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Impact of SARS-CoV-2 infection on human pregnancy

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Abstract

As pregnancy involves immunological and physiological changes, including pulmonary and cardiovascular changes and immune suppression, pregnant women are at increased risk for severe outcomes from Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection. Expression of SARS-CoV-2 receptor, the angiotensin-converting enzyme 2 (ACE2), increases during pregnancy and is found in the placenta, highlighting the importance of understanding the impact of the dual challenge pregnancy and infection on the mother and the newborn. Using blood samples from women who were SARS-CoV-2 positive within their gestational period, and blood from the respective umbilical cords, we evaluated both humoral and cellular responses to determine if immune protection was transferred *in uterus* and how frequently vertical transmission occurs. Through serological tests, we were able to detect anti-SARS-CoV-2 IgG antibodies in 34/52 cord samples (≈65%), while only 2/52 cord samples (≈4%) presented both IgA and IgM, indicating that vertical transmission might have occurred. We also determined that IgG transfer ratio was inefficient and that only 9/52 cord samples (≈17%) had weakly neutralizing activity. Flow cytometry analysis showed that umbilical cord samples presented lower percentages of Spike-specific $CD4^+$ T cells when compared to mothers. Interestingly, a pool of naïve B cells with the capacity to bind to the receptor binding domain (RBD) from the virus was found in umbilical cord samples. Altogether, our results suggest that maternal infection with SARS-CoV-2 leads to inefficient antibody transfer to the fetus. Nonetheless, we hypothesise that *in uterus* protection can still be conferred by the simultaneous placental transfer of Spike-specific IgG antibodies and possibly immune cells. In addition to contributing to new insights on the consequences of SARS-CoV-2 infection during pregnancy, this project generated data that might guide future studies in the area. Future work will also include the assessment of the *in uterus* infection impact in baby´s development.

Keywords:

SARS-CoV-2 infection; pregnancy; *in uterus* transfer of protection; SARS-CoV-2 vertical transmission; antibodies.

Resumo

O sistema imune desempenha um papel extremamente importante na defesa do nosso organismo contra diferentes ameaças. Estes pode ser dividido em dois subsistemas que estão interligados e têm como objetivo a produção de uma resposta imune eficiente que culmina na eliminação da ameaça. Os referidos subsistemas, o sistema imune inato e o sistema imune adaptativo, apresentam diferenças em termos de rapidez, especificidade e capacidade de produção de memória. O sistema imune inato consiste numa primeira resposta, não específica, que é composta por, entre outros, barreiras fisiológicas e células imunes inatas como as células natural killer (NK). O sistema imune adaptativo, por sua vez, é uma segunda linha de resposta, específica e composta essencialmente por células T, células B e anticorpos. Este entra em ação após a sua ativação pelo sistema imune inato através da apresentação de antigénio pelas células apresentadoras de antigénio (APCs) às células T e B. Contrariamente ao sistema imune inato, o sistema imune adaptativo tem a capacidade de gerar células de memória que irão atuar rapidamente e de forma mais eficaz caso a ameaça surja novamente.

Durante uma gravidez normal e saudável, o corpo da mulher necessita de passar por várias mudanças fisiológicas e imunológicas para que o feto se possa implementar, desenvolver e, finalmente, nascer. As mudanças fisiológicas associadas à gravidez incluem alterações pulmonares como diminuição da resistência respiratória, diminuição da capacidade pulmonar total e menor resistência à hipoxia, e alterações cardiovasculares como o aumento da frequência cardíaca. Relativamente às alterações imunológicas, estas ocorrem no sentido de permitir o desenvolvimento do feto e ao mesmo tempo proteger a mãe e o feto de possíveis infeções e da resposta imune materna. Para que isto aconteça, é necessário que os sistemas imunes inato e adaptativo sofram alterações como, por exemplo, o aumento das células NK uterinas e T regulatórias (Treg) em prol da implementação correta do embrião e manutenção da tolerância imunológica, respetivamente. Apesar do sistema imune materno e a placenta possuírem mecanismos de defesa contra as infeções, estas podem ocorrer e levar a graves repercussões na gravidez como aborto e nascimento prematuro.

A atual pandemia causada pelo novo coronavírus Síndrome Respiratório Agudo Grave Coronavírus 2 (SARS-CoV-2), um vírus altamente transmissível, já infetou mais de 200 milhões de pessoas e causou a morte a mais de 5 milhões. A doença associada à infeção, designada por coronavírus 2019 (COVID-19), pode manifestar-se sem quaisquer sintomas ou, por outro lado, causar insuficiência respiratória grave podendo, nos casos mais graves, levar à morte. Os sintomas mais comuns associados à COVID-19 são a febre, o cansaço e a tosse seca e o seu principal meio de transmissão é por gotículas respiratórias. Relativamente à sua estrutura, o SARS-CoV-2 é um betacoronavírus cujo envelope viral se encontra revestido pela glicoproteína Spike. Esta proteína tem como recetor alvo a enzima conversora da angiotensina 2 (ACE2) e é composta por duas subunidades, S1 e S2, sendo que é na subunidade S1 que se encontra o domínio de ligação ao recetor (RBD). Desde o início da pandemia, o principal método de deteção da infeção por SARS-CoV-2 têm sido os testes RT-PCR uma vez que estes detetam diretamente o RNA viral. No entanto, os testes serológicos, utilizados para detetar anticorpos específicos contra o vírus, permitem-nos ter uma estimativa mais precisa da prevalência cumulativa da infeção por SARS-CoV-2 uma vez que estes são capazes de detetar indivíduos assintomáticos. A infeção por SARS-CoV-2, caracteriza-se pela produção inicial de anticorpos IgA e IgM poucos dias após infeção e pela produção de anticorpos IgG cerca de duas semanas após infeção, podendo estes últimos durar até 8 meses.

Sendo a COVID-19 uma doença respiratória infeciosa que se manifesta de forma mais severa em idosos e/ou em indivíduos com comorbilidades pré-existentes, as alterações sofridas durante a gravidez referidas anteriormente fazem com que as grávidas apresentem, também, um risco mais elevado de desenvolver formas severas da doença. Para além das mudanças fisiológicas e imunológicas, existem outros fatores que fazem das mulheres grávidas um grupo de risco. Estes incluem o aumento da

expressão do ACE2 durante o período inicial da gravidez bem como a presença do mesmo nos tecidos da placenta. Até à data, já foram reportados casos de transmissão vertical do vírus, mas, no geral, estes podem ser considerados um acontecimento raro. Para além disto, estudos recentes mostraram que algumas complicações durante a gravidez, como o nascimento prematuro e o aborto espontâneo, podem estar relacionadas com a infeção por SARS-CoV-2. Tendo em conta o recente aparecimento da COVID-19 na população humana, a informação disponível é escassa em comparação com outras doenças infecciosas, nomeadamente no que toca ao impacto da mesma durante e após a gravidez. Assim sendo, é de extrema importância perceber qual o impacto do duplo desafio gravidez e infeção na mãe e, consequentemente, no recém-nascido para que se possa definir e proporcionar um acompanhamento obstétrico e pediátrico adequado e obter mais informação acerca deste novo vírus.

Utilizando amostras de sangue de grávidas que testaram positivo para infeção por SARS-CoV-2 durante o seu período gestacional e cujo parto se realizou na Maternidade Dr. Alfredo da Costa (MAC), bem como amostras de sangue dos respetivos cordões umbilicais, avaliámos tanto a resposta humoral como a resposta celular da mãe e do recém-nascido. Esta análise teve como objetivos: determinar a frequência com que a transmissão vertical ocorre através da deteção de anticorpos IgA e IgM específicos contra Spike no sangue do cordão umbilical; determinar o rácio de transferência de anticorpos IgG específicos contra Spike; e verificar se é conferida proteção ao recém-nascido no útero.

Através de testes serológicos foi possível detetar a presença de anticorpos IgG específicos contra o SARS-CoV-2 em 34 das 52 amostras de sangue de cordão umbilical (≈ 65%) enquanto que a presença de anticorpos IgA e IgM específicos contra o SARS-CoV-2 apenas foi detetada em 2 das 52 amostras (≈ 4%). Tendo conhecimento de que tanto os anticorpos IgA como IgM não têm a capacidade de atravessar a barreira placentária, a deteção dos mesmos no sangue do cordão umbilical sugere que estes anticorpos provavelmente terão sido produzidos pelo feto e, como tal, indicam possíveis casos de transmissão vertical do vírus. Para os anticorpos IgG específicos contra o SARS-CoV-2 determinámos que o rácio de transferência da mãe para o feto era ineficiente e que apenas 9 das 52 amostras de sangue de cordão umbilical (≈ 17%) apresentavam anticorpos com propriedades neutralizantes fracas. A análise de citometria de fluxo mostrou existir uma menor quantidade de células T $CD4^{+}$ específicas para Spike nas amostras de cordão umbilical em comparação com as amostras maternas, no entanto, estas encontravam-se mais ativadas, indicando que as células T são transferidas maioritariamente na forma ativada. Curiosamente, ainda que imatura, também foi possível verificar a existência de uma população de células B no sangue do cordão com capacidade de ligação ao RBD. Tendo em conta que a maior parte das amostras de sangue de cordão umbilical não apresentou sinais de infeção por SARS-CoV-2, podemos colocar a hipótese de que a presença de células T e B específicas para SARS-CoV-2 será devida à transferência materna.

De um modo geral, os resultados obtidos permitiram confirmar que, apesar de não ser frequente, a transmissão vertical do vírus pode ocorrer. Para além disto, sugerem que a infeção por SARS-CoV-2 durante a gravidez leva a uma transferência ineficiente de anticorpos IgG específicos contra Spike da mãe para o recém-nascido. Apesar de ineficiente, podemos considerar que a transferência simultânea, via placenta, de anticorpos e células imunes específicas para o SARS-CoV-2 pode conferir proteção ao recém-nascido, permitindo-lhe reagir de forma mais rápida e eficaz em caso de uma futura infeção. No seguimento deste projeto, o próximo passo será avaliar a produção de citocinas na mãe e no recém-nascido bem como o impacto da infeção uterina no desenvolvimento dos recém-nascidos. Para além de ter permitido tirar conclusões importantes acerca das consequências da infeção por SARS-CoV-2 na gravidez, este projeto também permitiu recolher uma grande quantidade de dados que irão ser essenciais para prosseguir com novos projetos dentro desta área de estudo.

Palavras-chave:

Infeção por SARS-CoV-2; gravidez; transferência de proteção no útero; transmissão vertical SARS-CoV-2; anticorpos.

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List of Abbreviations

ACE2	Angiotensin-converting Enzyme 2
AIDS	Acquired Immunodeficiency Syndrome
AIM	Activation Induced Marker
APC	Antigen Presenting Cell
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CEDOC	Centro de Estudos de Doenças Crónicas
COVID-19	Coronavirus Disease 2019
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
Fab	Antigen binding fragment
FBS	Fetal Bovine Serum
Fc	Crystallizable Fragment
FcRn	Neonatal Fc Receptor
H&E	Hematoxylin and Eosin
Ig	Immunoglobulin
MAC	Maternidade Dr. Alfredo da Costa
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
NK	Natural Killer
NT_{50}	Half-maximal Neutralization Titer
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PRR	Pattern Recognition Receptor
RBD	Receptor Binding Domain
RT	Room Temperature
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
Tc	T cytotoxic
TCR	T Cell Receptor
Tfh	T follicular helper
Th	T helper
TMPRSS2	Transmembrane Serine Protease 2
Treg	T regulatory
WHO	World Health Organization

1. Introduction

1.1 Immune Response

The immune system plays a major role at protecting our body against several threats, including pathogens, toxins and cancerous cells[1]. When functioning properly, the immune system is able to eliminate these threats and to distinguish "self" from "non-self" (cells/antigens/pathogens) without excessive inflammation or the development of autoimmunity[1,2]. It can be divided into two subsystems that are linked and work together to generate an effective immune response: the innate immune system and the adaptive immune system, that vary in the degree of specificity, capacity of establishing memory and rapidity of action[1–3]. Pathogens can be recognized by pattern recognition receptors (PRRs) in the case of the innate immune system or by antigen-specific receptors in the case of the adaptive immune system such as T cell and B cell receptors (TCR and BCR)[1–4]. Antigens are unique components of the pathogen that allow its recognition by the immune system[1]. Once the pathogen is detected, an immune response is initiated through the production of pro-inflammatory cytokines and further specialized immune cell recruitment[4].

The innate immune system is responsible for the first line response to the pathogen. Anatomical and physiological barriers, epithelial cells, macrophages, dendritic cells (DCs), mast cells, neutrophils, basophils, eosinophils, natural killer (NK) cells are some of the elements involved in this type of response[4] (Figure 1.1). NK cells are important effector cells that represent 5-20% of peripheral blood mononuclear cells and are known to be naturally cytotoxic[5–7]. When activated through their PRRs, NK cells undergo cytotoxic degranulation and production of inflammatory cytokines, such as interferon-γ and tumour necrosis factor-α, which have an important role in the activation and/or maturation of other innate immune mediators like DCs, macrophages and neutrophils[5,7,8]. In humans, most mature NK cells express CD56 and can be divided into two subpopulations according to their surface density expression, $CD56^{dim}$ and $CD56^{bright}$[9,10]. $CD56^{dim}$ NK cells have cytotoxic properties and correspond to most NK cells present in peripheral blood, whereas $CD56^{bright}$ NK cells are a small subset with immunoregulatory properties[5,9,10]. In addition to the cellular response, the innate immune system also has a humoral component which includes, among others, complement proteins and antimicrobial peptides[4] (Figure 1.1). Together, the cellular and humoral responses can produce a more efficient and faster first line response that will aid the activation of the adaptive immune system through antigen presentation by antigen-presenting cells (APCs) such as DCs[1,3].

The adaptive immune system comes into action after being activated by the innate immune system[4], therefore representing a latter response. The cellular response is essentially composed by antigen specific T and B cells [1,4,11] (Figure 1.1). T cell antigen recognition occurs through TCR recognition of a complex of peptide antigens embedded within class I or II Major Histocompatibility Complex (MHC) proteins, presented by APCs[1–3,11]. T cells can belong to distinct lineages, including $CD8^+$ T cells (T cytotoxic cells- Tc), which act to kill infected cells[1–3], and $CD4^+$ T cells (T helper cells-Th), which regulate the cellular and humoral immune responses[1–3]. When activated by APCs, these subsets differentiate into functionally distinct effector subsets depending on the cytokines and costimulatory signals they receive during the activation process[2,11].

The most studied subsets of Th cells are Th1 and Th2 cells. Th1 cells play a critical role in protective cell-mediated immunity against intracellular pathogens through the activation of macrophages, while Th2 cells are important for the orchestration of humoral immune responses, clearing extracellular pathogens and parasites through the induction of immunoglobulin class switching[2,11]. Besides the two previously mentioned subsets, there are other important subsets of Th cells: the T follicular helper (Tfh) cells that help establish connection between the cellular and humoral response by

aiding B cells in the production of antibodies[12] and the T regulatory (Treg) cells which control autoimmune responses and prevent uncontrolled immunity[11].

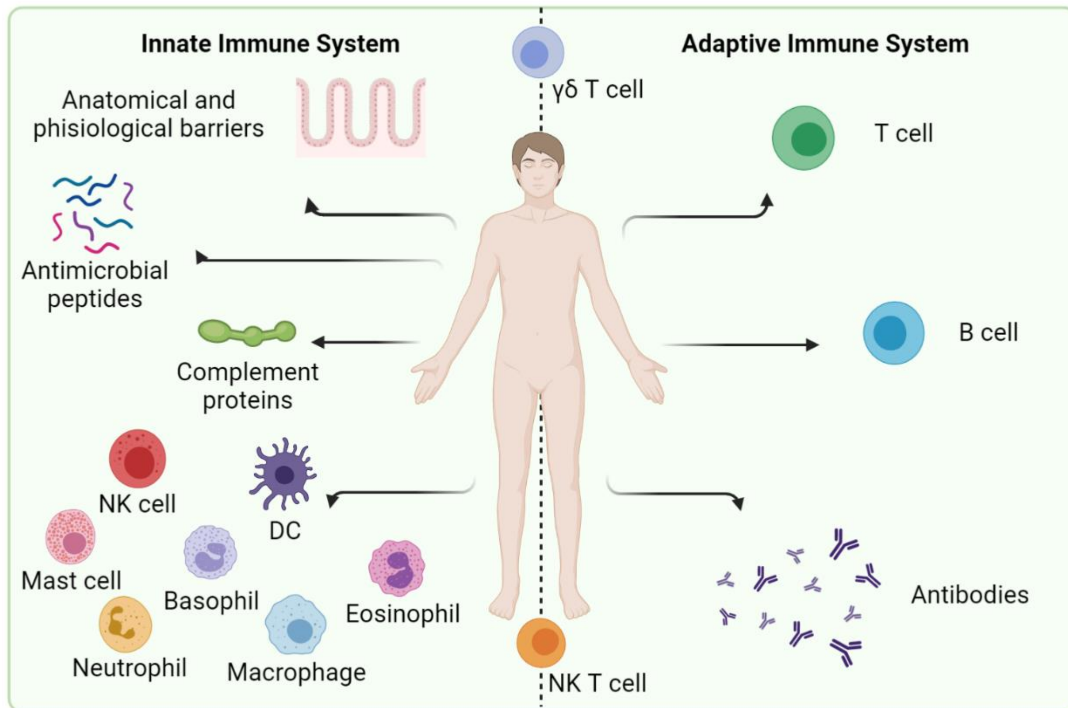


Figure 1.1 The human innate and adaptive immune systems. The human immune system is divided into the innate immune system and the adaptative immune system, two subsystems that work together to generate an immune response. The innate immune system is an early non-specific response to the pathogen which includes, among others, anatomical and physiological barriers, antimicrobial peptides, complement proteins, NK cells, DCs, mast cells, basophils, eosinophils, macrophages and neutrophils. The adaptative immune system is a late specific response essentially composed by T cells, B cells and antibodies. Contrarily to the innate immune system, the adaptative immune system has the capacity to generate memory cells that will respond rapidly and effectively upon subsequent reinfection. γδ T cells and NK T cells are two T cell subsets that work simultaneously in the innate and adaptative immune systems.

B cells can recognize intact antigens through their BCR and, when activated by Tfh cells, they proliferate into plasma cells to secrete specific antibodies against the pathogen[13–15]. These antibodies will then enter the host's circulation and tissues, bind to the specific antigens, and mark them for clearance, establishing a humoral response[13–15]. Antibodies, also known as immunoglobulins (Ig), are essential molecules for an effective clearance of the virus. They are composed by two antigen binding fragments (Fab), that confers them antigen specificity, and by one crystallizable fragment (Fc), that drives antibody function, generating a 'Y'-shaped molecule that can either exist as a monomer or form multimers[16,17]. The Fc domain changes between the five possible isotypes (IgG, IgA, IgM, IgD and IgE), each with different features according to the immune response needed[16]. IgG is the most abundant circulating antibody, represents 80% of total serum antibodies, provides most of the antibody-based immunity against pathogens and is the only antibody capable of crossing the placental barrier[15–18]. Monomeric IgA corresponds to 10-20% of total serum antibodies. IgA is present as a dimer in mucosal secretions in the respiratory, gastrointestinal and urogenital tracts as well as in saliva, tears, sweat and breastmilk[15,18], where it confers protection to mucosal surfaces by neutralizing bacterial toxins and inhibiting adhesion to epithelial cells[15–18]. IgM is secreted as a pentamer antibody, comprises 5-10% of total antibodies and is expressed on the surface of B cells as a monomer where it serves as a receptor for antigens[16–18]. IgD corresponds to less than 0.5% of total antibodies, is found on the surface of B lymphocytes, and, together with monomeric IgM, serves as antigen receptor for the activation of B

cells[15,17,18]. Lastly, IgE is a monomeric antibody with the lowest percentage in total serum antibodies (<0.01%)[18]. IgE is bound to tissue cells, especially mast cells, and associated with allergic reactions[15–18]. Contrarily to the innate immune system, the adaptative immune system has the capacity to generate memory cells that will respond rapidly and effectively upon subsequent reinfection[1–3].

Finally, there are two small subsets of T cells that act in the innate immune system and in the adaptative immune system, the γδ T cells and the NK T cells[11]. γδ T cells are T cells enriched in peripheral tissues that express a unique TCR composed by γ and δ chains and are involved in the initiation and propagation of immune responses in these sites[19]. On the other hand, NK T cells are T cells with important immunoregulatory functions that express a semi-invariant aβ TCR and cell surface molecules in common with NK cells[11,20].

1.2 Immune response during pregnancy

In a normal pregnancy, the woman's body undergoes several physiological and immunological changes in order to achieve a healthy and normal pregnancy. Physiological changes include pulmonary changes such as less airway resistance, total lung capacity and resistance to hypoxia[21], and cardiovascular changes such as the increase of cardiac output[22,23]. As for the immunological changes, the maternal immune system has, at the same time, to prevent infections of the mother and fetus and to allow fetal development. Therefore, a normal pregnancy oscillates between pro- (implantation and placentation; delivery) and anti-inflammatory (fetal growth) states[24]. For this to happen, both innate and adaptive arms of the maternal immune system must undergo alterations.

Pregnancy can be characterized by an increase in the number and activation state of circulating monocytes and granulocytes, which leads to a more aggressive attack on invading bacteria[25]. On the other hand, the maternal immune response is suppressed in order to protect the placenta and uterus from harmful immune responses. Studies have revealed that the numbers of T cells, B cells and NK cells are lower in pregnant women when compared to non-pregnant women[26,27]. However, this information varies according to the trimesters of pregnancy[25]. Tregs have a crucial role in maintaining immunological tolerance during pregnancy as they induce tolerance to fetal growth and non-inherited antigens such as paternal antigens, preventing the accumulation of effector T cells in the uterus[24,28]. Also, a specialized subset of NK cells, the decidual NK cells, assists and regulates implantation of the embryo and induces vascular growth[24,29]. Besides this suppression, the maternal immune system can still respond to pathogens and interfere with pregnancy outcomes.

The human placenta is a unique vascular organ that suffers several morphological changes throughout the gestation period. Full placental development is only achieved in the end of the first trimester and starts with the invasion of maternal tissues by the fetal trophoblast cells[24,30]. The basic units of the placenta are the chorionic villi which allow maternal-fetal exchanges of oxygen, nutrients and waste products[31–33]. When mature, these structures are composed by a layer of syncytiotrophoblast cells followed by a layer of cytotrophoblast cells[30,32]. Together, these two layers function as a placental barrier that impairs the direct mixing of the maternal blood present in the intervillous space and the fetal blood present in fetal vessels[30,31]. The decidua is a specialized layer of endometrium that forms the base of the placenta. In the end of the first trimester a haemochorial placenta is established where direct contact between maternal blood and the placenta occurs[30]. For this process to happen, another type of trophoblastic cells, the extravillous trophoblasts, are essential as they have the capacity to invade the decidua and the surrounding spiral arteries, allowing physiological remodelling of the vasculature, an important phenomenon for efficient and effective placental circulation[30,34]. A schematic representation of all these structures can be found in Figure 1.2.

In addition to the exchange of essential molecules, maternal antibodies can also be transferred to the newborn via placenta and/or breastfeeding through a process known as passive immunity[35]. Maternal antibodies protect neonates from infectious diseases, during their first months of life, when their immune system is not yet able to produce efficient amounts of antibodies themselves[35]. Transplacental transfer of IgG antibodies is mediated by the neonatal Fc receptor, FcRn, expressed in the syncytiotrophoblast cells[16,36,37]. Until 16 weeks of pregnancy, IgG transfer is minimal, but then starts to increase continuously throughout the second trimester and third trimester[33]. The highest transfer period occurs in the last 4 weeks before delivery and can result in higher levels in the fetus than in the mother[33,38]. The efficiency of IgG placental transfer can be affected by several factors such as maternal comorbidities, placental abnormalities, IgG levels, gestational age and infection, including cases of malaria and Acquired Immunodeficiency Syndrome (AIDS)[33,35,39].

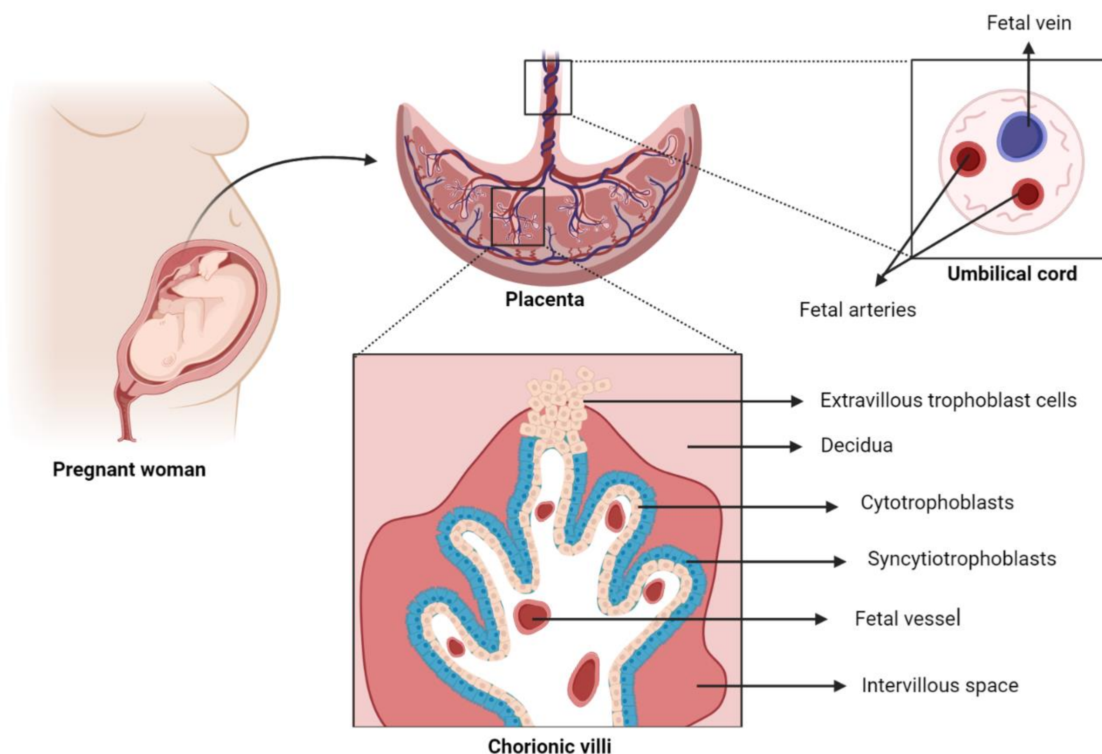


Figure 1.2 Schematic representation of the human placenta and umbilical cord. The basic functional units of the placenta are the chorionic villi. These structures are composed by fetal vessels, syncytiotrophoblast cells, cytotrophoblast cell, extravillous trophoblast cells and the intervillous space and are surrounded by decidua. The umbilical cord is composed by connective tissue, two fetal arteries and one fetal vein. Both placenta and umbilical cord allow maternal-fetal exchanges of oxygen, nutrients and waste products.

Besides antibody transfer, cell transfer from mother to fetus and vice-versa can also occur[28,40]. Transfer of T cells from the mother to fetus via placenta and breastfeeding have already been shown[28,41]. Moreover, trophoblast cells[42], lymphocytes[43] and erythrocytes[44] from fetal origin have also been detected in maternal blood.

Infection of the fetus from the mother, also known as vertical transmission, can occur, leading to preterm birth and spontaneous abortion in some cases[30,45,46]. The human placenta presents several defense mechanisms against fetal infection, including the syncytiotrophoblast layer that represents a

physical barrier against pathogens due to the lack of cell-to-cell junctions and a dense actin network that prevents pathogens' attachment[30]. Also, the decidua and chorionic villi are considered immunological barriers as the first is rich in immune cells including NK cells, T cells and macrophages and the second releases antimicrobial factors such as cytokines, chemokines and exosomes[30]. However, pathogens have evolved to overtake these barriers. Fetal infection can occur before birth, through the mother's urogenital tract, decidua or intervillous circulation, or after birth through vaginal delivery, subsequent contact with the mother and breastfeeding[30,46]. Vulnerability to infection oscillates during the gestational period being highest in late first trimester and second trimester[46].

1.3 COVID-19 and pregnancy

1.3.1 COVID-19 general information

An outbreak caused by a new type of coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), started in late December 2019 in the city of Wuhan[47]. Due to its high transmission, the number of cases quickly escalated all over the world, with the coronavirus disease 2019 (COVID-19) being considered a global pandemic on 11th March 2020, by the World Health Organization (WHO)[48].

SARS-CoV-2 is the third highly pathogenic betacoronavirus capable of infecting humans to emerge in the last two decades. The first, severe acute respiratory syndrome coronavirus (SARS-CoV), appeared in 2002 and the second, Middle East respiratory syndrome coronavirus (MERS-CoV), in 2012[49,50]. Since the beginning of SARS-CoV outbreak, at least 8 096 cases were reported including 774 deaths[51] while for MERS-CoV, 2 468 cases were reported including 851 deaths[52]. Until now (December/2021), there have been reported to WHO more than 260 million cases of SARS-CoV-2 infection, including more than 5 million deaths[53]. As the numbers keep rising, SARS-CoV-2 can be considered more contagious and infectious than the past two coronaviruses[54,55] even though presenting a lower mortality[56]. SARS-CoV-2 is an enveloped virus that shares 79% genome sequence identity with SARS-CoV and 50% with MERS-CoV[57]. Just like SARS-CoV, the envelope of the SARS-CoV-2 is coated by Spike glycoprotein that has as target receptor the angiotensin-converting enzyme 2 (ACE2)[58,59]. Spike protein is composed by two subunits, S1 and S2, with the S1 subunit containing the receptor binding domain (RBD) that binds to ACE2[59]. Besides having a long incubation period (2-14 days), SARS-CoV-2 presents a stronger binding between Spike protein and ACE2 and a more efficient invasion of host cells than SARS-CoV[59], which may justify the high transmissibility observed[55,59].

In humans, the pathology of SARS-CoV-2 infection spans from asymptomatic to severe respiratory failure, being the most common symptoms fever, fatigue and dry cough[60,61]. As it enters the human body, it binds to epithelial cells in the respiratory tract through its target receptor, ACE2, in synergy with the host's transmembrane serine protease 2 (TMPRSS2)[58,59], a cell surface protein that is mainly expressed in the airway epithelial cells and vascular endothelial cells[59], leading to membrane fusion and release of viral genome into the host cytoplasm, where it starts replicating[59]. Its rapid replication can drive an aberrant immune response leading to cytokine storm syndrome, causing acute respiratory distress syndrome and respiratory failure, some of the main causes of death of COVID-19 patients[59,61,62]. As it exits the host cell by the fusion of particle-loaded vesicles with the cell membrane, the virus is capable of infecting the neighbouring healthy cells and can be released into the surrounding environment via respiratory droplets, its main way of transmission [62] (Figure 1.3).

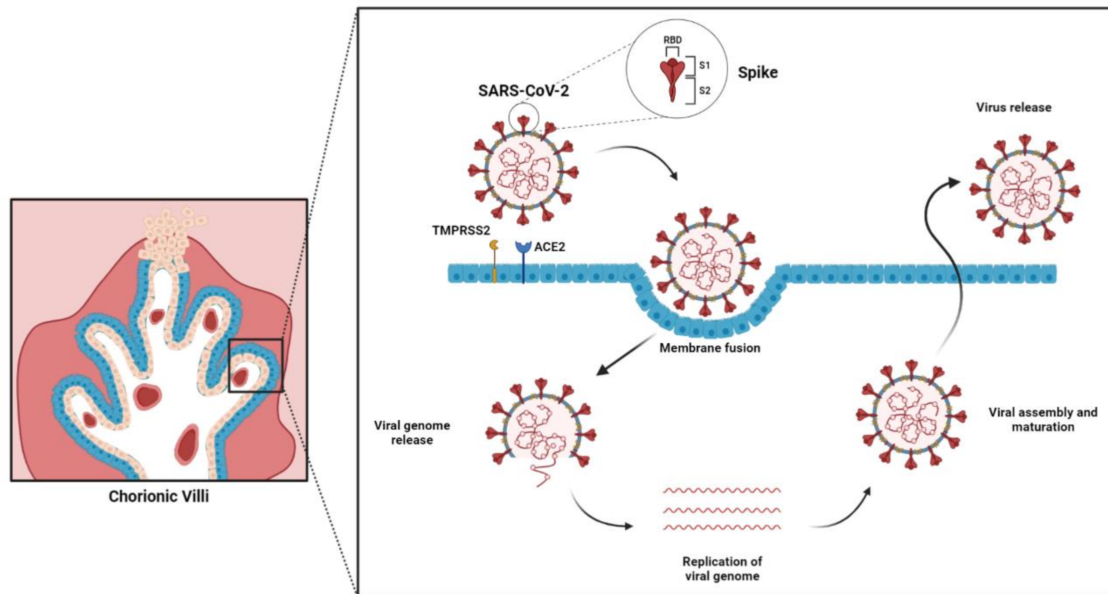


Figure 1.3 Schematic representation of SARS-CoV-2 infection cycle. After entering the human body, SARS-CoV-2 binds to epithelial cells in the respiratory tract through ACE2 in synergy with TMPRSS2, leading to membrane fusion and release of viral genome into the host cytoplasm. Following viral genome release, replication starts and, after viral assembly and maturation, the virus exits the host cell by the fusion of particle-loaded vesicles with the cell membrane.

Nowadays, the commonly used tests for SARS-CoV-2 infection diagnosis can detect specific viral gene regions or viral antigens[63]. RT-PCR tests are the most used as they directly detect the RNA viral genome rather than secondary biomarkers and present clinically validated results[63].

On the other hand, serological tests are used to detect specific antibodies and allow a more accurate estimate of the cumulative prevalence of SARS-CoV-2 infection and immunity in a population[64,65]. The most used antibodies for diagnosis are IgM, IgA and IgG. For SARS-CoV-2, both IgA and IgM antibodies start to be produced a few days after the infection, have a peak around 2 weeks and then start to decrease[64,66]. The production of IgG starts in the second week after infection and can last up to 6-8 months[64,67] (Figure 1.4). An important feature of IgG is to be the only antibody capable of crossing the placental barrier[13,15,16,18]. As neither IgA or IgM can cross the placenta, their presence in umbilical cord blood can only result from fetal production. Thus, IgA and IgM detection in umbilical cord blood can be used as a marker of *in uterus* infection of the fetus[68,69].

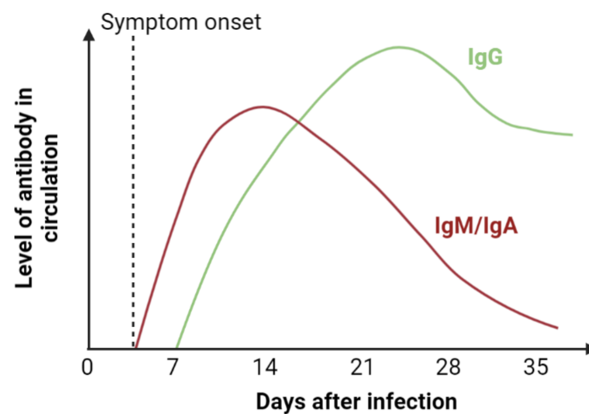


Figure 1.4 Levels of antibodies in circulation after SARS-CoV-2 infection. Anti-Spike IgA and IgM antibodies start to be produced a few days after the infection, have a peak around 2 weeks and then start to decrease. The production of anti-Spike IgG starts in the second week after infection and can last up to 6-8 months. Figure data based on references 64, 66 and 67.

1.3.2 Pregnant women as a risk group

Elderly individuals or/and with pre-existing comorbidities such as hypertension, diabetes, cardiovascular diseases, lung diseases and immunological disfunction are considered risk groups for developing severe or fatal COVID-19[70]. During pregnancy, the immunological and physiological changes in the woman's body make them prone to develop viral infections, including COVID-19[71,72]. On the past SARS-CoV and MERS-CoV outbreaks, pregnant women were susceptible to severe forms of the diseases associated with these viruses but only 12 and 11 cases were reported, respectively[73,74].

In the beginning of the SARS-CoV-2 outbreak less was known about the consequences of this infection in this risk group compared to other known viral diseases such as influenza, hepatitis and AIDS[46,75,76]. Now that more than two years have passed since the initial outbreak, the knowledge in this area has improved. Being SARS-CoV-2 positive while pregnant might have consequences for both the mother and the fetus considering that the hormonal profile of a normal pregnancy is characterized by an early increase of ACE2[77,78]. At the maternal-fetal interface of SARS-CoV-2 positive pregnant women, robust immune responses, including increased activation of T and NK cells and increased expression of markers associated with pregnancy complications have been found[78]. Concerning pregnancy complications, pre-eclampsia, pre-term birth and stillbirth/miscarriage have already been associated with SARS-CoV-2 infection during pregnancy[77,79–81]. Besides this, invasion of the placenta by SARS-CoV-2 virus has already been observed[82] and can lead to miscarriage[79]. In addition, previous studies have shown the expression of ACE2 in the placenta[83,84] and, even though rare, there have already been reported cases of confirmed vertical transmission[85,86]. Regarding newborn infection, a review of reported SARS-CoV-2 neonatal infections determined that only 30% of the infection cases were due to vertical transmission while 70% was due to environmental transmission post-partum, including direct contact with the mother[87].

Concerning antibody transfer, diseases, such as AIDS and malaria, are known to diminish antibody transfer of specific antibodies[33]. For SARS-CoV-2 infection, a study correlated the diagnostic time until delivery with the increase of SARS-CoV-2-specific IgG antibodies showing transfer ratios higher than 1, indicating an efficient transplacental transfer[88]. Contrarily, another study found a

compromised SARS-CoV-2-specific placental antibody transfer associated to women infected in the third trimester but an efficient transfer concerning SARS-CoV-2 non-specific antibodies [89].

Histopathological analysis of placentas from SARS-CoV-2 infected pregnant women from several studies revealed increased inflammation and fibrin deposition, maternal and fetal vascular malperfusion and villitis[90,91]. These features are related to placental injury and can lead to pregnancy complications and repercussions in the newborn's development[91].

1.4 Open questions and objectives

Over the past year, a huge effort has been done globally to fight the current SARS-CoV-2 pandemic with the development of several safe and effective vaccines for COVID-19 accomplished in a record time. Some of them include vaccines developed by Pfizer-BioNTech, AstraZeneca and Moderna[92]. Usually, pregnant women and newborns are excluded from clinical trials and, consequently, from vaccination campaigns until additional studies including these groups are made[93,94] which contributes to their risk group status. Even though more than 3 billion people have already been fully vaccinated[53], the number of cases is still rising, so it is still of great importance to evaluate the impact of SARS-CoV-2 infection during pregnancy has on the mother and, consequently, on the newborn. By increasing the knowledge on this subject, we can help define a proper obstetric and pediatric management, take conclusions about *in uterus* protection of the newborn and, possibly, understand more about this new coronavirus. Having this in mind, with this project we aim to: document the frequency of SARS-CoV-2 vertical transmission through detection of Spike-specific IgA and IgM antibodies in umbilical cord blood; determine the Spike-specific IgG transfer ratio associated to infection; assess both the mother's and the fetus' humoral and cellular immune responses; take conclusions about immunity and *in uterus* protection of the newborn and, finally, identify histological changes associated to SARS-CoV-2 infection. A long-term objective is to confirm vertical transmission through detection of viral particles in the placenta, assess cytokine production through functional assays and evaluate the impact of *in uterus* infection in baby´s development.

2. Materials and Methods

2.1 Study participants and human samples

Tissue and blood samples were collected between May 2020 and February 2021 from pregnant women who were SARS-CoV-2 positive (diagnosed by RT-PCR test from nasopharyngeal and/or oropharyngeal swabs in a laboratory certified by the Portuguese National Health Authorities) within their gestational period and whose delivery was performed at Maternidade Dr. Alfredo da Costa (MAC), in Lisbon, Portugal. Control samples were collected from mothers with no previous history of SARS-CoV-2 infection, no problems associated and whose delivery was also performed at MAC. All participants were non-vaccinated. In total, we received samples from 60 pregnant women including 58 blood samples from mothers, 52 blood samples from umbilical cords, 58 placenta tissue samples and 51 umbilical cords tissue samples. Different numbers of samples were used for the different parts of the project. Tissue samples, placenta and umbilical cord, were delivered to the histology facility from Centro de Estudos de Doenças Crónicas (CEDOC), after 48 hours in new formol media, where they were processed into paraffin blocks and H&E stained. Blood samples were delivered in heparin tubes and ficoll isolation was performed. Clinical data from all study participants can be found at Table 1 in Appendix. All participants provided informed consent and all procedures were approved by ethics committee (112/2021/CEFCM and 859/2020), in accordance with the provisions of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonization.

2.2 Peripheral blood cells isolation

Whole blood from heparin tubes was diluted with an equal volume of Phosphate Buffered Saline (PBS) 1x and then layered carefully over a ficoll layer. After 30 minutes 1200g centrifugation without brake at 20˚C, plasma and the peripheral blood mononuclear cells (PBMCs) layer were removed with a Pasteur pipette. Plasma was stored in cryotubes at -80˚C and in Eppendorfs at -20˚C and 4˚C for further analysis. PBMCs were washed two times with PBS 1x at 700g for 10 and 5 minutes, respectively and then resuspended in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (DMSO). PBMCs were stored in cryotubes at -80˚C for subsequent analysis[95,96].

2.3 Enzyme-Linked Immunosorbent Assay

The Enzyme-Linked Immunosorbent Assays (ELISA) performed for detecting antibodies binding to SARS-CoV-2 trimeric spike protein or its RBD domain were based on a published protocol[97] and customized in our laboratory[64]. Serum/plasma samples were heat-inactivated at 56˚C for 15 minutes before being loaded. 96-well ELISA plate (ThermoScientific) was coated with 50 µL of 0.5 mg/mL trimeric Spike protein or RBD diluted in PBS 1x and incubated overnight at 4˚C. After plate washing 3 times with PBS-Tween20 (PBST) 0,1% (washing buffer) using an automatic plate washer (ThermoScientific), 100 µL of 3% dry milk in PBST 0,05% (for IgG and IgA) or 3% Bovine Serum Albumin (BSA) in PBST 0,05% (for IgM) were added to each well to block. Blocking was performed for 1 hour at room temperature (RT). Serum/plasma samples were diluted 1:50 in 1% dry milk /BSA in PBST 0,05% and seriated dilutions of 1:3 were made reaching a final dilution of 1:109350. The calibrators included serum from PCR-tested SARS-CoV-2 infected individuals classified as low (1:150), moderate (1:450) and high ($\geq$ 1:1350) antibody producers according to their antibody titer[64]. Two individual samples from each group were used. As negative control we used a pre-pandemic sample and

one blank well. Serially diluted samples were incubated for 1 hour at RT. Following sample incubation, the plate was washed 3 times. Secondary antibody solutions of goat anti-human IgG/IgA/IgM-HRP 1:25000 diluted in 1% dry milk/BSA in PBST 0.05% were prepared and 50 µL distributed to each well. The secondary antibodies were incubated for 30 minutes at RT and then 3 washes were performed. Following incubation, 50 µL of TMB substrate were added to each well for approximately 7 minutes at RT. The reaction was stopped by adding 25 µL of 1M phosphoric acid and absorbance at 450 nm was measured using a plate reader (BioTek). Antibody titer was assessed using the values of optical density at 450 nm (OD_{450nm}) and considering a cut-off value of 0.15. Information relative to reagents and antibodies used is present in Appendix Table 2 and 3, respectively.

2.4 Neutralization assay

Following heat inactivation, plasma samples were four-fold serially diluted and incubated with spike pseudotyped lentiviral particles for 1h at 37˚C. The mix was added to a pre-seeded plate of 293T-Ace2 cells, with a final multiplicity of infection (MOI) of 0.2. After 48h, fluorescence was measured using the GloMax Explorer System (Promega). The relative fluorescence units were normalized to those derived from the virus control wells (cells infected in the absence of plasma), after subtraction of the background in the control groups with cells only.

2.5 Flow cytometry

For the detection of SARS-CoV-2 reactive T, B and NK cells, cryopreserved PBMCs were rested for 10 minutes at 37˚C and transferred to falcon tubes with RPMI1640 medium supplemented with 10% of FBS plus 1% of antibiotic/antimycotic plus 1% of glutamine. After 10 minutes 700g centrifugation, the supernatant was discarded and the pellet containing the PBMCs was resuspended in RPMI1640 media and transferred to a 24-well plate (Costar). We used an activation induced marker (AIM) assay in which following 1 hour incubation at 37˚C, PBMCs were stimulated overnight with either 1 mg/mL of spike protein plus 5 µg/mL of anti-CD28 cross-linked with 2.5 µg/mL of anti-mouse IgG (stimulated) or with medium alone (negative control- unstimulated). IL-2 (20 IU/mL) was added to all wells. On the following day, PBMCs were stained with a fixable viability dye eFluor™ 506 and surface labelled with the following antibodies: anti-CD3, anti-CD4, anti-OX40, anti-CD25, anti-CD69, anti-CXCR-5, anti-CCR6, anti-CD19, anti-IgD, anti-CD27 and anti-CD20. RBD labelling was performed using an available commercial kit according to manufactor's instructions. After labelling, cells were washed, fixed with 1% Paraformaldehyde (PFA) and acquired in BD FACS Aria III equipment (BD Biosciences). Results were then analysed with FlowJo v10.7.3 software (Tree Star). Information relative to reagents and antibodies used is present in Appendix Table 2 and 3, respectively.

2.6 Image acquisition and Histological score

H&E-stained tissue samples slides (SARS-CoV-2 and control samples) were observed using Zeiss Imager Z2 Microscope and the ZEN2012 Pro software. Processing of images was performed using Fiji Image J v1.53m software. Placental and umbilical cord H&E-stained tissues were also observed and scored by two independent pathologists blinded to patient SARS-CoV-2 infection state**.** Features scored included villitis (absent, low grade or high grade), intervillositis (absent or present), intervillous fibrin (absent, mild or moderate), deciduitis (absent or present), fetal and maternal vascular malperfusion (absent or present) and chorioamnionitis (absent or present) for the placentas analysis and

microcalcification (absent or present) and presence of inflammatory infiltrates in the amnios (absent or present) for the umbilical cords analysis.

2.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 software. The nonparametric Wilcoxon test and the parametric *t* test were used when appropriated. Spearman and Pearson correlation tests were used in correlation analysis for nonparametric data and parametric data, respectively. When comparing 3 groups, p-values were determined by ANOVA, post-hoc Friedman and Kruskal-Wallis, post-hoc Dunn's multiple comparison test and ANOVA post-hoc Holm-Šídák multiple comparison test to compare means between groups. Statistical significance was considered for $p\text{-}value < 0.05$. For the neutralization experiments the half-maximal neutralization titre (NT_{50}), defined as the reciprocal of the dilution at which infection was decreased by 50%, was determined using four parameter nonlinear regression (least squares regression without weighting; constraints: bottom=0).

3. Results

3.1 Humoral response evaluation through serological tests

3.1.1 Antibody production

Antibodies are key elements for an effective immune response; therefore, their presence indicates that an immune response against the pathogen has occurred or is occurring. Serological tests, usually used to detect pathogen-specific antibodies, allow confirmation of a previous infection and to take conclusions about the immune response and, consequently, antibody protection.

Using blood samples from 58 pregnant women who were SARS-CoV-2 positive within their gestational period and blood samples from the respective umbilical cords, we evaluated antibody production by mothers and newborns dyads. Detection of IgG, IgA and IgM SARS-CoV-2-specific antibodies was made through an ELISA assay based on a protocol from Krammer's laboratory[97] and customized in ours[64].

As mentioned before, IgG is the only type of antibody capable of crossing the placental barrier[15–18] so it is expected to find the presence of this antibody in umbilical cord samples. The same does not happen for IgA or IgM since they do not have the same capability. Thus, the detection of these two immunoglobulins in umbilical cord blood can be considered a marker of *in uterus* infection[68,69]. When looking into the antibody profile among our cohort (Figure 3.1) there is a higher percentage of triple positive (46%) and a lower percentage of seronegative mothers' samples (9%) when comparing to umbilical cord samples (4% and 31%, respectively). Also, for the umbilical cord samples there were no double positive samples, and the majority (65%) was positive only for IgG.

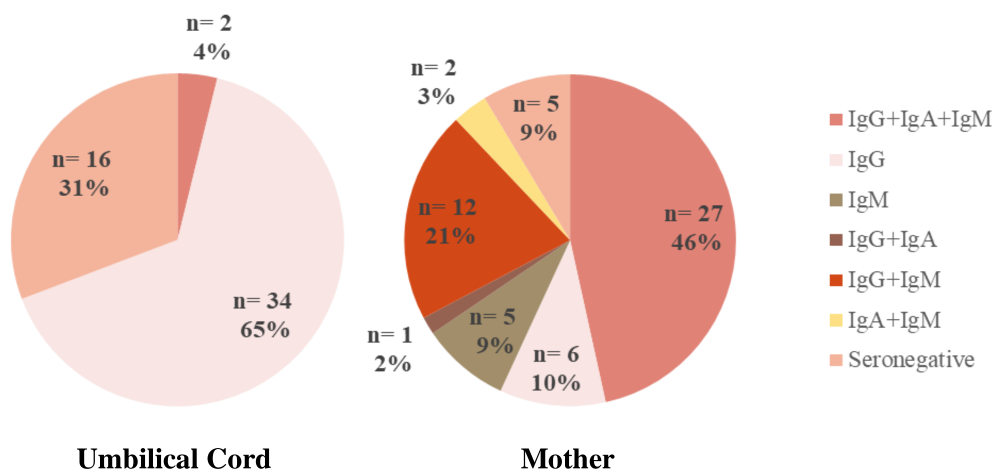


Figure 3.1 Antibody profile distribution among mother and umbilical cord samples. Antibody profile was divided into triple positive (IgG+IgA+IgM), double positive (IgG+IgA, IgG+IgM or IgA+IgM), single positive (IgG, IgA or IgM) and seronegative according to the OD_{450nm} values. n= 58 mother samples and n= 52 umbilical cord samples.

In order to make direct comparisons between mothers and umbilical cord samples, we decided to use only paired samples (mother samples that had the correspondent umbilical cord sample; n=50) for the following analysis. Considering the OD_{450nm} values of the mothers, the IgG and IgM values are higher than IgA values, being the highest values for IgM (Figure 3.2 A). In total we have 39/50 (78%), 27/50 (54%) and 41/50 (82%) mothers positive for anti-spike IgG, IgA and IgM antibodies, respectively. Given the fact that the three isotypes of antibodies analysed are produced at different time points during infection, the absence of any of them can be normal if we consider the time between the diagnosis and delivery. It is expected that mothers who were infected earlier in pregnancy will have higher levels of IgG while mothers who were infected close to birth will have higher levels of IgA and/or IgM.

As expected, there is a high number of umbilical cord samples positive for anti-spike IgG (32/50; 64%) but only 2/50 (4%) samples are positive for anti-spike IgA and IgM (Figure 3.2 A). By comparing mother and umbilical cord samples, in general, cord samples tend to have lower levels of SARS-CoV-2-specific antibodies (Figure 3.2 B). Interestingly, one of the umbilical cord samples is positive for both anti-spike IgA and IgM while the correspondent mother is not (black dot in Figure 3.2 B). In addition to knowing that both IgA and IgM cannot pass the placental barrier, the fact that the mother did not have these antibodies in circulation allows us to exclude completely the possibility of transfer from the mother to the fetus. Thereby, the presence of both IgA and IgM found in the umbilical cord blood indicates a possible infection of the fetus. Unfortunately, a SARS-CoV-2 PCR test was not performed to the newborn in question so we cannot be certain about his state of infection. As the presence of IgA and IgM in umbilical cord blood only occurred in two samples, we can say that vertical transmission is indeed rare, in agreement to previous reports[86,88,91].

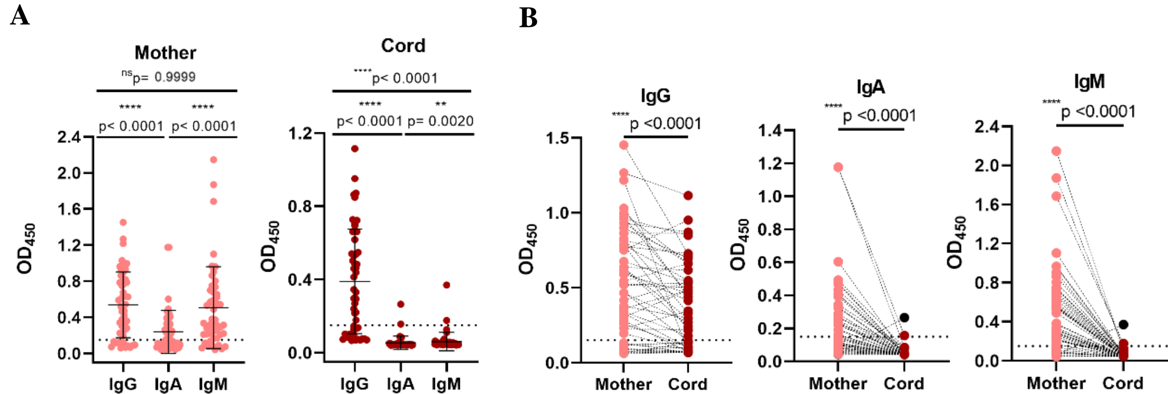


Figure 3.2 Levels of anti-Spike IgG, IgA and IgM are present at higher levels in mother samples. **(A)** Anti-Spike IgG, IgA and IgM levels in mother samples (left) and umbilical cord samples (right). **(B)** Comparisons between anti-Spike IgG, IgA and IgM levels in mother samples (pink) versus umbilical cord samples (dark red). Black dot indicates the umbilical cord sample with higher levels of anti-Spike IgA and IgM than the mother. Dashed line indicates the assay cut off. n= 50 dyads. p-values determined by ANOVA, post-hoc Friedman, post-hoc Dunn's multiple comparisons test when comparing 3 groups and by non-parametric paired Wilcoxon test when appropriate. ****p<0.0001, ***p<0.001, **p<0.01, ns: not significant.

To verify if the absence or presence of antibodies could be indeed related with the time spanned from diagnosis to delivery, we looked into the dates of the first SARS-CoV-2 positive test of the mothers, located it into the three pregnancy trimesters and related it with IgG abundancy. For this analysis all mother samples were considered (n=58). None of the mothers was infected in the first trimester of pregnancy and only 9 were infected in the second trimester (Figure 3.3). Since most of our study participants were infected in the third trimester we decided to divide it into diagnosed two weeks before birth, having in mind the two weeks incubation period for SARS-CoV-2 virus. Most of the mothers (39/58; ≈67%) were diagnosed as SARS-CoV-2 positive only 2 weeks before birth, being many of them diagnosed at the time of labor, and only 10 diagnosed more than 2 weeks before birth (Figure 3.3). Furthermore, only mothers diagnosed two weeks before birth were seronegative for IgG. This may explain why the levels of IgM are higher than the levels of IgG, since the last one only starts to be produced more or less 2 weeks after infection[64,67].

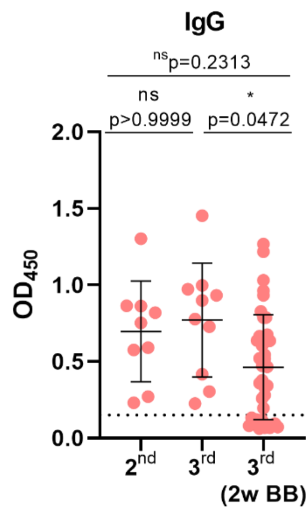


Figure 3.3 Time elapsing from diagnosis to delivery influences anti-Spike IgG levels in mother samples. 2nd: second trimester; 3rd: third trimester; 2wBB: two weeks before birth. Dashed line indicates the assay cut off. n= 58 mother samples. p-values determined by ANOVA post-hoc Kruskal-Wallis, post-hoc Dunn's multiple comparisons test. *$p<0.05$, ns: not significant.

3.1.2 Correlation between the age of the mother and antibody production

As age can be considered a risk factor for pregnancy[98] and SARS-CoV-2 infection[70,71], we wanted to see if there was a correlation between the age of the mothers and their antibody production. In this analysis, only paired and seropositive mothers were considered. For all three types of antibodies no correlation was found (Figure 3.4).

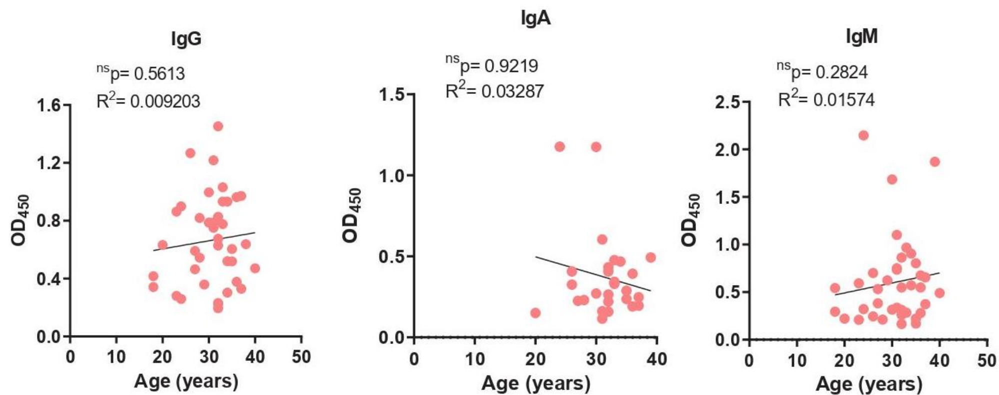


Figure 3.4 No correlation between the age of the mother and anti-Spike antibody production. Only paired and seropositive mother samples were considered. n=39 (IgG), n=27 (IgA) and n= 41 (IgM). p-values determined by Pearson and Spearman correlations. ns: not significant.

3.1.3 Antibody transfer

Transmission of maternal IgG antibodies to the newborn occurs prenatally via placenta and postnatally through breastfeeding[15] and allows protection of the newborn while their immune system is not fully capable[35]. Some diseases, such as AIDS and malaria, are known to diminish antibody transfer of specific antibodies[33]. Therefore, we wanted to evaluate the Spike-specific IgG transfer ratio from mother to newborn. We determined that the mean transfer ratio of IgG antibodies was below 1 in the third trimester, indicating an inefficient transfer of SARS-CoV-2-specific IgG antibodies to the newborn (Figure 3.5). Even though overall inefficient, there was transfer of SARS-CoV-2- specific IgG antibodies from the mother to the newborn indicating that protection might still be considered.

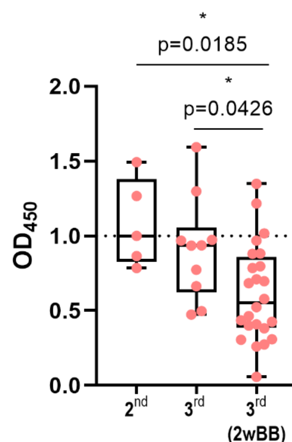


Figure 3.5 Transfer of anti-Spike IgG from mother to newborn during third trimester is inefficient. Only paired samples in which both mother and newborn were seropositive for IgG were considered. 2nd: second trimester; 3rd: third trimester; 2wBB: two weeks before birth. Dashed line indicates the efficient transfer cut-off. n= 33.p-values determined by ANOVA post-hoc Holm-Šídák multiple comparisons test. *p<0.05.

3.1.4 Antibody function

As said before, antibodies have an important role in the development of a specific and effective immune response against pathogens. Among antibodies functions, one of them is the capacity to neutralize the pathogen[15], in this case by blocking the interaction between the RBD of the SARS-CoV-2 spike glycoprotein and the ACE2 human cell surface receptor. When this happens, they are called neutralizing antibodies and they can be generated by infection or by vaccination. To test if the circulating SARS-CoV-2-specific IgG antibodies from mothers and newborns were neutralizing we performed a neutralization assay. As this assay is based on the blocking of the interaction between RBD and ACE2, an ELISA assay using RBD as antigen was also performed using the protocol described above in Materials and Methods. Just like for Spike, cord samples had lower levels of SARS-CoV-2-specific IgG antibodies, therefore no differences in specificity were found (Figure 3.6 A). For the neutralization assay, plasma samples, including non-paired samples, at different dilutions were incubated with spike pseudotyped lentiviral particles and fluorescence was measured. The results showed that 29/58 (50%) mothers and 9/52 (≈ 17%) umbilical cords had neutralizing antibodies (Figure 3.6 B). It was clear that some samples had stronger neutralizing antibodies then others. Therefore, a division according to the NT_{50} value, defined as the reciprocal of the dilution at which infection decreases 50%, was made and the samples were classified as strong neutralizers ($NT_{50} \geq 100$) or weak neutralizers ($NT_{50} < 100$). From the 29 neutralizing mother samples, 15 (≈52 %) were weak neutralizers and 14 (≈ 48%) were strong neutralizers. Contrarily, the few umbilical cord samples that were neutralizing were only weak neutralizers.

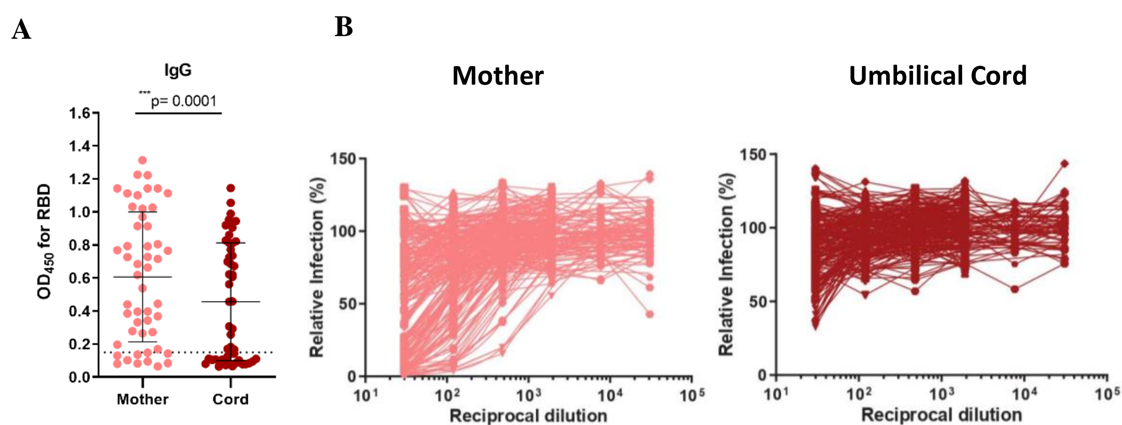


Figure 3.6 Most umbilical cord samples do not have anti-Spike IgG antibodies with neutralizing properties. (A) Levels of anti-RBD antibodies in mother (pink) and umbilical cord (dark red) samples **(B)** Plasma neutralization titers (NT_{50}) in mother (pink, left) and umbilical cord (dark red, right) samples. n=50 dyads (RBD ELISA assay). n=58 mother samples and n=52 umbilical cord samples (neutralization assay). Dashed line indicates the assay cut off. p-value determined by non-parametric paired Wilcoxon test. ***p<0.001

3.2 Cellular response evaluation through Flow Cytometry

As humoral and cellular responses are interconnected, we wanted to investigate what type of immune cells mothers and newborns had in circulation, especially T, B and NK cells. The detection of SARS-CoV-2 reactive cells was made by flow cytometry and using cryopreserved PBMCs. For this analysis, only paired samples and samples from which was possible to isolate PBMCs were used. As gating strategy, we decided to start from lymphocytes, then live cells and then proceed to the different types of cells (T, B and NK cells) using their characteristic markers (Figures 3.7, 3.9 and 3.11, respectively).

3.2.1 T cells

The percentage of CD4$^+$ T cells, identified by the co-expression of CD3 and CD4, in mother and umbilical cord samples did not vary (Figure 3.8 A). Contrarily, when talking about SARS-CoV-2-specific T cells (Spike T cells), identified by the co-expression of OX40 and CD25, mother samples presented a significant higher percentage than umbilical cord samples (Figure 3.8 B). To evaluate the profile of these Spike T cells, CCR6, CXCR5 and CD69 markers were used to identify CD4$^+$ T cells migrating to infections sites, Tfh cells and activated CD4$^+$ T cells, respectively. We determined that most umbilical cord samples had almost no specific Tfh cells migrating to infection sites (CXCR5$^+$CCR6$^+$ cells) when comparing to mother samples (Figure 3.8 C). Interestingly, even though having almost no Spike T cells, umbilical cord cells were more activated than mother cells (Figure 3.8 D).

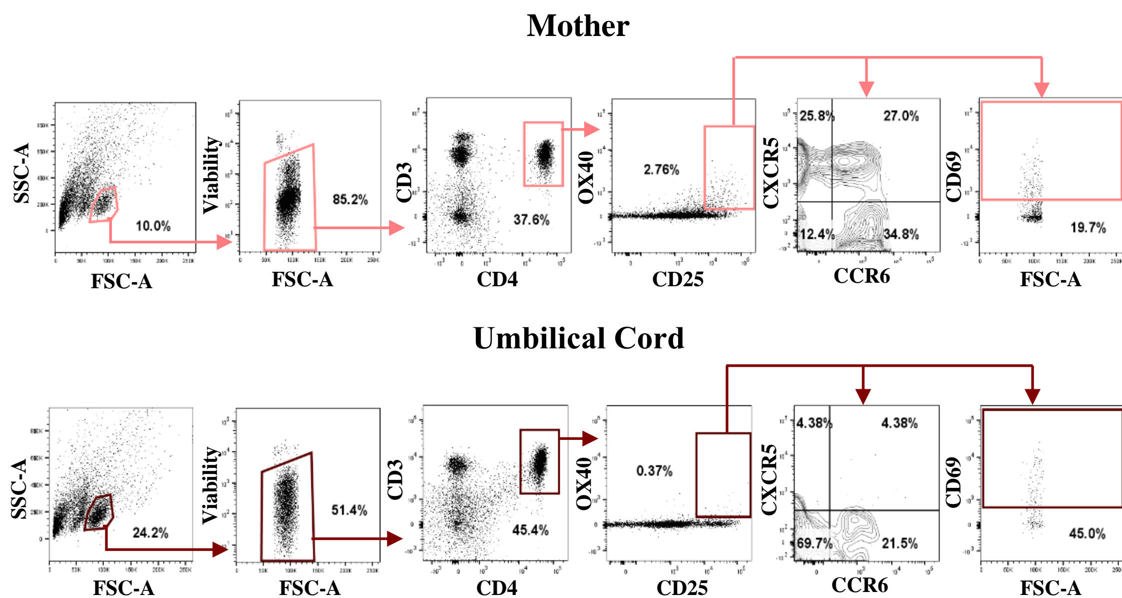


Figure 3.7 Gating strategy applied for T cell analysis of mother and umbilical cord samples. For analysing T cells, we started by gating the lymphocytes, then live cells and then T cells, identified by the co-expression of CD3 and CD4. Among T cells, Spike T cells were identified by the co-expression of OX40 and CD25 markers. Spike T cells profile was then analysed using the markers CXCR5, CCR6 and CD69 to identify Tfh cells, CD4$^+$ T cells migrating to infections sites and activated CD4$^+$ T cells, respectively. The same gating strategy was applied in mother (pink) and umbilical cord (dark red) samples. Samples used include only paired samples with PBMCs and in stimulated conditions (n= 27 dyads).

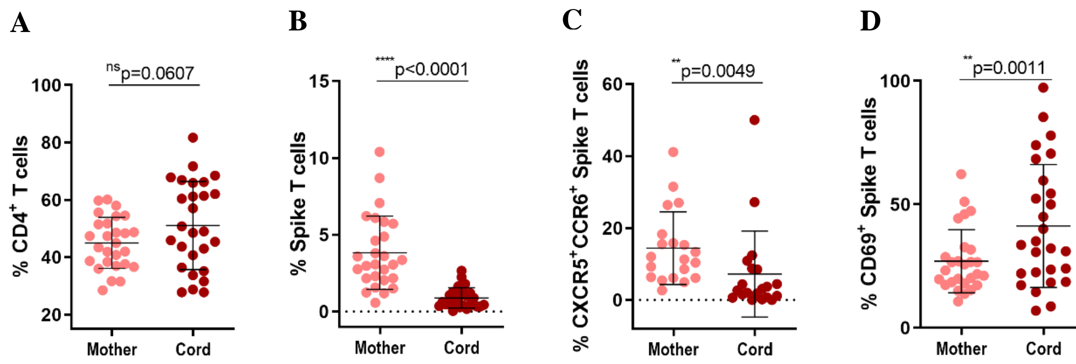


Figure 3.8 Spike T cells are present in umbilical cord samples in lower levels than in mother samples. Cumulative frequency of circulating (**A**) $CD4^+$ T cells, (**B**) Spike T cells, (**C**) $CXCR5^+CCR6^+$ T cells and (**D**) $CD69^+$ T cells in mother (pink) and umbilical cord (dark red) samples. n= 27 dyads. p-values determined by parametric paired t test and by non-parametric paired Wilcoxon test when appropriate. ****p<0.0001, **p<0.01, ns: not significant.

3.2.2 B cells

Just like for T cells, there were no significant differences in the percentages of B cells from mothers and umbilical cords (Figure 3.10 A). After identifying B cells by the expression of CD19 marker and absence of CD3 marker, we wanted to see if there were differences in the naïve and memory B cell populations. The co-expression of CD20 and CD27 identifies memory B cells while the expression of CD20 and the absence of CD27 identifies naïve B cells. As expected, there were significant differences in both populations. Mothers presented higher percentages of memory B cells (Figure 3.10 B) while umbilical cords presented mainly naïve B cells (Figure 3.10 C). For identifying reactive B cells, we used the RBD marker. Interestingly, umbilical cord samples presented a higher percentage of RBD^+ B cells when compared to mother samples (Figure 3.10 D). However, these cells represent a pool of naïve B cells with the potencial to bind RBD. IgD is expressed by naïve B cells[3] and the absence of this molecule identifies class-switched B cells, meaning B cells that were activated to produce antibodies. In agreement with the previous results, cord samples presented lower percentages of IgD^- cells (Figure 3.10 E) showing that most of the umbilical cord B cells are indeed naïve.

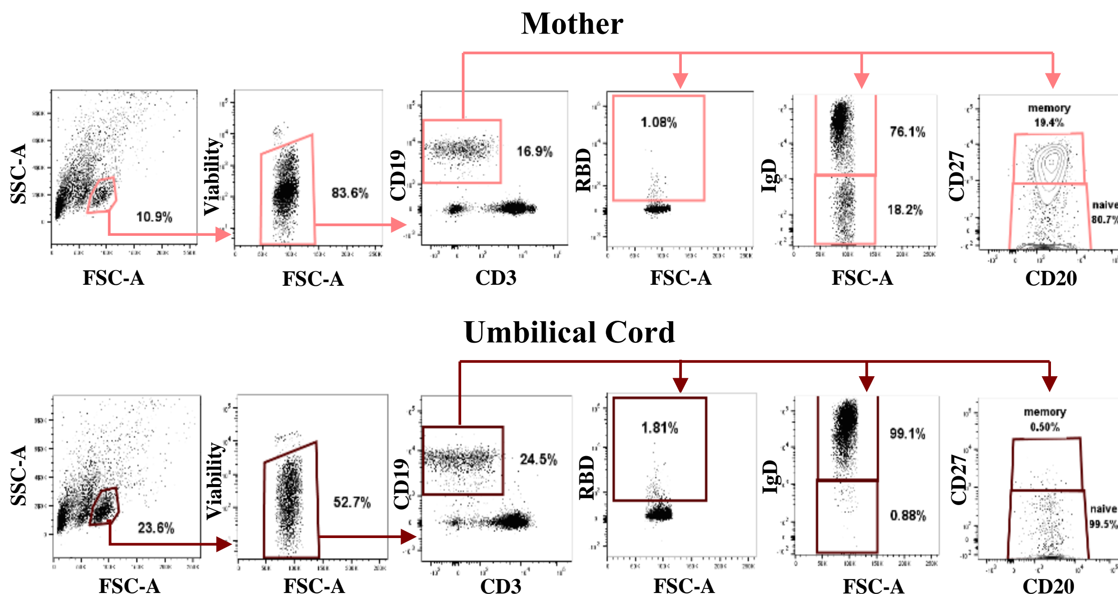


Figure 3.9 Gating strategy applied for B cell analysis of mother and umbilical cord samples. For analysing B cells, we started by gating the lymphocytes, then live cells and then B cells, identified by the expression of CD19 and the absence of CD3. Among B cells, reactive B cells were identified with the marker RBD, and naïve and memory B cells were identified using the markers CD27 and CD20. Expression of CD20 and the absence of CD27 identifies naïve B cells and co-expression of CD20 and CD27 identifies memory B cells. Class-switched B cells were identified by the absence of IgD. The same gating strategy was applied in mother (pink) and umbilical cord (dark red) samples. Samples used include only paired samples with PBMCs and in unstimulated conditions (n= 32 dyads).

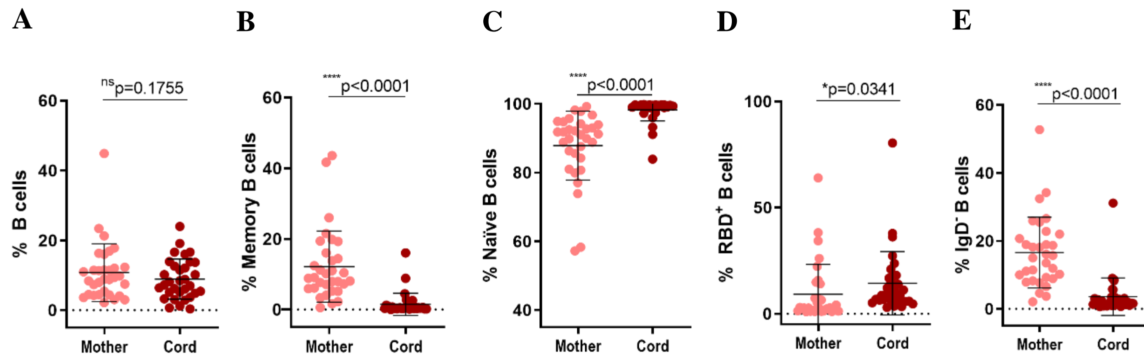


Figure 3.10 Naïve B cells in umbilical cord samples have the potencial to bind RBD. Cumulative frequency of circulating **(A)** B cells, **(B)** memory B cells, **(C)** naïve B cells, **(D)** RBD$^+$ B cells and **(E)** IgD$^-$ B cells in mother (pink) and umbilical cord (dark red) samples. n= 32 dyads. p-values determined by parametric paired t test and by non-parametric paired Wilcoxon test when appropriate. ****p<0.0001, *p<0.05, ns: not significant.

3.2.3 NK cells

To identify NK cells, their characteristic marker, CD56, was used. Instead of only identifying the NK general population we decided to look into differences between the CD56^{dim} and CD56^{bright} NK cell populations from mothers and newborns. No significant differences were found either in CD56^{bright} (Figure 3.12 A) or CD56^{dim} (Figure 3.12 B) populations.

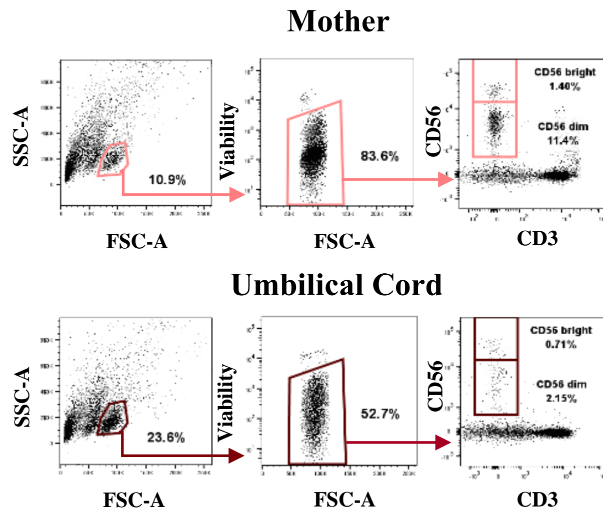


Figure 3.11 Gating strategy applied for NK cell analysis of mother and umbilical cord samples. For analysing NK cells, we started by gating the lymphocytes, then live cells and then NK cells, identified by the expression of CD56 and the absence of CD3. NK cell population was divided into CD56^{bright} and CD56^{dim} according to CD56 expression. The same gating strategy was applied in mother (pink) and umbilical cord (dark red) samples. Samples used include only paired samples with PBMCs and in unstimulated conditions (n= 32 dyads).

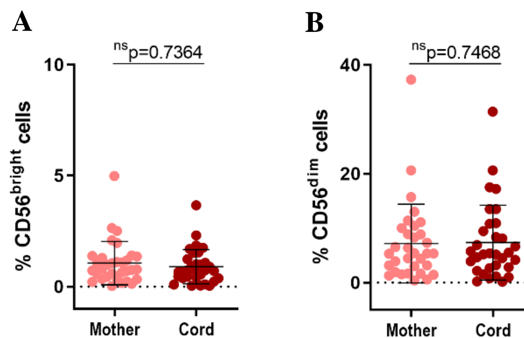


Figure 3.12 No differences in NK cell populations between mother and umbilical cord samples. Cumulative frequency of circulating **(A)** CD56^{bright} cells and **(B)** CD56^{dim} cells. Mother samples (pink), umbilical cord samples (dark red). n= 32 dyads. p-values determined by non-parametric paired Wilcoxon test. ns: not significant.

3.3 Histopathological analysis

Since the beginning of pregnancy until birth, the woman's immune system and tissues suffer several adjustments so that the fetus can have a healthy development and, lastly, be born. The first contact between mother and fetus occurs through the placenta and umbilical cord, both important structures to pass nutrients and oxygen. Besides their main functions, it is also through these structures that infection of the fetus can occur[30,46] since it is the only contact route between mother and fetus. Therefore, we aimed to determine if there were differences in the tissues from placentas and umbilical cords correspondent to SARS-CoV-2 infection compared to normal and healthy pregnancies. For this, H&E-stained tissue samples slides (SARS-CoV-2 and control samples) were observed using Zeiss Imager Z2 Microscope and images were processed using Fiji Image J. In general, no differences were found between SARS-CoV-2 and controls (Figure 3.13). The slides were also evaluated by two independent pathologists, blinded to state of infection of the samples, in terms of different features that included villitis, intervillositis, intervillous fibrin, deciduitis, chorioamnionitis, fetal and maternal vascular malperfusion (placentas analysis), microcalcification and presence of inflammatory infiltrates in the amnios (umbilical cord analysis). Again, most of the abnormalities were not predominant in tissue samples from women with SARS-CoV-2 infection (Figure 3.14). Only for intervillous fibrin and villitis (placentas) and microcalcification (umbilical cords) SARS-CoV-2 tissues had a higher frequency (Figure 3.14), but this might be explained by the low number of control samples (n=13) compared to the number of infected samples (n=58 placentas and n=51 umbilical cords).

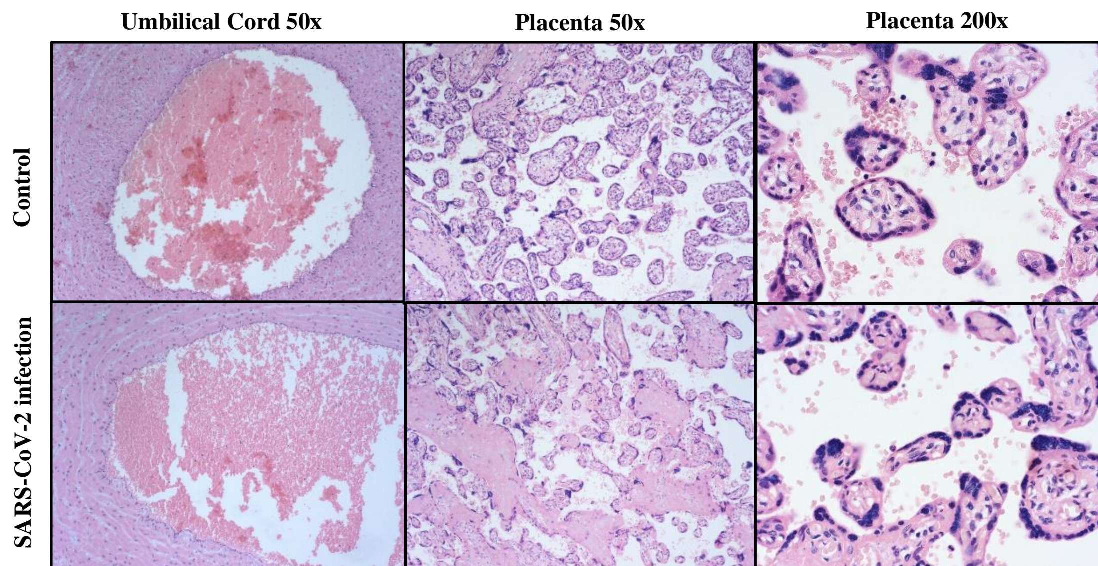
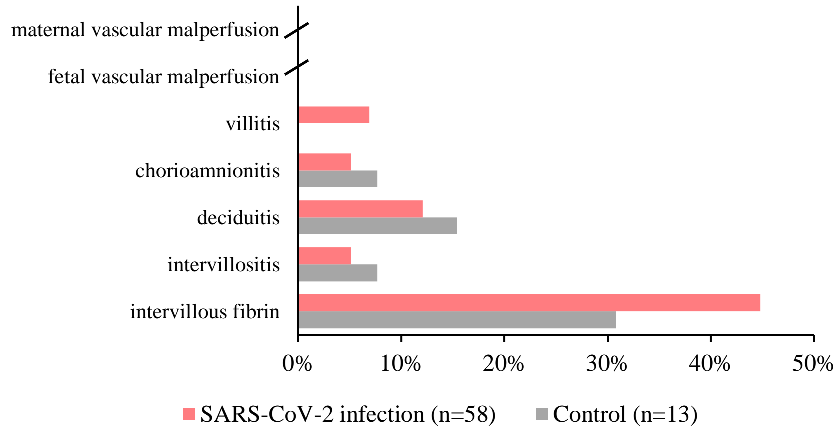


Figure 3.13 No histological differences were found between tissues from SARS-CoV-2 positive samples versus controls. Pictures of placenta and umbilical cord tissues from SARS-CoV-2 infection cases and matched controls were taken using Zeiss Imager Z2 Microscope. Umbilical cords were observed with a magnification of 50x while placentas were observed with 50x and 200x magnifications. n= 58 placenta tissue SARS-CoV-2 samples, n= 51 umbilical cord tissue SARS-CoV-2 samples, n= 13 placenta tissue control samples and n=13 umbilical cord tissue control samples.



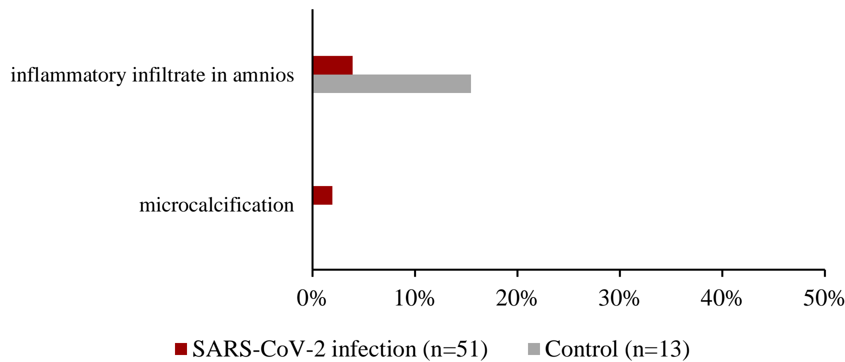


Figure 3.14 No histological features differences between tissues from SARS-CoV-2 positive samples versus controls. Placenta tissue samples (pink) were analysed for maternal and fetal vascular malperfusion, villitis, intervillositis, intervillous fibrin, deciduitis and chorioamnionitis. Umbilical cord tissue samples (dark red) were analysed for inflammatory infiltrates in the amnios and microcalcification. All tissue samples were analysed by two independent pathologists blinded to sample state of infection. n=58 placenta tissue SARS-CoV-2 samples, n= 51 umbilical cord tissue SARS-CoV-2 samples, n= 13 placenta tissue control samples and n=13 umbilical cord tissue control samples.

4. Discussion

Human pregnancy is a complex and unique process that involves physiological and immunological changes of the woman's body. These changes are crucial for the development of a healthy and normal pregnancy but are known to elicit pro- and anti-inflammatory strong immune responses[24] and increase the probability to develop viral infections, including COVID-19[71,72]. Considering the current SARS-CoV-2 pandemic and the risk associated to pregnant women, understanding the impact of the dual challenge pregnancy and infection on the mother and, consequently, on the newborn, is of extreme importance. The number of studies in this area has been continuously increasing; however, some questions still require further analysis. Here we investigate the impact of SARS-CoV-2 in human pregnancy through the analysis of blood and tissue samples from pregnant women infected by SARS-CoV-2 during their gestational period and correspondent umbilical cords using several techniques including serological tests, flow cytometry and histopathological analysis. We demonstrate that from 50 umbilical cord samples, 64% were seropositive for Spike-specific IgG but the overall transfer of antibodies from the mother to newborn was considered inefficient. Moreover, in two umbilical cord samples was detected the presence of Spike-specific IgA and IgM antibodies indicating a possible *in uterus* infection. Functionally, only 17% of the total umbilical cord samples had neutralizing antibodies even though with weak neutralizing properties. Contrarily, half of the mothers had neutralizing Spike-specific IgG antibodies in circulation with strong (48%) or weak (52%) neutralizing properties. Spike T cells and naïve RBD$^+$ B cells were found to be present in umbilical cord blood, suggesting a protective cell transfer from mother to newborn. Lastly, our results showed no significant histopathological differences between placentas from SARS-CoV-2 infected mothers and healthy mothers.

SARS-CoV-2 antibody response can be characterized by the production of IgA and IgM soon after infection, reaching a peak around two weeks following which they start to decrease[64,66]. Immunological memory is provided by IgG production that starts in the second week after infection and can last up to 6-8 months[64,67]. With different production time points, the detection of IgA, IgM and IgG depends on the initial date of infection. This means that serological tests performed at the beginning of infection will have higher levels of IgA and IgM while tests performed in latter stages of infection will have higher levels of IgG. At the same time, levels of antibodies also depend on the severity of the disease, with higher levels being associated with severe cases of COVID-19[59,99,100]. When distributing our study participants into the typical three trimesters, we determined that none of the mothers was diagnosed during the 1st trimester, 9 (≈16%) were diagnosed in the 2nd and 49 (84%) in the 3rd trimester, mostly two weeks prior to giving birth. Accordingly, assessment of antibody production showed that the levels of IgM SARS-CoV-2-specific antibodies were higher than IgG antibodies. The assessment of antibody production in newborns indicated that 64% were seropositive for Spike-specific IgG and only two samples presented levels of Spike-specific IgA and IgM antibodies. Through a neutralization assay we determined that only 17% of umbilical cord samples had neutralizing antibodies and were all weak neutralizers. Contrarily, half of the mothers had SARS-CoV-2-specific IgG antibodies in circulation with strong (48%) or weak (52%) neutralizing properties. Even though most umbilical cords did not present neutralizing antibodies, the presence of Spike- specific IgG antibodies in 64% of the samples suggests protection of the newborn since, besides neutralizing properties, antibodies can also generate an immune response through the recruitment of other immune effector cells[101].

Protection of newborns, while their immune system is still immature, can be achieved by placental transfer of maternal IgG antibodies or immune cells to the fetus[35]. Antibody transfer is mediated by the FcRn receptor present in the syncytiotrophoblast cells[16,36,37] and increases throughout

pregnancy, being minimal until 16 weeks of pregnancy and maximal 4 weeks before birth[33]. The efficiency of placental transfer depends on the maternal levels of IgG, gestational age, IgG subclass, nature of antigen and placental integrity with viral infections playing a major role[35,39]. For SARS-CoV-2 infection, Flannery *et al.* determined an efficient transfer of maternal SARS-CoV-2-specific IgG antibodies to the fetus with transfer ratios higher than 1 positively correlated with the time between diagnostics and birth[88]. In contrast, we determined an inefficient transfer during the third trimester (mean transfer ratio bellow 1) for Spike-specific IgG maternal antibodies. This result goes according to Atyeo *et al.* findings where SARS-CoV-2 non-specific antibodies were efficiently transferred while transfer of SARS-CoV-2-specific antibodies in third-trimester infected mothers was decreased and linked to altered glycosylation profiles[89]. Regardless of the inefficient transfer of Spike-specific IgG antibodies, levels of these antibodies were indeed passed to newborns and can confer future protection in case of infection. Nonetheless, disaggregating transfer ratios by time elapsing from diagnosis to delivery might provide a more nuanced and conclusive view.

Transfer of active immune cells from the mother to the fetus can occur during pregnancy via placenta and after birth via breastmilk[35]. The majority of transferred cells are T cells, and they can be retained until adulthood[35]. Through flow cytometry analysis, we were able to detect Spike- specific T cells in the umbilical cord blood, but much less prevalently than in mother's circulation. These cells were more activated than mother cells, probably indicating that transfer of T cells is enriched for activation status. Concerning B cells, we were also able to detect in the umbilical cord blood a pool of naïve B cells with potential to bind RBD. Since most newborns were not positive for SARS-CoV-2 infection, we can presume the presence of these cells was due to transplacental transfer. Even though presenting lower levels than mothers, the amount of Spike- specific T cells and the presence of naïve B cells with RBD binding potential will possibly be enough to induce a faster immune response against SARS-CoV-2 in case of newborn infection[35].

Together, our humoral and cellular responses analysis suggests that having COVID-19 while pregnant might confer a level of protection to the newborn through placental transfer of SARS-CoV-2-specific antibodies and immune cells.

It is known that diseases, including Influenza, AIDS and malaria, disturb the equilibrium of pregnancy being sometimes associated with pre-term birth, pre-eclampsia and stillbirth/miscarriage[30,46,75], and the same has already been described for SARS-CoV-2 infection[77,79–81]. Besides causing pregnancy complications, viruses can be vertically transmitted to newborns through placental infection[46]. The risk of placental infection is critically increased in the late 1st trimester and 2nd trimester due to the lack of a mature syncytiotrophoblast layer, an effective barrier against pathogens[30,46]. Interestingly, the timing corresponds simultaneously with the early increase of the ACE2 receptor in the human placenta [78,83]. This suggests that women are at higher risk of being infected throughout the late 1st trimester and 2nd trimester and, if so, the fetus also has an increased risk of vertical transmission due to placental infection. In addition to previously reported cases of SARS-CoV-2 vertical transmission[85,86], we present a case of possible vertical transmission confirmed by the presence of IgA and IgM SARS-CoV-2-specific antibodies in umbilical cord blood and absence in maternal blood. As IgA and IgM cannot pass through the placental barrier, their presence in umbilical cord blood indicates production by the fetus. The fact that the mother did not have these antibodies in circulation allows us to exclude completely the possibility of transfer from the mother to the fetus. In addition, for this sample the first positive SARS-CoV-2 RT-PCR test from the mother occurred in the beginning of the 2nd trimester, which supports our view. This only gives us an insight on a possible vertical transmission since the newborn in question did not perform the PCR test after birth and several points need to be taken in consideration when confirming a placental infection, including the detection and localization of the virus

in placental tissue[102]. Thereby, further studies concerning the detection of SARS-CoV-2 viral particles in placental tissues from our samples need to be assessed.

Pregnancy complications can be associated with maternal comorbidities and viral infections that result in histopathological abnormalities in placental tissues[46]. Features such as fetal and maternal vascular malperfusion, fibrin deposition and fetal thrombotic vasculopathy are often observed in cases of viral infections during pregnancy[46]. For SARS-CoV-2 infection, increased intervillous and subchorionic fibrin deposition, inflammation, villitis and fetal and maternal vascular malperfusion have already been reported[90]. Our analysis of H&E-stained tissue samples slides (SARS-CoV-2 and control samples) revealed no differences between SARS-CoV-2 and controls. Also, the histopathological analysis by two independent pathologists blinded to state of infection of the samples showed no differences between infected samples and control samples concerning features such as villitis, intervillositis, intervillous fibrin, deciduitis, chorioamnionitis, fetal and maternal vascular malperfusion, microcalcification and presence of inflammatory infiltrates in the amnios. Only for intervillous fibrin and villitis (placentas) and microcalcification (umbilical cords) SARS-CoV-2 samples had a higher frequency. These observations go according to the augmented fibrin deposition and villitis in SARS-CoV-2 cases reports[90,91]. Regarding the two samples with possible vertical transmission, intervillous fibrin deposition was observed. Throughout gestation, the syncytiotrophoblast cell layer loses integrity and small breaches start to appear. Due to the continuous discard of syncytiotrophoblast cell debris into the maternal circulation, hypoxic injury, or even immune-mediated injury from maternal antibodies, these breaches are filled with fibrinoid (composed by maternal blood fibrin, fibrin degradation products and cytotrophoblasts-derived matrix-type fibrinoid) alloying the separation between maternal and fetal circulation therefore preventing maternal-fetal haemorrhage[32,46]. We hypothesize that the increase of intervillous fibrin deposition can be considered a defense mechanism used by the maternal immune system to prevent virus infection through maternal blood. Moreover, calcification of umbilical cords has been considered a possible effect of intrauterine infections[103,104], which goes according to our findings. The decreased number of control samples also needs to be considered as a possible reason for the differences found between control and infected samples.

This project provided important results regarding the impact of SARS-CoV-2 in human pregnancy and tools for future studies. However, some limitations must be taken into consideration. RT-PCR tests from nasopharyngeal and/or oropharyngeal swabs are the most used SARS-CoV-2 diagnosis method due to their direct identification of viral genome and clinically validated results[63]. Nevertheless, in the newborn, the diagnostics efficiency of these tests has not been established yet. This could be due to the fact that *in uterus* infection of the newborn is not necessarily associated with the migration of SARS-CoV-2 virus into the upper respiratory tract of the newborn[105]. Also, is difficult to perform a nasopharyngeal swab in a neonate and obtain a sufficient amount of viral RNA to diagnostic. Other study limitations include the low number of control samples comparing to infected samples and the low quantity of blood, specially from the umbilical cord, which reduced the numbers of cells for flow cytometry analysis. This last limitation only made it possible to carry out one flow cytometry assay for each sample.

Overall, the study results allowed a general insight of the impact of SARS-CoV-2 infection in human pregnancy. Assessment of humoral and cellular responses suggested that immunity can be conferred to newborns via placenta and that, even though possible, vertical transmission is rare. Further studies will include the detection of SARS-CoV-2 viral particles within the placenta through immunohistochemistry techniques, assessment of cytokine production through functional assays and the

evaluation of the *in uterus* infection impact in baby´s development. Also, the high number of samples gathered will help develop other projects concerning SARS-CoV-2 infection.

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Appendix

Table 1- Clinical data from study participants.

	Symptomatic (n=30)	Asymptomatic (n=30)
Age of the mother (years)		
18-26	7	9
27-33	15	11
34-40	8	10
Ethnicity		
Caucasian	21	11
African	4	9
Asian	3	10
ROMA	1	0
Unknown	1	0
Comorbilities		
Yes	15	11
No	14	17
Unknown	1	2
Pregnancy Complications		
Yes	8	4
No	22	24
Internment due to COVID-19		
Yes	4	0
No	26	30
Gestational Age at time of delivery (weeks)		
25-34	3	2
35-37	4	3
38-39	14	9
40-41	8	16
Unknown	1	0
Gestational Age at time of COVID-19 diagnosis (weeks)		
14-23	5	3
24-32	3	0
33-35	4	2
≥ 36	18	25
Type of delivery		
Vaginal Delivery	17	14
Caesarean	13	15
Unknown	0	1
PCR test result from the newborn		
Positive	1	2
Negative	20	26
Unrealized	9	2

Table 2- Reagents used and respective information.

Reagent	Supplier	Identifier
Alexa Fluor™ 488 Antibody Labeling Kit	Life Technologies	A20181
Antibiotic/antimycotic	Gibco	15240-062
BSA	GE Healthcare Life Sciences	SH30574.02
DMSO	Sigma-Aldrich	D8418
Dry milk	Santa Cruz	sc-2325
FBS	Merck Millipore	S0615
Fixable Viability dye eFluor™ 506	Invitrogen	65-0866-18
IL-2	NIH	136
L-Glutamine	Gibco	2916801
Lymphosep	Biowest	MS00K01002
PBS 10x	Alfa Aesar	J62036/K183
PFA	Sigma-Aldrich	P6148
Phosphoric acid	Sigma-Aldrich	P5811
RPMI1640 medium	Gibco	21975-034
SARS-CoV-2 RBD	iBET Bioproduction Unit	N/A
SARS-CoV-2 Spike CS + PP	iBET Bioproduction Unit	N/A
TMB	BioLegend	421101
Tween 20	Sigma-Aldrich	P1379

Table 3- Antibodies used and respective information.

Antibody	Supplier	Identifier	Clone
Anti-human CD3 (Alexa Fluor 700)	BioLegend	300424	UCHT1
Anti-human CD4 (PE-Fire 700)	BioLegend	344666	SK3
Anti-human CD28	BioLegend	302914	CD28.2
Anti-human CD25 (PE-Cy7)	BioLegend	356107	M-A251
Anti-human CD69 (BV421)	BioLegend	310910	FN50
Anti-human CXCR-5 (PE)	BioLegend	356904	J252D4
Anti-human CCR6 (APC-Cy7)	BioLegend	353432	G034E3
Anti-human CD19 (BV650)	BioLegend	363026	SJ25C1
Anti-human CD56 (PE/Dazzle)	BioLegend	318348	HCD56
Anti-human CD27 (APC)	BioLegend	302810	O323
Anti-human CD20 (BV711)	BioLegend	302342	2H7
Anti-human IgD (PE-Cy5)	BioLegend	348250	IA6-2
Anti-human OX40 (BV 605)	BioLegend	350028	Ber-ACT35
Goat anti-human IgA	Abcam	ab97215	ab97225
Goat anti-human IgG	Abcam	ab97225	ab97215
Goat anti-human IgM	Abcam	ab97205	ab97205
Purified anti-mouse IgG1	BioLegend	406601	RMG1-1