 cases (35.7%) had gestational hypertension. Demographic and clinical data, as well as perinatal and neonatal outcomes, are shown in Table 1. There were no significant differences in maternal age, maternal weight, risk of PE at 1º trimester, nulliparity rate, and diabetes mellitus between the 2 groups. The vast majority of patients, 8 pairs, represent pregnant women who were diagnosed with COVID-19 during the third trimester, whereas 5 pairs in the second trimester and only 1 pair in the first trimester. The mean gestational age at delivery was 268.1 days in the case group and 278.9 days in the control group ($p = 0.005$). Women with HPD also exhibited lower birth weight than those in the control group (2,852.5 g SD 467.0 vs. 3,282.9 g SD: 435.9; $p = 0.005$). There were no significant differences for small for gestational age, umbilical arteria ph <7.1. Induced labor and caesarean were significantly more frequent in the HDP group than in controls ($p = 0.003$ and $p = 0.015$, respectively).

The chronology of diagnosis of HDP and SARS-CoV-2 infection of each patient is summarized in Table 2. There was no significant difference in the severity of initial SARS-CoV-2 symptoms between the groups ($p = 0.341$).

Table 1. Demographic data and obstetrical and SARS-CoV-2 infection characteristics

	Case group (n = 14)	Control group (n = 14)	p value
Demographic maternal data			
Maternal age (SD), years	31.93 (6.6)	30.36 (5.8)	0.506
Maternal weight (SD), kg	69.32 (10.5)	68.57 (13.2)	0.868
Caucasian, %	9 (64.3)	4 (28.6)	0.063
1º trimester high risk for PE, %	5 (35.7)	3 (21.4)	0.194
Diabetes mellitus, %	0 (0)	1 (7.14)	0.317
Nulliparous, %	4 (28.6)	7 (50)	0.317
Maternal and neonatal outcome at delivery			
Gestational age at birth mean (SD), days	268.1 (10.8)	278.9 (7.8)	0.005
Birth weight, mean (SD), kg	2,852.5 (467.0)	3,282.9 (435.9)	0.018
SGA (<10th percentile), %	4 (28.6)	1 (7.2)	0.146
Induced labor, %	12 (85.1)	4 (28.6)	0.003
Caesarean delivery, %	5 (35.7)	0 (0)	0.015
pH <7.1, %	2 (14.3)	0 (0)	0.541
Pre-term birth (<37 wk gestation), %	3 (21.4)	0 (0)	0.072
Description of SARS-CoV-2			
Trimester of SARS-CoV-2 infection			
1º, %	1 (7.2)	1 (7.2)	
2º, %	5 (35.7)	5 (35.7)	
3º, %	8 (57.1)	8 (57.1)	1
COVID-19 symptoms			
No	6 (42.9)	7 (50)	
Mild	6 (42.9)	7 (50)	
Severe	2 (14.2)	0 (0)	0.341
Interval from diagnosis of SARS-CoV-2 to delivery (SD), d	61.86 (54.79)	72.64 (57.7)	0.616
Positive SARS-CoV-2 RT-PCR placenta tissue (CT <37), %	9 (64.3)	1 (7.2)	0.009
Neonatal IgG,* %	6 (42.9)	7 (50)	0.688

SD, standard deviation; CT, cycle threshold value RT-PCR; SGA, small for gestational age; PE, preeclampsia.
* Analyzed 24 of 28.

Thirteen had no symptoms, 13 patients had mild and only 2 patients had severe SARS-CoV-2 infection (Table 2). No patient required admission to the intensive care unit.

We analyzed placentas delivered by 28 women who had tested positive for SARS-CoV-2 at some point during pregnancy using a sensitive RT-PCR assay. Ten placentas (35.7%) tested positive for SARS-CoV-2 RNA at birth. In the case group, we found 9 positive placentas (9/14, 64.3%) while in the control group only 1 placenta (1/14, 7.2%) was positive (p = 0.009) (shown in Fig. 1). Moreover, 7 of 9 placentas (77.8%) belong to women diagnosed with PE. Focusing on positive placentas, we have 3 cases with high loads of viral RNA (CTs ≤28) (see below), and the remaining 7 cases with low loads of viral RNA (median CT of 30.6; interquartile range 28.7–33.9) (Table 2).

While only 6 pairs of pregnancies with positive SARS-CoV-2 tests during the first or the second trimester (GA ≤27 weeks) were analyzed (Table 2), several remarkable results deserve mentioning. First, out of the 6 cases with HDP, 6 out of 6 had a positive placental SARS-CoV-2 test at birth. In contrast, among the corresponding 6 control cases without HDP, only 1 tested positive for placental SARS-CoV-2 (Table 2). It is possible therefore that the continued presence of SARS-CoV-2 in the placenta promotes HDP. Moreover, out of 10 pregnancies with these early SARS-CoV-2 infections for which data are available, only 1 mother remained positive at delivery (Nº 6), while 7 placentas tested positive. This latter data suggest that infection with SARS-CoV-2 during early pregnancy may commonly result in persisting placental presence of SARS-CoV-2.

Finally, antibody quantification was performed for 87.5% neonates with umbilical cord blood samples. Thirteen infants tested positive for IgG on the first 10 days of life, and none tested positive for IgM + IgA. There was no significant difference between the groups in the number

Fetal Diagn Ther
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Table 2. Chronology of diagnosis of HDP and SARS-CoV-2 infection

	N	Diagnosis SARS-CoV-2		Diagnosis HDP			Delivery					Neonatal antibodies SARS-CoV-2	
	°	SYN	GA	HDP	GA	interval SARS-HDP (w + d)	GA	interval DEL – SARS (w + d)	RT-PCR result naso-pharyngeal*	RT-PCR result placenta tissue	CT RT-PCR placenta tissue	IgG	IgM + IgA
Case	1	No	8 + 0	S-PE	35 + 0	27 + 0	35 + 2	27 + 5	Negative	Positive	28.7	Negative	N/A
Control	2	Mild	9 + 3				38 + 5	29 + 2	Negative	Negative	≥40	Negative	N/A
Case	3	No	21 + 3	S-PE	36 + 3	15 + 0	36 + 5	15 + 2	N/A	Positive	33.5	Negative	Negative
Control	4	Mild	20 + 4				39 + 5	19 + 1	N/A	Negative	≥40	Positive	Negative
Case	5	Mild	24 + 4	GHT	37 + 1	12 + 3	39 + 5	15 + 4	Negative	Positive	32.4	Positive	Negative
Control	6	Mild	23 + 4				40 + 2	16 + 4	Positive	Negative	≥40	Positive	Negative
Case	7	Mild	24 + 6	PE	36 + 4	11 + 5	37 + 1	12 + 2	Negative	Positive	31.7	Positive	Negative
Control	8	Mild	25 + 1				39 + 2	14 + 1	Negative	Positive	33.9	Positive	Negative
Case	9	Mild	25 + 6	GHT	38 + 0	12 + 1	40 + 1	14 + 2	Negative	Positive	33.1	Positive	Negative
Control	10	Mild	25 + 5				41 + 2	15 + 3	Negative	Negative	≥40	Positive	Negative
Case	11	Mild	26 + 6	S-PE	35 + 0	8 + 1	36 + 5	9 + 6	Negative	Positive	23.1	Positive	Negative
Control	12	Mild	27 + 4				40 + 2	12 + 4	Negative	Negative	≥40	Positive	Negative
Case	13	Mild	29 + 4	GHT	35 + 3	5 + 6	39 + 6	13 + 2	Negative	Negative	≥40	N/A	N/A
Control	14	No	30 + 0				39 + 1	9 + 1	Negative	Negative	≥40	N/A	N/A
Case	15	Severe	30 + 0	PE	33 + 3	3 + 3	37 + 2	7 + 2	N/A	Negative	≥40	Positive	Negative
Control	16	No	29 + 4				41 + 0	11 + 3	N/A	Negative	≥40	Positive	Negative
Case	17	No	31 + 0	GHT	36 + 5	5 + 5	40 + 4	10 + 4	Negative	Negative	≥40	N	N/A
Control	18	No	31 + 2				39 + 6	8 + 4	Negative	Negative	39.5	N	N/A
Case	19	No	36 + 2	PE	37 + 5	1 + 3	38 + 0	1 + 5	Positive	Positive	35.2	N	N/A
Control	20	No	36 + 4				40 + 2	3 + 4	Positive	Negative	≥40	N	N/A
Case	21	No	37 + 6	PE	37 + 6	0 + 0	38 + 1	0 + 2	Negative	Positive	33.9	N	N/A
Control	22	No	37 + 5				37 + 5	0 + 0	Positive	Negative	≥40	Positive	Negative
Case	23	Severe	38 + 3	S-PE	38 + 3	0 + 0	38 + 4	0 + 1	Positive	Positive	15.1	Positive	Negative
Control	24	Mild	38 + 3				38 + 3	0 + 0	Positive	Negative	≥40	N	N/A
Case	25	No	38 + 5	PE	38 + 5	0 + 0	38 + 6	0 + 1	Positive	Negative	≥40	Negative	N/A
Control	26	No	38 + 0				40 + 4	2 + 4	Positive	Negative	≥40	Negative	N/A
Case	27	No	39 + 0	GHT	39 + 0	0 + 0	39 + 2	0 + 2	Positive	Negative	≥40	N/A	N/A
Control	28	No	38 + 5				41 + 2	2 + 5	Negative	Negative	≥40	N/A	N/A

Syn, symptoms of SARS-COV-2, infection; GA, gestational age (week + days); HDP, hypertensive disorders of pregnancy; GHT, gestational hypertension; PE, preeclampsia; S-PE, severe preeclampsia; DELIV-PE (days):interval from diagnosis of COVID-19 to HDP, diagnosis, days; DELIV-SARS (days), interval from diagnosis of COVID-19 to delivery, days; CT, cycle threshold value RT-PCR; R, result; P, positive; N, negative; N/A, not available. The squares marked in gray color correspond to controls without HDP. * RT-PCR, was only performed if the latest negative RT-PCR, test was more than 3 months ago.

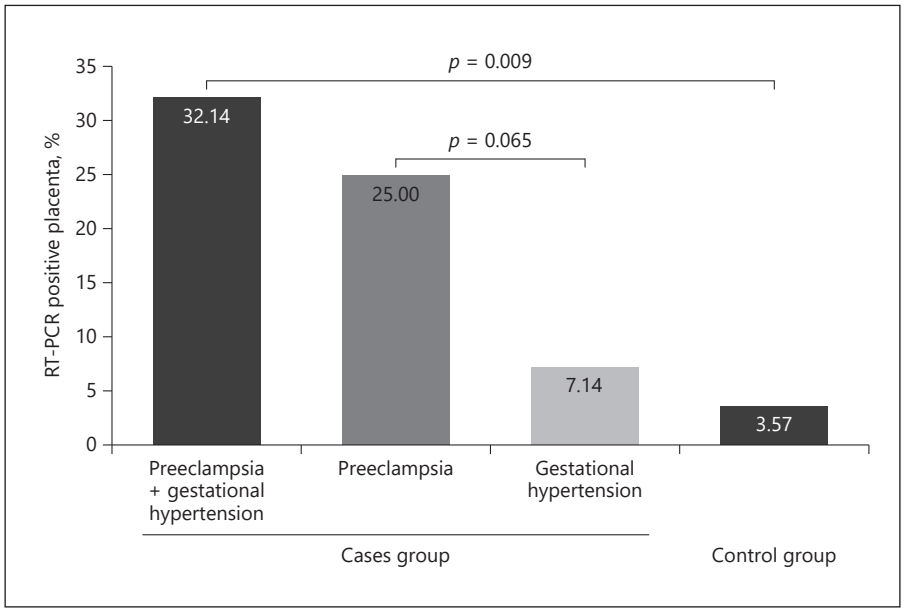

Fig. 1. % RT-PCR-positive placenta: case group versus control group. N total = 28. case group = 14 and control group = 14. Ten placentas (35.7% = 3.57% + 32.14%) tested positive for SARS-CoV-2 RNA. Nine placentas were positive in the case group (9/28, 32.14%), while in the control group, only 1 placenta was positive (1/28, 3.57%). Seven (7/28, 25%) belong to women diagnosed with PE versus 2 of 9 belong to women diagnosed with gestational hypertension (2/28, 7.14%). PE, preeclampsia; LMWH, low molecular weight heparin; IUGR, intrauterine growth restriction; CT, cycle threshold.

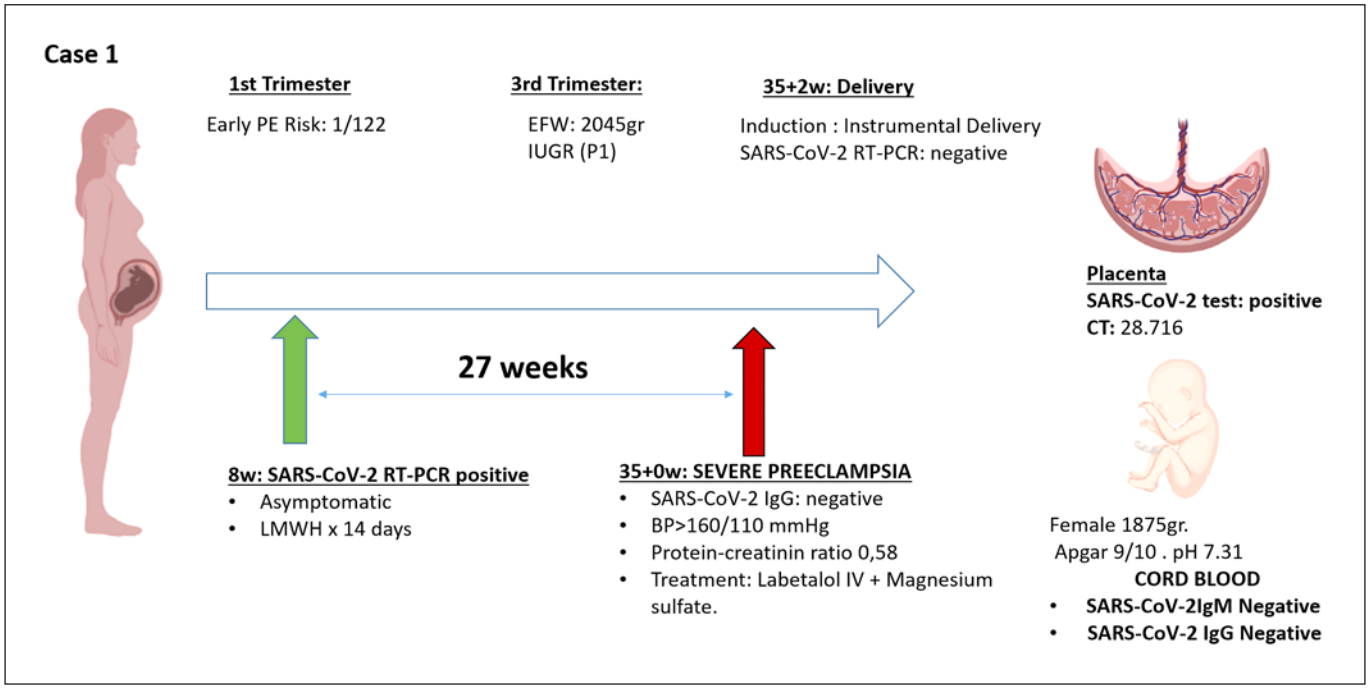

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Fig. 2. Time line of case 1. TPAL, term, pre-term, abortion, life; LMWH, low molecular weight heparin; PAPP-A, pregnancy-associated plasma protein-A; MoM, multiple of median; BP, mean arterial blood pressure; UtA PI, uterine artery resistance pulsatility index; w, weeks; EFW, estimated fetal weight; PI UA, umbilical artery pulsatility index; PI MCA: fetal middle cerebral artery pulsatility index; IUGR, intrauterine growth restriction; BP, blood pressure; CT, cycle threshold.

of neonates that tested positive for SARS-CoV-2 IgG (p = 0.688).

Among the placentas that tested positive, 3 cases with high loads of viral RNA (CTs = 15; 23; 28, respectively) represented cases of severe PE (Table 2). Case number 1 (shown in Fig. 2) was a woman with no medical history of interest. The estimated risk of early-onset PE in the first trimester was 1/122. She presented positive SARS-CoV-2

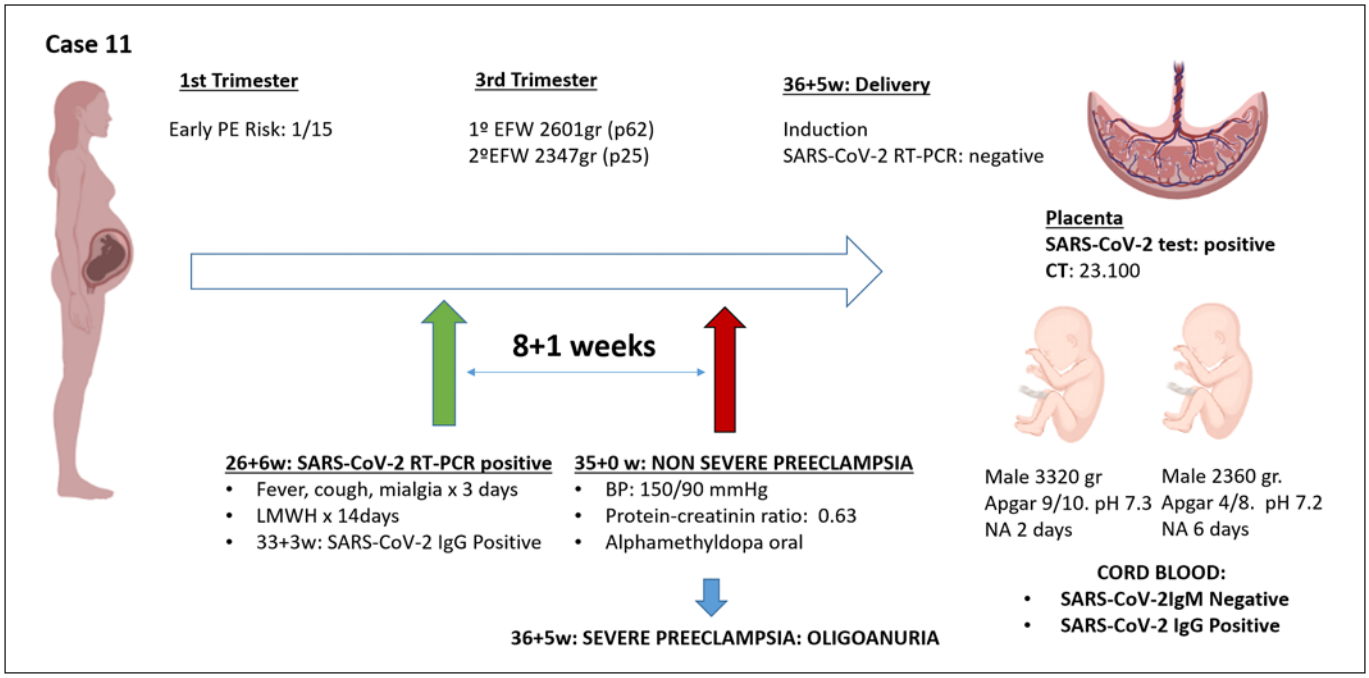

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Fig. 3. Time line of case 11. TPAL, term, pre-term, abortion, life; LMWH, low molecular weight heparin; PAPP-A, pregnancy-associated plasma protein-A; MoM, multiple of median; BP, mean arterial blood pressure; UtA PI, uterine artery resistance pulsatility index; w, weeks; EFW, estimated fetal weight; PI UA, umbilical artery pulsatility index; PI MCA, fetal middle cerebral artery pulsatility index; IUGR, intrauterine growth restriction; BP, blood pressure; NA, neonatal admission; PE, preeclampsia; CT, cycle threshold.

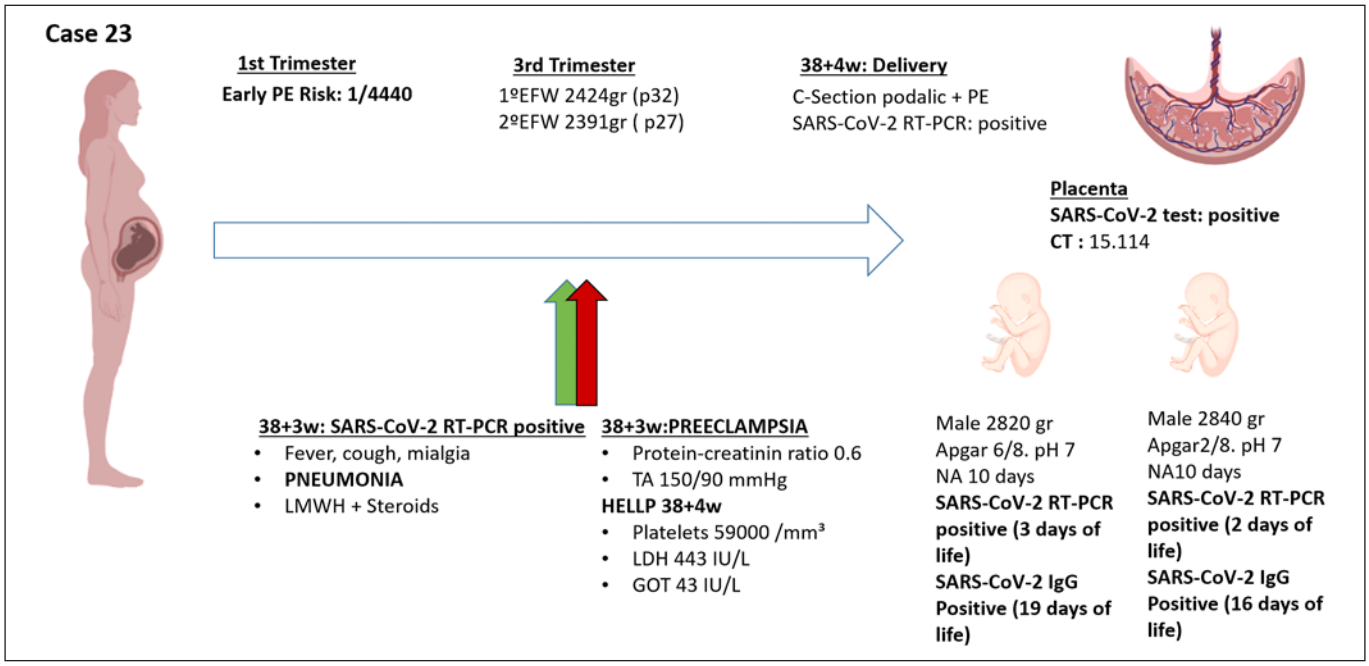

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Fig. 4. Time line of case 23. TPAL, term, pre-term, abortion, life; LMWH, low molecular weight heparin; PAPP-A, pregnancy-associated plasma protein-A; MoM, multiple of median; BP, mean arterial blood pressure; UtA PI, uterine artery resistance pulsatility index; w, weeks; EFW, estimated fetal weight; PI UA, umbilical artery pulsatility index; PI MCA, fetal middle cerebral artery pulsatility index; IUGR, Intrauterine growth restriction; BP, blood pressure; NA, neonatal admission; PE, preeclampsia; CT, cycle threshold.

RT-PCR (CT: 18.7) at week 8 of gestation and infection courses asymptomatic. Two weeks later, she tested negative for SARS-CoV-2 by RT-PCR. At 35 week of gestation, she debuted with severe PE, which required admission to the hospital and treatment with intravenous labetalol and magnesium sulfate. RT-PCR for SARS-CoV-2 infection was performed at the time of admission, with a negative result. Termination of pregnancy was in week 35 + 2 for this reason. A female of 1,875 g, APGAR test was 9 and 10 at 1 and 5 min, respectively, and pH 7.31 without antibodies against SARS-CoV-2 was born. Placental analysis for SARS-CoV-2 was positive, with a high viral load by RT-PCR.

Case number 11 (shown in Fig. 3) was a nulliparous woman with a spontaneous monochorionic diamniotic twin. The estimated risk of early-onset PE in the first trimester was 1/15. Pregnancy of normal course until week 26 + 5, when she debuted with fever, cough, and myalgias. RT-PCR results for SARS-CoV-2 was positive (CT: 5.9). At 34 + 0 she tested negative for SARS-CoV-2 by using RT-PCR. In week 35, the patient presented nonsevere PE, well controlled with oral treatment with alfamethyldopa. At 36 + 5 weeks was decided an elective delivery due to the onset of severe PE, ending in an intrapartum caesarean section. The first male twin was born: 3,320 g, the APGAR test was 9 and 10 at 1 and 5 min, respectively, and was admitted for observation for 2 days. Second twin, male, 2,360 g, with the APGAR test of 4 and 8 at 1 and 5 min, and he was admitted for observation for 6 days. Cord blood serology was positive for IgG and negative for IgM. The placenta was clearly positive for SARS-CoV 19 by using RT-PCR.

Case 23 (shown in Fig. 4) was a woman with a bi-chorionic biamniotic twin pregnancy. The estimated risk of early-onset PE in the first trimester was 1/4,440. Gestation of normal course until onset of fever, cough, and malaise, with positive result in RT-PCR SARS-CoV-2 (CT: 20.2) at 38 + 3 weeks of gestation. The patient presented blood pressure around 150/90 mm Hg, with positive creatinine protein ratio and pneumonia on chest X-ray that required treatment with low molecular weight heparin, steroids, and oxygen therapy. Laboratory tests were performed in week 38 + 4, HELLP syndrome was detected (59,000 mm^3 platelets, LDH 443 U/L and GOT 43 U/L), and it was decided to elective delivery at that time by caesarean section due to HELLP and breech presentation. The birth weight of the first male twin was 2,820 g, with the APGAR test 6 and 8 at 1 and 5 min, respectively. The birth weight of the second male twin was 2,840 g, the APGAR test was 2 and 8 at 1 and 5 min, respectively. Both were admitted to the neonatal unit and the mother had no contact with the newborns after the caesarean section. RT-PCR SARS-CoV-2 was performed on both, positive at 3 and 2 days of life, respectively, as well as SARS-CoV-2 serologies, which were positive at 19 and 16 days of life, respectively. The analysis of the placenta yielded a clear positive result for SARS-CoV-2 (CT 15.1) [6].

Discussion/Conclusion

Placental histopathology at term in women suffering from COVID-19 has been examined in relation to disease symptoms such as systemic inflammatory responses, hypercoagulation, and intervillous thrombi [30, 31]. The presence of SARS-CoV-2 in the placenta has been studied in the context of horizontal transmission of the virus [22] with an early study reporting 7.7% positivity (2/26). However, only a very limited number of studies report data that allow a direct comparison between the placental presence of SARS-CoV-2 and HDP. An early prospective cohort study [16] describes the inability to detect SARS-CoV-2 in the placenta by RNA in situ hybridization in 44 women with SARS-CoV-2 diagnosis in the third trimester. Afterward, since the first report describing the detection of SARS-CoV-2 fetal membrane samples [32], similar reports have followed. Hosier et al. [33] mention high viral load in the placenta (CT <16) of a single woman with severe PE and SARS-CoV-2 infection during the late second trimester of pregnancy. In a posterior study, a rate of 47% SARS-CoV-2-positive placenta tissue was reported in 21 women who were diagnosed (using RT-PCR) between 35 and 40 weeks of gestation [23]. In this latter study, a woman who exhibited severe placental damage delivered a baby with neurological manifestations. Based on these combined 2 cases, the possibility was raised that the severity of SARS-CoV-2-mediated placental pathology might be directly related to placental SARS-CoV-2 viral load.

Following up on these studies, we further assessed the relationship between the SARS-CoV-2 viral load in placental tissue and the development of HDP in 14 HDP cases and paired controls, all diagnosed with COVID-19. Out of 28 samples tested in our study, we identified 10 placental tissue samples that tested positive for SARS-CoV-2 (35.7% of total), 9 of them belonging to the HDP group. We observed that, among patients with SARS-CoV-2 infection during gestation, the frequency of a positive placental SARS-CoV-2 RT-PCR test was much high-

er in the HDP group than the non-HDP group. Furthermore, we detected that the percentage of cases with a positive placental SARS-CoV-2 RT-PCR test trend differently between PE and AHT ($p = 0.065$). In fact, the 3 most severe cases of PE (cases Nº 1, 11 and 23), showed higher placental viral load than all the others. Moreover, each of the 3 tested positive for SARS-CoV-2 in a different trimester of gestation. It has been argued that localization of SARS-CoV-2 to fetal cells such as the syncytiotrophoblast as opposed to maternal uterine cells requires detection by immunohistochemistry or RNA in situ hybridization [16]. We only applied RT-PCR and can therefore not exclude that the placenta samples we have analyzed do contain maternal tissue. Considering that maternal tissue was avoided when taking samples, and for the sake of simplicity, we interpret positive placental RT-PCR results as representative of viral load in villous placenta. This might indicate that in women susceptible to HDP, the presence of placental SARS-CoV-2 may contribute to the severity of the disorder. We suggest that a relationship between a higher viral load in placenta tissue and the severity of the hypertensive disorder deserves consideration.

Placental characteristics described in SARS-CoV-2 infection include maternal vascular perfusion and inflammation [30, 34]. It has been postulated that the detrimental health outcomes associated with HDP are the result of generalized endothelial and vascular dysfunction [35]. Similarly, a consequence of SARS-CoV-2 infection is endothelial injury in different organs resulting directly or indirectly from the host inflammatory response [9]. Alternatively [36], it has been proposed that direct infection of syncytioprophoblast cells in the placenta by SARS-CoV-2 may cause placental dysfunction and pregnancy complications [37]. The pathophysiological mechanisms that connect placental SARS-CoV-2 with HDP remain to be established.

The main limitation of our study is the small sample size. Further studies with a large number of patients are required to confirm the association between high SARS-CoV-2 viral load in placenta tissue and the development of HDP. Moreover, our hospital's protocols changed as the pandemic progressed. Therefore, different methodologies have been used for viral RNA detection. As not all tests proved to be equally sensitive and specific, the use of distinct tests may have caused a discrepancy with studies published previously [13, 16, 22]. In addition, we do not have sequencing data from nasopharyngeal maternal swabs available, but all pregnant women were diagnosed before the alpha (B.1.1.7 UK) variant and delta (B.1.617.2 India) were routinely detected in our area. A final limitation to address is the fact that for each placenta, we tested only a single sample. As SARS-CoV-2 is not necessarily homogenously present (or absent) throughout the placenta, the identification of samples as positive or negative should be interpreted in this context.

Among the strengths of the study, we highlight the inclusion of women who tested positive for SARS-CoV-2 in each of the 3 trimesters of pregnancy. We demonstrate a higher viral load in placental tissue in the case group than in the control group and a higher frequency of infected placentas. Besides, as a SARS-CoV-2 RT-PCR test carried out at the time of birth showed all mothers tested negative, we could rule out the possibility of acute maternal infection during delivery. Some cases we describe with high placenta SARS-CoV-2 levels, specifically patient 1 and patient 11, had negative RT-PCR results at the time of delivery. This data shows that placenta infection may persist in the absence of maternal infection. In the limited of cases we describe, this persistent viral presence is associated with the development of adverse perinatal outcomes.

Previous viral epidemics have confirmed that pregnant women are at increased risk for severe virus infection [38–41]. SARS-CoV-2 is not an exception, as pregnant patients with COVID-19 are at increased risk for severe illness [42] and adverse pregnancy outcomes [8]. We showed that the frequency of persistent SARS-CoV-2 in the placenta is increased among mothers with HDP, and that 6 out of 6 mothers who tested positive for SARS-CoV-2 before week 27 of gestation and also showed persistent placental infection at birth developed HDP. Combined, these results suggest that chronic COVID might increase the risk of PE. We therefore suggest that healthcare professionals should monitor pregnant women after SARS-CoV-2 infections even more closely, since a possible placental infection cannot be tested until delivery.

In summary, this study assesses the relationship between SARS-CoV-2 viral load in placental tissue and the maternal risk of developing gestational hypertensive disorders. We find that the presence of SARS-CoV-2 (although we do not formally prove viral infection) was more frequent in the placentas of those in the HDP group than in the placentas of COVID-19 mothers without HDP. We detect placental RNA representative of SARS-CoV-2 infection at high levels in a limited number of PE cases, and at lower levels in about 25% of placentas from COVID-19 positive mothers. There are different ways in which the infant of a RT-PCR-positive mother could be affected by SARS-CoV-2: maternal inflammation and endothelial damage may be transmitted to the placenta in

the form of cytokines which might modulate the fetal immune system [43]. Alternatively, SARS-CoV-2 infection of the placenta may directly influence the fetal environment. The presence of placental viral RNA needs to be investigated in a much larger cohort to clarify if and in what frequency if SARS-CoV-2 infection during pregnancy does trigger gestational hypertensive disorders through placenta-related mechanisms. Our work contributes to the understanding of how SARS-CoV-2 virus affects pregnancy, which includes the possibility that placental infection contributes to pregnancy complications. Better and more detailed understanding of the placental disease process may aid or could be essential for appropriate monitoring during pregnancy and providing appropriate postnatal care.

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Statement of Ethics

The study was approved by the Research Ethics Committee of the Community of Aragon (C.I. PI21/155 and COL21/000). Written informed consent was obtained from participants.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.F., J.S., and D.O. were responsible for the design of the study and the writing of the manuscript. M.F., M.P., P.C., S.R.M., C.P., J.S., and D.O. were responsible for the analysis and interpretation of the data. PC and SRM contributed to the writing of the manuscript. M.F., M.P., M.S., A.M.M., J.S., and R.B. were responsible for the laboratory analysis. P.C., S.R.M., C.P., and D.O. were responsible for the collection of clinical data of the patients. All authors reviewed and approved the final version of the manuscript.

Data Availability Statement

The data described in this article are openly available in medRxiv https://www.medrxiv.org/content/10.1101/2021.09.07.21261607v1?rss=1#disqus_thread.

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	Nº	Rt-PCR result by nasopharyngeal swabs	CT	Technology
CASE	1	Positive	20.2	M2000 (Abbott)
CONTROL	2	Positive	36.6	M2000 (Abbott)
CASE	3	Positive	18.2/20.1	Viasure (CerTest)
CONTROL	4	Positive	24.14/24.94/24.41	TaqPath (Thermo Fisher)
CASE	5	Positive	30.4/31.4	Viasure (CerTest)
CONTROL	6	Positive	18.3/19.7	Viasure (CerTest)
CASE	7	Positive	33.3/34.3	Viasure (CerTest)
CONTROL	8	Positive	11.3	M2000 (Abbott)
CASE	9	Positive	31.7/33.3	Viasure (CerTest)
CONTROL	10	Positive	15.49/14.67/14.57	TaqPath (Thermo Fisher)
CASE	11	Positive	5.9	M2000 (Abbott)
CONTROL	12	Positive	17.3/18.6	Viasure (CerTest)
CASE	13	Positive	Antigen test	PanBio (Abbott)
CONTROL	14	Positive	20.78	Alinity (Abbott)
CASE	15	Positive	14.87	M2000 (Abbott)
CONTROL	16	Positive	31.2	Alinity (Abbott)
CASE	17	Positive	15.89	M2000 (Abbott)
CONTROL	18	Positive	21.24	Alinity (Abbott)
CASE	19	Positive	34.3/33.8	Viasure (CerTest)
CONTROL	20	Positive	22.63	Alinity (Abbott)
CASE	21	Positive	0/36.6	Viasure (CerTest)
CONTROL	22	Positive	0/37.3	Viasure (CerTest)
CASE	23	Positive	21.3/18.7	Viasure (CerTest)
CONTROL	24	Positive	30.2	Alinity (Abbott)
CASE	25	Positive	0/30.5	Viasure (CerTest)
CONTROL	26	Positive	18.35	Alinity (Abbott)
CASE	27	Positive	36.9	M2000 (Abbott)
CONTROL	28	Positive	31.5/31.5/31.9	TaqPath (Thermo Fisher)
Supplemental 1. Diagnosis of SARS-CoV-2 by nasopharyngeal swabs				
CT: cycle threshold value; RT-PCR;Viasure (CerTest Biotec. Zaragoza. Spain) limit of detection 40 copies/mL and target sequence: ORF1ab and N genes; M2000 SARS-CoV-2 Assay (Abbott RealTime SARS-CoV-2 Assay. Abbott Molecular. Abbott Park. IL. USA) limit of detection 100 copies/mL and target sequence: RdRp and N genes; TaqPath COVID-19 (Thermo Fisher Scientific. USA-FDA) limit of detection 40 copies/mL and target sequence: S. N AND ORF1ab genes; Alinity SARS-CoV-2 (Abbott Alinity. Abbott Molecular. Abbott Park. IL. USA) limit of detection 40 copies/mL and target sequence: ORF1ab and N genes; Panbio™ COVID-19 Ag Rapid Test (Abbott Rapid Diagnostics Jena GmbH. Germany) Positive >2.5 ng/mL SARS-CoV-2				

Supplemental 1. Diagnosis of SARS-CoV-2 by nasopharyngeal swabs

viruses

MDPI

Article

Placental Infection Associated with SARS-CoV-2 Wildtype Variant and Variants of Concern

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Abstract: The original SARS-CoV-2 lineages have been replaced by successive variants of concern (VOCs) over time. The aim of this study was to perform an assessment of the placental infection by SARS-CoV-2 according to the predominant variant at the moment of COVID-19 diagnosis. This was a prospective study of SARS-CoV-2-positive pregnant women between March 2020 and March 2022. The population was divided into pregnancies affected by COVID-19 disease during 2020 (Pre-VOC group) and pregnancies affected after December 2020 by SARS-CoV-2 variants of concern (VOC group). The presence of virus was assessed by RT-PCR, and the viral variant was determined by whole genome sequencing. A total of 104 placentas were examined, among which 54 cases belonged to the Pre-VOC group and 50 cases belonged to the VOC group. Sixteen positive placental RT-PCR tests for SARS-CoV-2 were reported. The NGS analysis confirmed the SARS-CoV-2 lineage in placenta tissue. All samples corresponded to the Pre-VOC group, whereas no placental presence of SARS-CoV-2 was detected in the VOC group (16, 29.6% vs. 0, 0.0% $p = 0.000$). Preterm birth (9, 16.7% vs. 2, 4%; $p = 0.036$) and hypertensive disorders of pregnancy (14, 25.9% vs. 3, 6%; $p = 0.003$) were more frequent in the Pre-VOC group than in the VOC group. Finally, the VOC group was composed of 23 unvaccinated and 27 vaccinated pregnant women; no differences were observed in the sub-analysis focused on vaccination status. In summary, SARS-CoV-2-positive placentas were observed only in pregnancies infected by SARS-CoV-2 wildtype. Thus, placental SARS-CoV-2 presence could be influenced by SARS-CoV-2 variants, infection timing, or vaccination status. According to our data, the current risk of SARS-CoV-2 placental infection after maternal COVID disease during pregnancy should be updated.

Keywords: SARS-CoV-2; SARS-CoV-2 variants of concern; pregnancy; waves of COVID-19

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1. Introduction

Since the coronavirus disease (COVID-19) outbreak caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus has been constantly changing [1–3]. The initial period of the pandemic up to December 2020, when SARS-CoV-2 wildtype was the predominant variant, has been referred to as pre-variants of concern (Pre-VOC) era. Then,

the original SARS-CoV-2 lineages have been replaced by successive variants of concern (VOCs) (Alpha, Beta, Gamma, Delta, Omicron) over time [4]. These variants exhibit higher transmissibility and cause COVID-19 disease of lower severity (with associated lower mortality rates) compared to SARS-CoV-2 wildtype [5,6].

Apart from this evolution, since May 2021, the COVID-19 vaccines have become available for pregnant women, and their effectiveness has been demonstrated in the pregnant population [7–9].

The pregnancy complications associated with SARS-CoV-2 infection have become an issue of concern. Reports have shown an increase in maternal and neonatal morbidity and mortality in pregnant women with COVID-19 diagnosis during the first months of the pandemic [10,11]. On the other hand, the literature is undecided about the relationship between VOCs, vaccination and maternal outcomes [7,12–14].

In the context of maternal disease severity or vertical transmission to the fetus or newborn, several studies have studied the presence of SARS-CoV-2 virus in placenta. Whereas some studies have detected SARS-CoV-2 in 95–100% of analyzed placentas [15,16], others have only detected around 6–20% [17–19] and some cannot detect SARS-CoV-2 at all in placentas [20,21]. This variability can be explained, because different studies describe pregnant women with different grades of COVID-19 severity, and different methodologies have been used for viral RNA detection. In addition, variability might depend on the viral linage and on the vaccination status of women suffering COVID-19 disease. Although the SARS-CoV-2 lineages have changed in the course of the pandemic, the relationship between the predominant variant causing COVID-19 disease and either placental infection or the lineage actually infecting the placenta has hardly been reported on.

The aim of this study was to analyze the frequency of placental infection by SARS-CoV-2 in SARS-CoV-2-positive women during pregnancy. We analyzed samples of SARS-CoV-2-positive women during 2020, when the Pre-VOC predominated, and samples collected during waves of VOC (Supplemental Figure S1).

2. Materials and Methods

We performed a prospective study in a Spanish tertiary care hospital (Hospital Clínico Lozano Blesa, Zaragoza, Spain) between March 2020 and March 2022. The inclusion criteria were SARS-CoV-2 infection during the pregnancy and placenta tissue available for analysis. The study was divided into two periods according to the date of their positive RT-PCR test and the periods of dominance of the SARS-CoV-2 variants. The Pre-VOC group was defined as women who tested positive for SARS-CoV-2 during 2020 (when the wildtype strain was predominant). The VOC group was defined as women who tested positive during 2021 and 2022 (era of all the other variants).

SARS-CoV-2 infection was diagnosed based on the positive RT-PCR test for SARS-CoV-2 from nasopharyngeal swabs. RT-PCR test kits from different companies were used: Viasure (CerTest Biotec, Zaragoza, Aragón, Spain), M2000 SARS-CoV-2 Assay (Abbott RealTime SARS-CoV-2 Assay, Abbott Molecular, Abbott Park, Green Oaks, IL, USA), TaqPath COVID-19 (Thermo Fisher Scientific, Waltham, MA, USA) and Alinity SARS-CoV-2 (Abbott Alinity, Abbott Molecular, Abbott Park, Green Oaks, IL, USA). As suggested by the manufacturer for nasopharyngeal specimens, cycle threshold (CT) values below 37 were taken as positive. Symptoms of COVID-19 have been divided into 3 types [22]: asymptomatic infection refers no clinical symptoms or signs; mild infection refers to symptoms such as fever, cough, headache, anosmia and asthenia; and severe infection refers dyspnea and hypoxemia accompanied by chest imaging compatible with pneumonia and respiratory infection. To determine the percentage of predominant variants of SARS-CoV-2 circulating in our patient recruitment area during sample collection, data were downloaded from the official local and national public sequence database [23] (Supplementary Figure S2).

The collection of placental tissue samples, posterior treatment and storage in RNAtm-Later, and subsequent purification of RNA and SARS-CoV-2 RT-PCR analysis was carried out as described in a previous study [24]. The RNA from placentas with a positive in SARS-

CoV-2 RT-PCR was analyzed by NGS to identify SARS-CoV-2 lineage, using a modified version of the COVID-19 ARTIC v4 Illumina library construction and sequencing protocol v4. Details on sequencing procedures and subsequent data analysis are provided in the Appendix A. Typical coverage of the SARS-CoV-2 genome obtained by whole genome sequencing is shown in Supplementary Figure S3.

Small for gestational age (SGA) was defined as birthweight below the 10th centile according to local standards [25]. Hypertensive disorders of pregnancy (HDPs) were divided into 4 categories, which were defined according to the criteria proposed by International Society for the Study of Hypertension in Pregnancy (ISSHP) [26]: preeclampsia (PE), chronic hypertension, chronic hypertension with superimposed PE and gestational hypertension.

Clinical characteristics, laboratory results, and maternal and neonatal outcomes were collected from medical records. All patients provided written informed consent. The study was approved by the Research Ethics Committee of the Community of Aragon (C.I. PI21/155 and COL21/000), and all patients provided written informed consent.

Statistical analysis was performed using SPSS 22.0. Categorical variables were presented as frequencies or percentages. Continuous variables were presented using mean ± standard deviation (SD), median, or range. For continuous variables, Shapiro–Wilk tests of normality were used to evaluate the distributions. Data were analyzed using Student's *t* test or Mann–Whitney U test for continuous variables and the Pearson $\chi 2$ test for the categorical ones. Statistical significance was considered $p < 0.05$.

3. Results

Fifty-four women in the Pre-VOC group and fifty women in the VOC group were included. Demographic and clinical data, as well as a description of SARS-CoV-2, are shown in Table 1. The study group was comparable regarding maternal age, maternal weight, Caucasian ethnicity and primiparous. There were no significant differences for gestation age at birth, birth weight, SGA, umbilical arteria pH < 7.1, induced labor and caesarean. Nevertheless, preterm birth and HDP were more frequent in the Pre-VOC group than in the VOC group ($p = 0.036$, and $p = 0.003$, respectively).

Table 1. Demographic and obstetrical data and SARS-CoV-2 infection characteristics.

	Pre-VOC (*n* = 54)	**VOC (*n* = 50)**	***p***
Demographic maternal data			
Maternal age (SD), years	31.5 (5.9)	32.8 (5.4)	0.311
Maternal weight (SD), kg	69.5 (12.6)	67.9 (13.1)	0.330
Caucasian (%)	30 (55.5)	32 (64.0)	0.381
Primiparous (%)	24 (44.4)	18 (36.0)	0.381
Maternal and neonatal outcome at delivery			
Gestational age at birth mean (SD), days	272.2 (20.3)	274 (7.4)	0.365
Birth weight, mean (SD), grams	3114.5 (609.3)	3136.7 (343.6)	0.224
Small for gestational age neonate (%)	7 (13.0)	4 (8.0)	0.411
Preterm birth (%)	9 (16.7)	2 (4.0)	0.036
Hypertensive disorders of pregnancy (%)	14 (25.9)	3 (6.0)	0.003
Labor induction (%)	23 (42.6)	16 (52.0)	0.338
Cesarean delivery (%)	8 (14.8)	12 (24.0)	0.235
Umbilical artery pH < 7.10 (%)	2 (3.7)	2 (4.0)	0.984

Table 1. *Cont.*

	Pre-VOC (*n* = 54)	VOC (*n* = 50)	*p*
Description of SARS-CoV-2 infection			
Trimester of SARS-CoV-2 infection			
1° (%)	4 (7.4)	0 (0.0)	0.083
2° (%)	19 (35.2)	14(28)	
3° (%)	31 (57.4)	36 (72)	
Time between SARS-CoV-2 diagnosis and delivery, days (%)			
<10 days	7 (13.0)	23 (46.0)	0.001
11–84 days	23 (42.6)	14 (28.0)	
>84 days	24 (44.4)	13 (26.0)	
Interval from diagnosis of SARS-CoV-2 to delivery (SD), days	75.0 (54.2)	50.3 (57.8)	0.027
COVID-19 Symptoms			0.184
No (%)	23 (42.6)	29 (58.0)	0.085
Mild (%)	27 (50.0)	20 (40.0)	0.204
Severe (%)	4 (7.4)	1 (2.0)	0.206
RT-PCR-positive placenta tissue (CT < 37), (%)	16 (29.6)	0 (0.0)	0.000

SD, standard deviation; CT, cycle threshold value RT-PCR.

Description of SARS-CoV-2 infection is presented in Table 1. No significant differences between trimester of SARS-CoV-2 maternal infection among cohorts were found ($p = 0.083$). Maternal diagnosis of COVID-19 was most frequent during the third trimester, 67 mothers (64.4%), whereas 33 women (31.7%) were in the second trimester and only four women (3.8%) were in the first trimester. However, we found significant differences according to the timing of maternal SARS-CoV-2 infection ($p = 0.001$) and the interval average from diagnosis of SARS-CoV-2 to delivery (Pre-VOC: 75.0 ± 54.2 days vs. VOC group: 50.3 ± 57.8, $p = 0.027$). Although no significant differences were found in the overall severity of SARS-CoV-2 symptoms between the groups ($p = 0.184$), the vast majority of patients with severe symptoms, four of five, were found in the Pre-VOC group.

We analyzed placental tissue from 104 patients by RT-PCR. Sixteen placentas were positive to SARS-CoV-2, and all were included in the Pre-VOC group. To confirm the presence and lineage of SARS-CoV-2 in the 16 positive placental samples identified, we performed whole viral genome sequencing by NGS. The assigned lineage in analyzed samples by whole genome sequencing corresponded to the predominant variant in our recruitment area at the time of infection of our study subjects. These results are shown in Table 2.

Additionally, we examined the vaccination status of study subjects. In the Pre-VOC group period, vaccination was still not available. In the VOC group, 27 women were vaccinated, whereas 23 women were unvaccinated (Figure 1). About vaccinated women, they were given viral vector (ChAdOx1-S (AstraZeneca)) or mRNA vaccines (mRNA-1273 (Moderna) or BNT162b2 (Pfizer-BioNTech)). Six women had only a single dose, whereas 20 women were completely vaccinated, and one had a booster vaccination. The average time between the last dose received and the COVID-19-positive result was 129.2 ± 85.1 days (Supplementary Table S1). Ten women had received the first dose before pregnancy, five women received the first dose during the first trimester, nine received the first dose during the second trimester and three received the first dose during the third trimester. Finally, we performed a sub-analysis to compare the Pre-VOC group with either the unvaccinated or the vaccinated VOC group (Table 3). Despite that in our sample we did not find differences among the overall maternal COVID-19 symptoms ($p = 0.218$), none of the cases or no severe cases occurred in the vaccinated VOC group.

Table 2. Description of sixteen positive placental SARS-CoV-2 cases. Assignment of SARS-CoV-2 lineage in each sample positive for placental SARS-CoV-2 based on the results of whole genome sequencing (see Supplementary Methods for details).

Diagnosis SARS-CoV-2				Delivery and Placenta Tissue Results						
N	**GA at Diagnosis**	**PS**	**SYM**	**GA at Delivery**	**Interval SARS-CoV-2—Delivery (d)**	**RT-PCR Result Nasopharyngeal at Delivery Time**	**CT RT-PCR Nasopharyngeal**	**CT RT-PCR Placenta Tissue**	**Lineage**	**Correlation between PS and Placenta Tissue**
1	38 + 3	Pre-VOC	S	38 + 4	1	Positive	33.5	15.1	B.1.177	Yes
2	25 + 1	Pre-VOC	M	39 + 2	99	Negative		33.9	B.1	Yes
3	31 + 4	Pre-VOC	A	41 + 0	74	Negative		30.2	B.1	Yes
4	24 + 6	Pre-VOC	M	37 + 1	86	Negative		31.7	B.1.177	Yes
5	25 + 6	Pre-VOC	M	40 + 1	100	Negative		33.1	-	-
6	32 + 0	Pre-VOC	A	40 + 1	85	Negative		31.1	B.1	Yes
7	21 + 3	Pre-VOC	A	36 + 5	107	N/A		33.4	B.1.177	Yes
8	24 + 4	Pre-VOC	M	39 + 5	106	Negative		32.4	B.1	Yes
9	36 + 2	Pre-VOC	A	38 + 0	12	Positive	34.8	35.2	B.1.177	Yes
10	30 + 0	Pre-VOC	A	40 + 2	72	Negative		24.5	B.1	Yes
11	37 + 6	Pre-VOC	A	38 + 1	2	Positive	35.3	33.9	-	-
12	17 + 5	Pre-VOC	M	38 + 2	151	Negative		32.0	B.1	Yes
13	27 + 5	Pre-VOC	M	37 + 0	68	Negative		23.3	B.1.177	Yes
14	32 + 3	Pre-VOC	A	41 + 2	64	Negative		32.5	B.1	Yes
15	8 + 0	Pre-VOC	A	35 + 2	191	Negative		28.7	B.1.177	Yes
16	41 + 0	Pre-VOC	A	41 + 1	8	Positive	35.5	33.7	-	-

GA, gestational age (week + days); PS, predominant strain at the moment of SARS-CoV-2 diagnosis; d, days; SYM, symptoms of SARS-CoV-2 infection; S, severe; M, mild; A, asymptomatic; -, absence of lineage assignment; CT, cycle threshold value RT-PCR; N/A, not available.

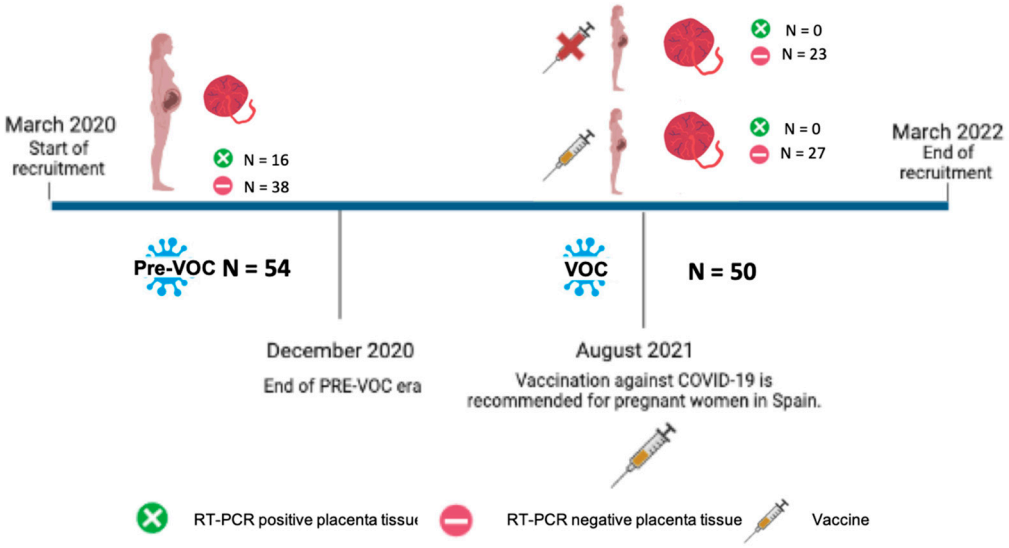

Figure 1. Analysis of association of SARS-CoV-2 placental infection with variant of concern era and vaccination. Pre-VOC group N = 54. VOC group = 50, including 23 unvaccinated women and 27 vaccinated women. Overall, 16 placentas were positive to SARS-CoV-2. All were included in the Pre-VOC group, no women were included in the VOC group.

Table 3. Results of sub-analysis focused on vaccination status.

	Pre-VOC (*n* = 54)	**VOC Unvaccinated (*n* = 23)**	**VOC Vaccinated (*n* = 27)**	***p***
Hypertensive disorders of pregnancy, (%)	14 (25.9)	2 (8.7)	1 (3.7)	0.022
Preterm birth, (%)	9 (16.7)	1 (4.3)	1 (3.7)	0.110
Small for gestational age, (%)	7 (13.0)	1 (4.3)	3 (11.1)	0.528
Birth weight, mean (SD), g	3114.5 (609.3)	3168.3 (308.4)	3290.7 (367.2)	0.331
Trimester of SARS-CoV-2 infection 1° (%) 2° (%) 3° (%)	 4 (7.4) 19 (35.2) 31 (57.4)	 0 (0.0) 4 (17.4) 18 (78.3)	 0 (0.0) 10 (37) 17 (63)	0.148
Interval from diagnosis of SARS-CoV-2 to delivery (SD), d	75.0	43.9	55.8	0.07
COVID-19 Symptoms No (%) Mild (%) Severe (%)	 23 (42.6) 27 (50) 4(7.4)	 12 (52.2) 10 (43.5) 1 (4.3)	 17 (63) 10 (37) 0 (0.0)	0.370 0.218 0.534 0.338
COVID-19 symptomatic, (%)	31 (57.4)	11 (47.8)	10 (37)	0.218
RT-PCR-positive placenta tissue (CT < 37), (%)	16 (29.6)	0 (0.0)	0 (0.0)	0.000

SD, standard deviation; CT, cycle threshold value RT-PCR.

4. Discussion

Our findings suggest that the frequency of SARS-CoV-2-positive placental tests may differ based on SARS-CoV-2 variants. We found a significantly higher frequency of positive placental RT-PCR SARS-CoV-2 in the placentas from women infected with SARS-CoV-2 wildtype compared with women infected with variants of concern of SARS-CoV-2, even adjusting for the vaccination status of the mother.

There are very few studies published describing the relation between SARS-CoV-2 variants of concern and placental health. Shanes et al. [27] detailed that maternal vascular malperfusion is a feature of SARS-CoV-2 infection during pregnancy and proposed that

the lesion frequency changed with the predominant circulating variant. These findings are consistent with our results, given that positive placental SARS-CoV-2 could vary depending on the SARS-CoV-2 variants.

In fact, only a very limited number of studies combine data about the relationship between SARS-CoV-2 variants and positive placental SARS-CoV-2 tests. Wierz et al. [28], reporting on an isolated case, detected the presence of the Alpha variant in placental tissue using MALDI-TOF technology. Argueta et al. [29] described a cohort of placental samples from mothers positive for SARS-CoV-2 at delivery, where forty-two percent had detectable RNA, and underscored two placentas from mothers infected with the Alpha variant. A single case of SARS-CoV-2 variant Delta in a placenta after two successive COVID-19 episodes in unvaccinated woman in the same pregnancy has also been reported [30]. These isolated cases carried SARS-CoV-2 variants of concern in placenta tissue in contrast to our cohort, in which we only detected the SARS-CoV-2 wildtype. This observation could be explained by the frequency of SARS-CoV-2 infection early during pregnancy, vaccination status or herd immunity. Independent of these variables, in our limited analyses, no placental infection of SARS-CoV-2 was detected starting in 2021 (Table 1). Altogether, very few cases of placental presence of SARS-CoV-2 VOC have been described, suggesting it is rare. However, whether the frequency of placental infection, replication and/or symptoms in placenta are different between Pre-VOC on the one hand, and VOC and posterior variants on the other, remains to be established. Altered angiogenesis and enhanced vascular alterations are associated with both COVID-19 and PE [31]. We hypothesize that the resulting increased permeability of the blood vessels may allow more infiltrations of SARS-CoV-2 infected cells into the placenta and subsequent placental infection. A recent study provides evidence that disease severity is reduced in pregnant women infected by newer variants such as the omicron strain compared to women infected with wildtype SARS-CoV-2 at the beginning of the pandemic [32]. Our data suggest that the presence of placental SARS-CoV-2 is less frequent in mothers infected with VOC compared to SARS-CoV-2 wildtype. Therefore, if placental infection was a function of disease severity, the reduced frequency we observe after 2020 could be explained by the more limited vascular damage associated with less severe disease.

Pregnant women and their fetuses represent a high-risk population during infectious disease outbreaks [33]. For several viral diseases, severity is dependent on the trimester of infection. In the case of Rubella [34], the risk of fetal infection is highest in the first trimester especially prior to 10 weeks of gestation. On the other hand, severe disease was more prevalent among women in the third trimester of pregnancy during an influenza pandemic (H1N1). Moreover, from implantation and trophoblast invasion during early pregnancy onwards, successful pregnancy requires an environment that is tolerant toward maternal/fetal immunological differences [35]. In light of these combined observations, we analyzed the frequency of placental SARS-CoV-2 as a function of the trimester of infection. We included women infected by SARS-CoV-2 during all stages or trimesters of pregnancy in contrast to many studies that focus on women with a positive SARS-CoV-2 diagnosis at labor on hospital admission [36,37]. The vast majority of SARS-CoV-2-positive placentas (12 out of 16) were delivered by women who were diagnosed with COVID-19 disease more than 10 days before delivery (Table 2). Indeed, the seven cases that correspond to women who were diagnosed with COVID-19 before the third trimester all belong to the Pre-VOC group. While we cannot exclude the possibility that these mothers were re-infected later during pregnancy, an equally plausible explication resides in persistent infection. This timeframe would define these cases as post-COVID-19 syndrome, according to most actual definitions [38]. Glynn et al. [39] studied placental pathology, differentiating between acute or nonacute SARS-CoV-2 based on infection <14 or ≥14 days from delivery admission. This study provides evidence that histologic lesions in the placenta may differ based on the timing of SARS-CoV-2 infection during pregnancy. Furthermore, a significantly lower placental weight was reported in the non-acute cohort, suggesting long-term sequelae in response to SARS-CoV-2 infection. As SARS-CoV-2 infection may

be persistent after infection during early pregnancy, and distinct placental lesions may develop during this time, further research is called for to mechanistically understand the effects on both placental and fetal health and to evaluate potential effects on newborns.

Whereas it was not the primary outcome of this study, we showed worse maternal and perinatal outcome in the Pre-VOC group compared to the VOC group. In fact, the association with preeclampsia that was reported early in the pandemic [24,40] is confirmed by our data (Pre-VOC group 14/54 HDP cases vs. VOC group 3/50 HDP cases, $p = 0.003$). Several studies correlate a worse perinatal outcome such as preterm birth, small for gestational age or ICU admission with hypertensive disorder of pregnancy [41,42]. Consistent with these reports, we observed higher rates of preterm birth in the Pre-VOC group than in the VOC group (9, 16.7% vs. 2, 4%; $p = 0.036$). Our results support reported findings suggesting that successive waves of SARS-CoV-2 variants of concern are associated with decreased severity in pregnant women [31,43,44], while other studies show that the VOC cases of severe COVID-19 disease during pregnancy are limited to women that are unvaccinated [9,45–47]. We also addressed the potential influence of vaccination on the presence of SARS-CoV-2 in the placenta. We did not detect SARS-CoV-2 in placenta tissue from 27 vaccinated women. Our data do not show significant differences between vaccination status and COVID-19 complications. This was probably due to the small sample size as well as because the vast majority of vaccinated women contracted the virus when Omicron was the most dominant strain. All vaccinated women in our study received vaccines not adapted to BA.4 and BA.5 Omicron variants. However, several studies published support the vaccine effectiveness in the pregnant population, showing a reduced risk for severe symptoms and complications in vaccinated women [9,48,49].

We assume that the sample size of this study is insufficient to provide evidence about the clinical outcomes associated with SARS-CoV-2 variants of concern, but it was not the main objective of this study. We consider that the prospective sampling of 106 placentas during the course of the pandemic provides enough consistency to evaluate the risk of placental infection over time as a function of different variants and clinical scenarios. Moreover, to collect the placentas of all SARS-CoV-2-positive pregnancies during the period of study, we needed to know at delivery time which women had passed COVID-19 during pregnancy and to have research staff available for sample collection and preservation. These requirements were very difficult to meet at certain times of the pandemic, and a much more limited number of samples was collected. This may have caused selection bias. It is important to note that the detection of the viral genome in the placental tissue does not necessarily mean that active infectious particles have been detected. However, the genome-wide coverage that we obtain in most positive samples (see Supplementary Figure S3) would suggest that replication occurs and active infection may take place. Moreover, we now confirm that the lineage infecting the placenta corresponds to the predominant lineage at the moment of SARS-CoV-2 infection and not to rare variants with tropism for placenta.

In summary, SARS-CoV-2-positive placentas were observed only in pregnancies infected by the SARS-CoV-2 wildtype. The frequency of placental SARS-CoV-2 may be determined by the SARS-CoV-2 variant, infection timing, or vaccination status of the mother. According to our data, the current risk of SARS-CoV-2 placental infection after maternal COVID disease during pregnancy should be updated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15091918/s1, Figure S1: Description of the SARS-CoV-2 pandemic in Aragón (Spain) and the placental samples collected in the timeframes indicated; Figure S2: SARS-CoV-2 variants and The ongoing evolution of variants of concern and interest of SARS-CoV-2 in Aragón, Spain; Figure S3: Representation of coverage of the SARS-CoV-2 genome obtained by whole genome sequencing. Three representative images are shown Table S1: Temporal trends in COVID-19 vaccine in our 27 vaccinated women.

Author Contributions: A.M.-M., M.F., J.S. and D.O. were responsible for the design of the study and the writing of the manuscript. M.F., M.P., P.C., S.R.-M., C.P., J.S. and D.O. were responsible for the analysis and interpretation of the data. P.C. and S.R.-M. contributed to the writing of the manuscript. M.F., M.P., M.S., A.M.-M., J.S., A.C.-S., M.O.C. and M.S. were responsible for the laboratory analysis. P.C., S.R.-M., C.P. and D.O. were responsible for the collection of clinical data of the patients. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Whole genome sequencing

RNA was analyzed using a modified version of the COVID-19 ARTIC v4 Illumina library construction and sequencing protocol v4. Briefly, cDNA was synthesized by incubating SuperScript IV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) with random hexamers and deoxynucleotide triphosphates. The reaction mixture was firstly incubated at 65 °C for 5 min and then at 42 °C for 50 min and at 70 °C for 10 min. Once the cDNA had been synthesized, amplicons were generated with ARTIC v3 two primer pools that covered the whole SARS-CoV-2 genome (Integrated DNA Technologies, Coralville, Iowa, USA). PCR conditions were 98 °C for 30 s, followed by 35 cycles of amplification for 98 °C for 15 s and 63 °C for 5 min, with a final 65 °C extension for 5 min. Amplicons from both pool reactions were mixed and purified with AMPure XP beads (Beckman Coulter, Brea, California, USA) and eluted in 10 Mm Tris-HCl (pH 8.0). Libraries were generated using the Illumina DNA Prep library preparation kit that uses transposomes with adapter sequences to bind fragmented DNA and introduces Illumina index adaptors by the amplification of tagmented DNA (Illumina, Spain Ref #20018705, San Diego, California, USA). Libraries were finally purified and quantified with the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit 4 Fluorometer reader (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were sequenced on a MiSeq instrument (Illumina, San Diego, CA, USA using 150 bp paired-end read). Analysis was performed using DRAGEN COVID Lineage application V3.5.6 (Ilumina, San Diego, CA, USA) and results were downloaded from the BaseSpace Platform. Consensus sequences were assigned to lineages by Pangolin v.2.0.4. According to the settings of the BaseSpace Platform, lineage assignment required a coverage $\geq$10$\times$ over 50% using standard settings. For all except samples 5, 11 and 16, sufficient reads and coverage were obtained to assign the variant present. The variant present in placentas samples was assigned as the pango

lineage B.1.177 (six samples), whereas other samples could be only identified as parent lineage B.1. due to limited coverage (seven samples) (Table 2).

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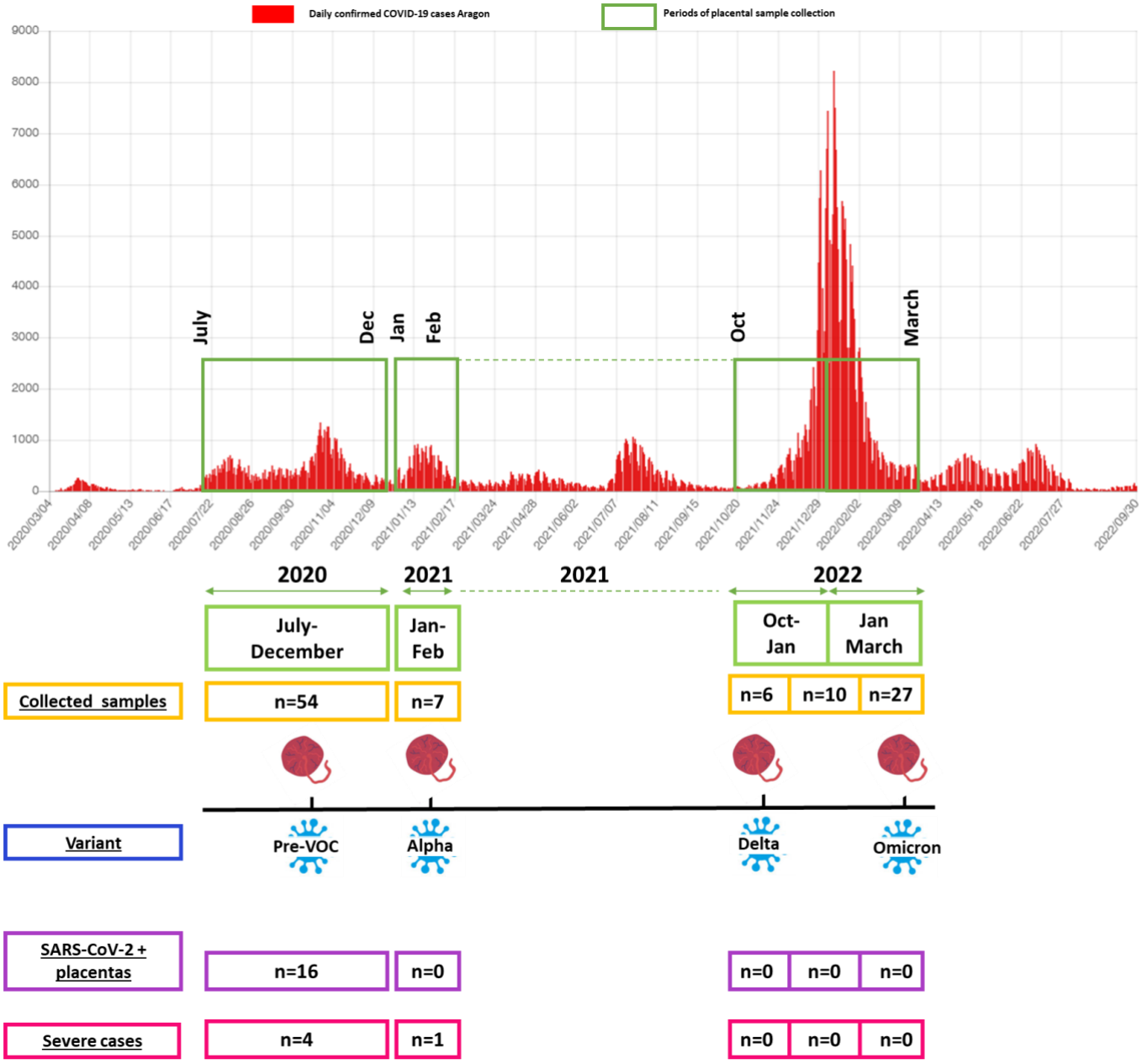
Supplementary Figure 1:



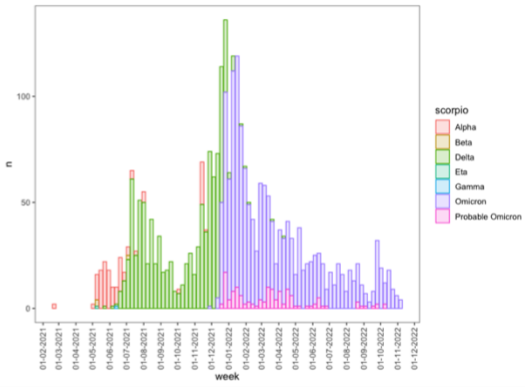
Supplementary Figure 1: Description of the SARS-CoV-2 pandemic in Aragón (Spain) and the placental samples collected in the timeframes indicated.

Supplementary Figure 2:

A

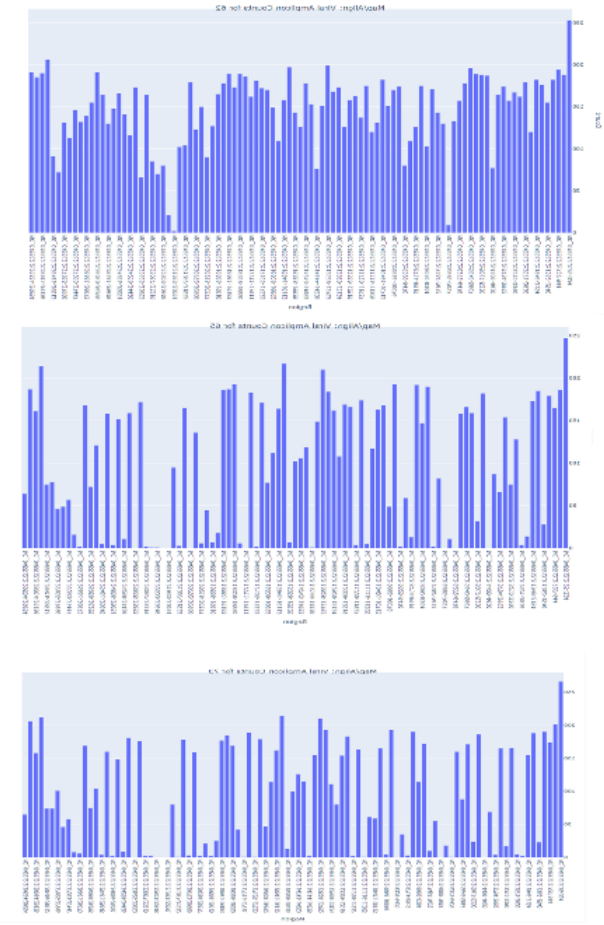
Variant, OMS designation	Lineage	Description
Pre-VOC	B.1.177	First detected in February 2020
Alpha	B.1.1.7	First detected in September 2020. Designated by OMS on 18 December 2020
Beta	B.1.351	First detected in May 2020. Designated by OMS on 18 December 2020
Gamma	P.1	First detected in November 2020. Designated by OMS on 11 January 2021
Delta	B.1.617.2	First detected in October 2020. Designated by OMS on 4 April 2021
Omicron	B.1.1.529	First detected in November 2021. Designated by OMS on 24 November 2021
Lambda	C.37	First detected in December 2020. Designated by OMS on 11 June 2021
Mu	B.1.621	First detected in January 2021. Designated by OMS on 30 August 2021

B



Supplementary figure 2: A) SARS-CoV-2 variants. History of principal designated SARS-CoV-2 variants over time as designated by WHO (https://www.who.int/activities/tracking-SARS-CoV-2-variants) during preVOC (March-December 2020) and VOC era (January 2021-present). The period in which samples for this study were taken is indicated on the left. B) The ongoing evolution of variants of concern and interest of SARS-CoV-2 in Aragón, Spain. Number of samples subjected to sequencing to identify the variant/lineage of SARS-CoV-2 by time period (January 2021-present) in our area. Note that the number of samples sequenced to identify the SARS-CoV-2 variant is not representative of the number of infections reported.

Supplementary figure 3.



Supplementary figure 3: Representation of coverage of the SARS-CoV-2 genome obtained by whole genome sequencing. Three representative images are shown.

FIRST DOSE	SECOND DOSE		SARS-CoV-2 INFECTION		
Trimester	**Days after 1º dose**	**Trimester**	**Days between last dose and SARS-CoV-2 diagnosis**	**SYN**	**PS**
3º	21	3º	61	A	Omicron
Preconception	21	Preconception	59	A	Delta
1º	-	-	142	A	Delta/Omicron
1º	-	-	133	A	Delta/Omicron
1º	21	1º	235	M	Delta/Omicron
2º	22	2º	136	A	Delta/Omicron
1º	21	1º	159	M	Delta/Omicron
2º	28	2º	100	M	Delta/Omicron
Preconception	21	1º	325	A	Omicron
2º	-	-	131	A	Omicron
Preconception	-	-	135	M	Omicron
3º	28	3º	3	M	Omicron
Preconception	344	3º	22	A	Omicron
3º	-	-	28	M	Omicron
2º	21	2º	134	A	Omicron
Preconception*#	92	Preconception	7	A	Omicron
Preconception	21	Preconception	177	M	Omicron
Preconception	291	3º	41	A	Omicron
Preconception	21	Preconception	181	M	Omicron
2º	21	2º	112	A	Omicron
2º	28	2º	104	A	Omicron
2º	21	2º	120	M	Omicron
Preconception	21	Preconception	195	M	Omicron
2º	21	2º	96	A	Omicron
Preconception*	-	-	354	A	Omicron
2º	21	2º	103	A	Omicron
1º	21	1º	195	A	Omicron

Supplementary Table 1: Temporal trends in COVID-19 vaccine in our 27 vaccinated women. Description of vaccination received by study subjects, the number of doses, the interval between dose, and timing between last dose and SARS-CoV-2 diagnosis. SYN, symptoms of SARS-CoV-2 infection; S, severe; M, mild; A, asymptomatic. PS, Predominant strain (in Aragón) at the moment of SARS-CoV-2 diagnosis. All subjects received mRNA-based vaccines except for study subjects marked*, who received Viral vector-based vaccines. Only the subject marked # received a third dose: 290 days after the first dose, during the 2º trimester of pregnancy mRNA.