كليـــــــة الـطـب
و الصيدلة - مراكش
FACULTÉ DE MÉDECINE
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Detection of SARS-CoV-2 particles and assessment of the ACE2 receptor protein distribution in different tissue samples in the pathology department at the Mohammed 6th university hospital of Marrakech.

THESIS

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BY

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TO OBTAIN THE DEGREE OF DOCTOR OF MEDICINE

KEYWORDS

Coronavirus disease 2019 (COVID-19), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), immunohistochemistry, Nucleocapsid protein (NP), Angiotensin Converting Enzyme II (ACE2), lungs, placenta, pregnancy, vertical transmission, transplacental infection.

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

قَالُوا۟ سُبْحَٰنَكَ لَا عِلْمَ لَنَآ إِلَّا مَا عَلَّمْتَنَآ ۖ إِنَّكَ أَنتَ ٱلْعَلِيمُ ٱلْحَكِيمُ ﴿٣٢﴾

صَدَقَ ٱللَّهُ ٱلْعَظِيمُ

Hippocratic Oath

I swear to fulfill, to the best of my ability and judgment, this covenant:
I will respect the hard-won scientific gains of those physicians in whose steps I walk, and gladly share such knowledge as is mine with those who are to follow.
I will apply, for the benefit of the sick, all measures that are required, avoiding those twin traps of overtreatment and therapeutic nihilism.
I will remember that there is art to medicine as well as science, and that warmth, sympathy, and understanding may outweigh the surgeon's knife or the chemist's drug.
I will not be ashamed to say "I know not," nor will I fail to call in my colleagues when the skills of another are needed for a patient's recovery.
I will respect the privacy of my patients, for their problems are not disclosed to me that the world may know. Most especially must I tread with care in matters of life and death. If it is given me to save a life, all thanks. But it may also be within my power to take a life; this awesome responsibility must be faced with great humbleness and awareness of my own frailty. Above all, I must not play at God.
I will remember that I do not treat a fever chart, a cancerous growth, but a sick human being, whose illness may affect the person's family and economic stability. My responsibility includes these related problems, if I am to care adequately for the sick.
I will prevent disease whenever I can, for prevention is preferable to cure.
I will remember that I remain a member of society, with special obligations to all my fellow human beings, those sound of mind and body as well as the infirm.

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DEDICATIONS

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A mes amis de l'AEM et IFMSA-Morocco

A tous ces patients qui ont nourri ma passion.

Aux morts nés, ayant séjourné entre les parois rouges d'un utérus, leur sol natal et funérarium.

À ces nouveau-nés, aussitôt nés, aussitôt morts, nouveaux morts. Un cri, des larmes, un silence. Leurs cris résonnent une fois dans ce monde, des cris de rage.

Aux expérimentés de la vie, à ceux qui ont survécu à la coupure du cordon, et qui ont résisté, en cris ou en silence.

Sous le toit d'un hôpital, à quelques corridors du nouveau-né, des âmes âgées s'étiolent, puis se volatilisent, des souffles sont abrogés, ces corps estropiés à jamais. Leurs esprits sont désormais intemporels, confondus, partageant leur vécus d'un souvenir utérin, aux mémoires émoussées d'un millénaire terrestre.

Sous le toit d'un hôpital, une vie, une survie, une mort, et moi. A vous toutes, âmes souffrantes, je vous dédie ce travail.

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Head of department at the University Hospital Mohammed 6th of Marrakech and professor of pathology

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Professor of histology-embryology-cytogenetics

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Professor of gynecology-obstetrics

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To professor and jury member Mr. A. HACHIMI

Head of department at the University Hospital Mohammed 6th of Marrakech and professor of intensive care medicine

We are very honored to have you as part of the jury for our thesis. We have had the privilege of working with you and we appreciated your advice throughout this work. Please find here the expression of our respectful consideration and deep admiration.

To professor and jury member Mr. A. FAKHRI

Professor of histology-embryology-cytogenetics

We are very honored to have you as part of the jury for our thesis. You are the model professor who has always been close to his students, either through the listening cell or other initiatives. I am very grateful for having you as a professor and, now, as a jury member. Please find here the expression of our respectful consideration and deep admiration.

To Doctor Mrs. S. SELLAMI

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Thank you for taking the time to provide your expertise in order to revise and improve this research work.

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ABREVIATIONS

LIST OF ABBREVIATIONS

SARS-CoV	: severe acute respiratory syndrome coronavirus
WHO	: World Health Organization
COVID-19	: coronavirus disease 2019
DAD	: diffuse alveolar damage
+ssRNA	: single stranded positive-sense RNA
NP	: nucleocapsid protein
RBD	: receptor binding domain
ACE2	: angiotensin-converting enzyme 2
TMPRSS	: transmembrane protease serine protease
ACE	: angiotensin-converting enzyme
RAAS	: renin-angiotensin aldosterone system
Ang I	: angiotensin-1
AngII	: angiotensin-2
Ang1-7	: angiotensin 1-7
ICH	: immunohistochemistry
PCR	: polymerase Chain Reaction
HE	: hematoxylin-eosin
ARDS	: severe acute respiratory distress syndrome
GLUTs	: glucose transporters
hPL	: placental lactogens
hGH	: placental growth hormone
AT1R	: angiotensin II type 1 receptor
RAS	: renin-angiotensin-system

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  -From maternal origin: accelerated villous maturation, intervillous thrombosis
  -From fetal origin: dilation of chorioallantoic vessels.
  -Hypoxia-ischemia lesions in the villi.
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-Multiple (6) central and paracentral infarctions measuring from 1.2 cm to 3 cm next to decidual arteriopathy lesions and a basal decidual hematoma extending over 6 cm.

-An intervillous thrombosis measuring 1 cm.

-Hypoxia-ischemia lesions in the villus.

- ✓ **Figure 15 (case 5-placenta- ACE2 IHC x4):** intense and diffuse cytoplasmic expression of cytotrophoblast, villus-axis, syncytiotrophoblast and decidual cells.
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  -Hypoxia-ischemia-villous lesions.
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OUTLINE

INTRODUCTION

In the late 1960s, human coronavirus was identified for the first time in History, under the names of 229E and OC43, and it was declared as not life threatening for humans. In the winter of 2002, southern China declared the outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV), bats were identified as natural reservoirs, and the virus was spreading rapidly and worldwide. Until 2012, a total of six coronaviruses had been identified and scientists had invested time and resources to analyze the phenomenon, its physiopathology and potential future outbreaks. (1)

On 31st December 2019, another outbreak occurred, when clusters of patients were diagnosed with pneumonia of unknown etiology and were reported to the World Health Organization (WHO) China Country Office and then associated with a seafood market in Wuhan, Hubei Province, China. (2) On 30th January 2020, WHO announced the outbreak as a "public health emergency of international concern". (3) (4) Currently, on 19th December 2023, the number of confirmed cases according to the WHO, exceeds 772 838 745 people with approximately 6 988 679 deaths. (5)

Coronavirus disease 2019 (COVID-19) has been characterized by the diversity of the clinical manifestations from mainly targeting the respiratory system by causing diffuse alveolar damage (DAD) and acute respiratory failure to a multisystemic disease leading to severe gastrointestinal, hepatic, and neurological complications. (6)

This complexity invites us to have a look at the virus structure and physiopathology.

In fact, a single stranded positive-sense RNA (+ssRNA), known as the largest RNA virus, forms the CoVs (27-32 kb) genome. It is covered by a capsid made of the nucleocapsid protein (NP), then by an envelope made of three structural proteins: membrane protein (M), spike protein (S), and envelope protein (E). Spike glycoprotein has a primordial role in the SARS-CoV-2 entry into host cells; S1 subunit binds to the receptor on host cell, S2 subunit fuses the virus and host cell membranes. (7)

Specifically, the receptor binding domain (RBD) is contained in S1. Thereby, S1 proteolytic cleavage is mandatory to start off the interaction with the angiotensin-converting enzyme 2

(ACE2) receptor peptidase domain and is operated by one or several host proteases (cathepsins, transmembrane protease serine protease (TMPRSS)2, TMPRSS4, human airway trypsin-like protease). (8) To sum up, SARS-CoV-2 entry into host cells happens after the virus recognition of the receptor ACE2 on host cells by means of RBD. (7)

Indeed, the infection is related to the dipeptidyl carboxypeptidase angiotensin converting enzyme (ACE) and the mono-carboxypeptidase ACE2. ACE function in the renin-angiotensin aldosterone system (RAAS) is cleaving the decapeptide angiotensin-1 (Ang I) into the potent vasoconstrictor angiotensin-2 (AngII). (9) Then, ACE2 cleaves the AngII to form angiotensin 1-7 (Ang1-7) , and the AngI to form Ang 1-9. (10)

Studying receptors' expression was compelling to better understand the COVID-19 infection process. Some studies show that the expression levels of ACE2 varies in the human body, from one organ to another. It has different levels: high (the small intestine, testis, kidneys, heart, thyroid and adipose tissue); intermediate (the lungs, colon, liver, bladder and adrenal glands); low (the blood, spleen, bone marrow, brain, blood vessels and muscle). (9)

Other studies point out the high expression of ACE2 in the human placenta which initiated a hypothesis about SARS-CoV-2 maternal-fetal transmission.(11)

Our aim was to detect SARS-CoV-2 particles through the NP and assess the ACE2 receptor protein distribution in different fixed and paraffin-embedded tissue samples recruited from the pathology department at the Mohammed 6th university hospital of Marrakech.

MATERIALS AND METHODS

I. Type, aim and duration of the study:

Type: this is a retrospective, descriptive case study.

Aim: Detection of SARS-CoV-2 viral particles and ACE2 receptor protein distribution on different fixed and paraffin-embedded tissue samples from our pathology department at the Mohammed 6th university hospital of Marrakech involving 6 cases positive to SARS-CoV-2.

Duration: 6 months

II. Study population:

Three lung biopsies were performed between May 4 and June 9, 2020, and three placenta surgical specimens were performed between June 29 and September 14, 2020.

1. Inclusion criteria:

- Patients with a diagnosis of COVID-19, with a positive nasopharyngeal Polymerase Chain Reaction (PCR) test.

2. Exclusion criteria:

None

III. Collected data:

Information was collected by accessing data for each case from archived pathology reports.

- Age; gender; nature of the specimen; site of the specimen; clinical data; detectable histological lesions.

IV. Study methods and materials:

Material:

The IHC technique was performed with the Dako OMNIS apparatus using EnVision FLEX and FLEX+ visualization systems, on formalin-fixed, paraffine- imbibed specimens; from 3 placental surgical specimens and 3 lungs biopsies from COVID-19 positive patients (view table II).

IHC was performed on 4µm paraffin sections. Slides were incubated overnight in the oven at 37°C for the sections' fixation. For each case, 2 white slides were prepared to study the detection of SARS-CoV-2 particles and the evaluation of a receptor protein in the different tissue samples: one slide for the study of ACE2 receptor protein, and another for SARS-CoV-2 Nucleocapsid protein; to have a total of 12 white slides. One slide was prepared for the positive control for the study of ACE2 receptor protein in a placenta sample.

We used ACE2 and Nucleocapsid antibodies (view table I).

Table I: Antibodies used in the IHC technique

Article	Conditioning	Dilution	Quantity
Anti-SARS-CoV antibody/SARS-CoV-2 (COVID-19) Nucleocapsid antibody [Clone: 6H3]	100 µl	1/100	1
Rabbit Anti-Human SARS Receptor/ACE2	100 µg	1/50	1

Methods:

- Sections of 4µm.
- Incubation in the oven at 37 °C for 24h.

Protocol for ACE2 antibody:

Automation:

- Deparaffinization- rehydration – unmasking at 97 °C for 30min.
- The IHC technique:
  - ✓ Washing with a wash buffer at pH=7.
  - ✓ Primary antibody: ACE2, for 15min.
  - ✓ Washing with a wash buffer at pH=7.
  - ✓ Enzymatic blockade of endogenous peroxidase.
  - ✓ Two washing with a wash buffer at pH=7.
  - ✓ Labeled polymer: EnV FLEX/ HRP, for 20 min

- ✓ Three washings with a wash buffer at pH=7, and 1 with distilled water.
- ✓ Chromogenic substrate: env flex DAB, for 5min
- ✓ Two washings with a wash buffer at pH=7, and 1 with distilled water.
- ✓ Counterstaining: hematoxylin.
- ✓ Two washing with a wash buffer at pH=7.

Protocol for SARS-CoV-2 antibody:

We used the same protocol as ACE2, however, few changes and additional steps were applied. The incubation time for unmasking and for the primary antibody was 40min, Mouse Linked was added for 10min to increase the number of peroxidase molecules in the antigen-antibody reaction. Eventually in the protocol, DAB enhancer was added for 5min to enhance the coloration.

Manually:

- ✓ Dehydration.
- ✓ Clearing.
- ✓ Mounting.
- ✓ Drying.
- ✓ Microscopy.

V. Ethical considerations:

This is an observational study, the data collection respected patients' anonymity. In this case we were not required to obtain the research ethics.

RESULTS

The population characteristics and specimen data were included in table II and figures (2,5,8,11,14,17). The age, gender, nature and site of the sampling, clinical data and histological lesions using hematoxylin-eosin (HE) stain were reported.

The positive control for ACE2 in a placenta sample using IHC is reported in figure 1. The SARS-CoV-2 Nucleocapsid and ACE2 receptor expressions in all tissue samples were reported in table III and figures (3,4,6,7,9,10,12,13,15,16,18,19).

Table II: Population characteristics and specimen data

Case	Age	Gender	Nature of the sampling	Site of the specimen	Clinical data	Detectable histological lesions
1	57	M	Biopsy	Lung	- Past medical history: smoking - Admitted in the reanimation department for severe acute respiratory distress syndrome (ARDS) due to COVID-19 confirmed with a PCR. - Day 4 of Intubation, ventilation, sedation. - Thoracic CT scan: 30-45% parenchymal involvement on thoracic. - Biopsy at day 9 of hospitalization.	- Inflammatory and hemorrhagic remodeling of dystrophic alveolar parenchyma compatible with SARS-COV2 infection.
2	72	M	Biopsy	Lung	- Past medical history: arterial hypertension, hyperthyroidism. - Admitted in the reanimation department for a viral pneumopathy due to COVID-19 confirmed with a PCR. - Day 13 of Intubation, ventilation, sedation.	- Mild mononuclear inflammatory lesion with DAD that may be part of the COVID-19 infection.

					- Thoracic CT scan: lesion extent of 25-50%. - Biopsy at day 13 of hospitalization.	
3	78	M	Biopsy	Lung	- Admitted in the reanimation department for severe ARDS due to COVID-19 confirmed with a PCR. - Day 1 of intubation, ventilation, sedation. - Thoracic CT scan: bilateral ground-glass aspect. - Biopsy at day 5 of hospitalization.	- Mild mononuclear inflammatory lesion with DAD that may be part of the COVID-19 infection.
4	28	F	Surgical specimen	Placenta	- Admitted in the obstetrics and gynecology department for delivery in a pregnancy estimated to be at 39 weeks of pregnancy, with COVID-19 infection confirmed with a PCR. - Vaginal delivery, newborn weight= 3kg400. - Placenta, stool and newborn samplings tested positive.	- Placental vascular malperfusion lesions: - From maternal origin: accelerated villous maturation, intervillous thrombosis - From fetal origin: dilation of chorioallantoic vessels. - Hypoxia-ischemia lesions in the villi.
5	18	F	Surgical specimen	Placenta	- Past medical history: hospitalized in the reanimation department at 12 weeks of pregnancy for COVID-19 confirmed with a PCR, and left after a discharge against medical advice. -	- Placental vascular malperfusion lesions: - Multiple (6) central and paracentral infarctions measuring from 1.2 cm to 3 cm next to decidual arteriopathy lesions and a basal decidual hematoma extending

					- Readmitted with HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome with severe preeclampsia, retroplacental hematoma, intrauterine fetal death and had a Cesarean section for maternal rescue in a pregnancy of 32 weeks and 2 days, that required a hospitalization in the reanimation department. - Positive rapid test and PCR for SARS-CoV-2. -	over 6 cm. - -An intervillous thrombosis measuring 1 cm. - -Hypoxia-ischemia lesions in the villus. -
6	28	F	Surgical specimen	Placenta	- Admitted in the obstetrics and gynecology department for a caesarean section for acute fetal distress and COVID-19 infection confirmed with a PCR in a pregnancy of 4 - Day 3 of treatment.	- Placental vascular malperfusion lesions of maternal origin: - Accelerated villous maturation lesion. - Lesion of intermediate chorioamnionitis (stage 2) and early funiculitis (stage 1). - Hypoxia-ischemia-villous lesions.

Table III: SARS-CoV-2 Nucleocapsid and ACE2 receptor expression in the tissue samples

Marker / Tissue sample	Rabbit Anti-Human SARS Receptor/ ACE2	Antibody anti-SARS-CoV/SARS-CoV-2 (COVID-19) Nucleocapsid antibody [Clone: 6H3]
Case 1- lung	Intense cytoplasmic expression of endothelial cells, alveoli (pneumocytes), and bronchioles.	Low and diffuse cytoplasmic expression in the respiratory epithelium (bronchi and alveoli)
Case 2- lung	Intense cytoplasmic expression of endothelial cells, alveoli (pneumocytes), and bronchioles.	Absence of expression
Case 3-lung	Intense cytoplasmic expression of endothelial cells, alveoli (pneumocytes), bronchioles and mesothelial cells.	Low and diffuse cytoplasmic expression in the respiratory epithelium (bronchi and alveoli)
Case 4-placenta	Intense and diffuse cytoplasmic expression of cytotrophoblast, villus-axis, syncytiotrophoblast and decidual cells.	Intense and diffuse cytoplasmic expression of cytotrophoblastic cells.
Case 5-placenta	Intense and diffuse cytoplasmic expression of cytotrophoblast,villus-axis, syncytiotrophoblast and decidual cells.	Absence of expression
Case 6-placenta	Moderate to intense and diffuse cytoplasmic expression of cytotrophoblast, villus-axis, syncytiotrophoblast and decidual cells.	Absence of expression

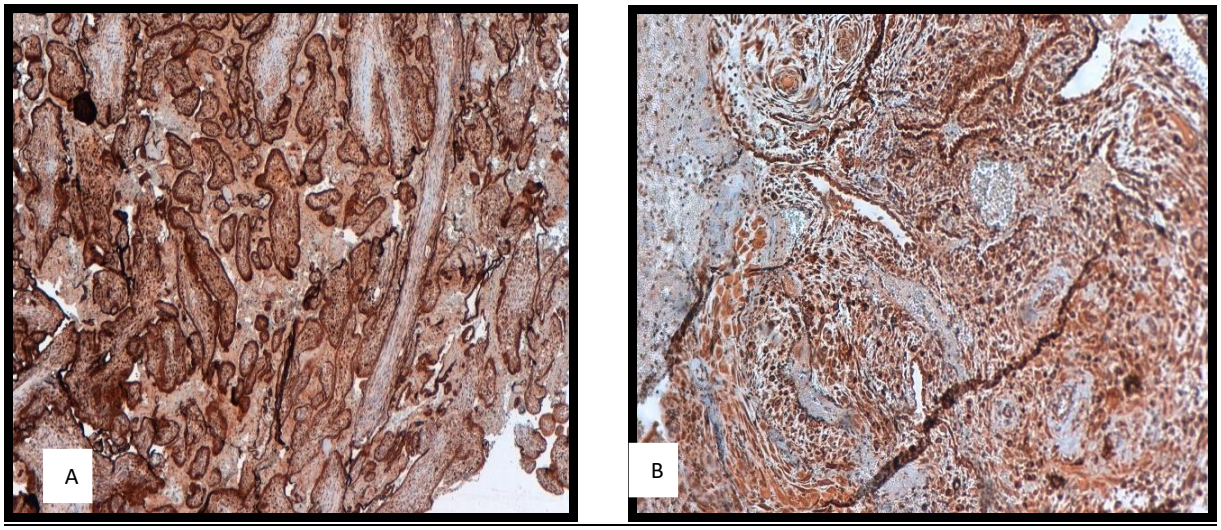

Figure 1 (positive control-placenta – ACE2 IHC): (A) intense and diffuse cytoplasmic expression of trophoblastic cells **(x4).** (B) Intense and diffuse cytoplasmic expression of decidual cells **(x10).**

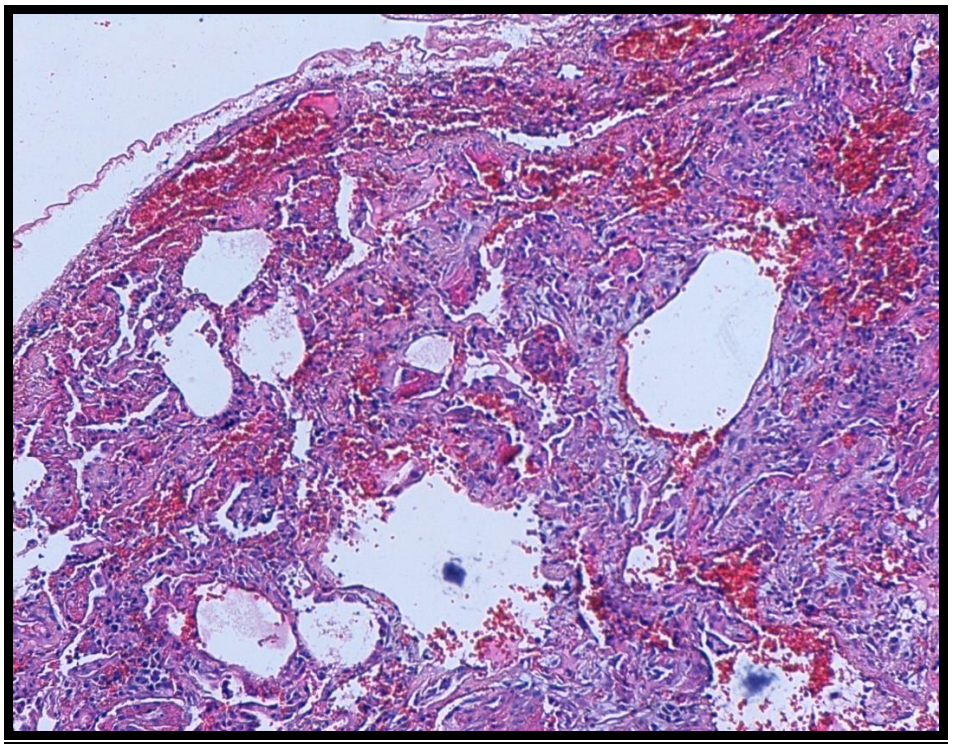

Figure 2 (case 1- lung- HE x4): inflammatory and hemorrhagic remodeling of dystrophic alveolar parenchyma compatible with SARS-COV2 infection.

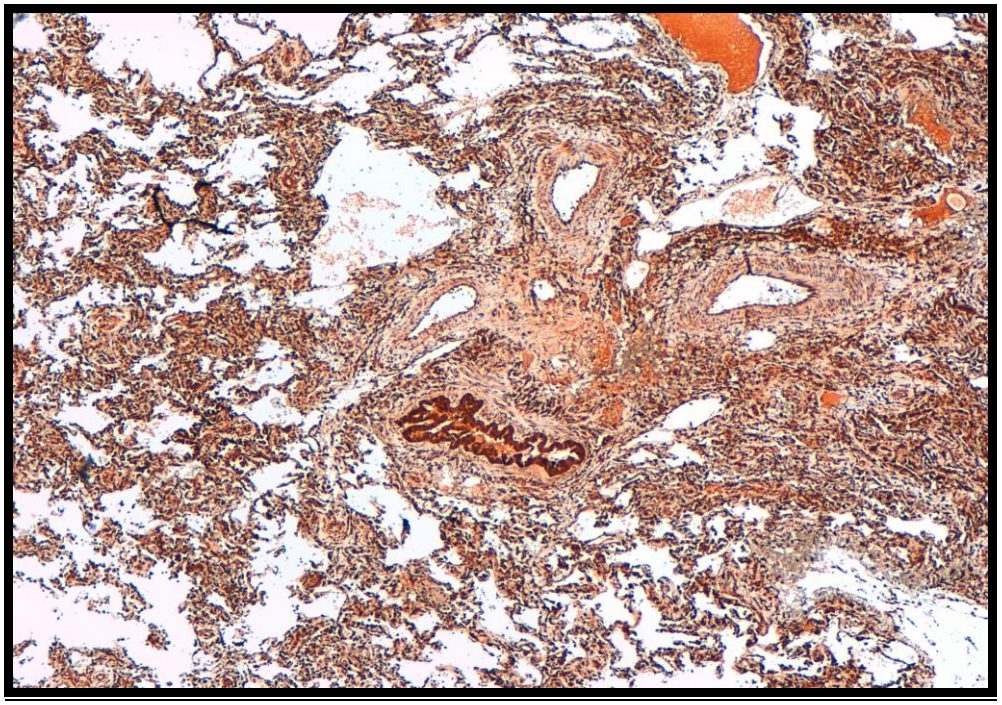

Figure 3 (case 1-lung- ACE 2 IHC x4): intense cytoplasmic expression of endothelial cells, alveoli (pneumocytes), and bronchioles.

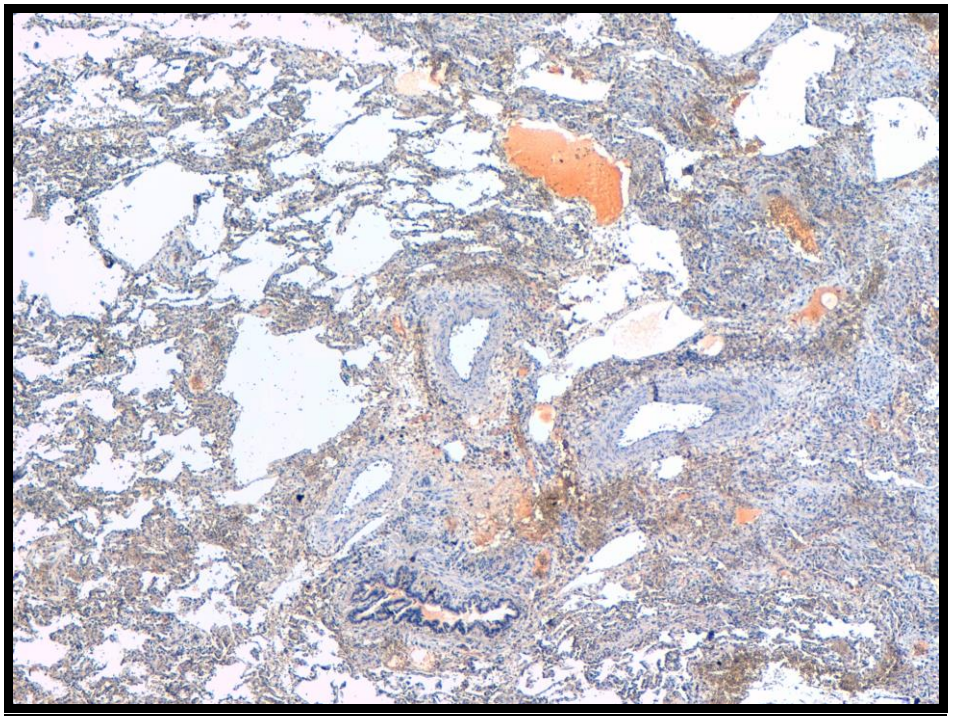

Figure 4 (case1-lung-NP IHC x4): low and diffuse cytoplasmic expression.

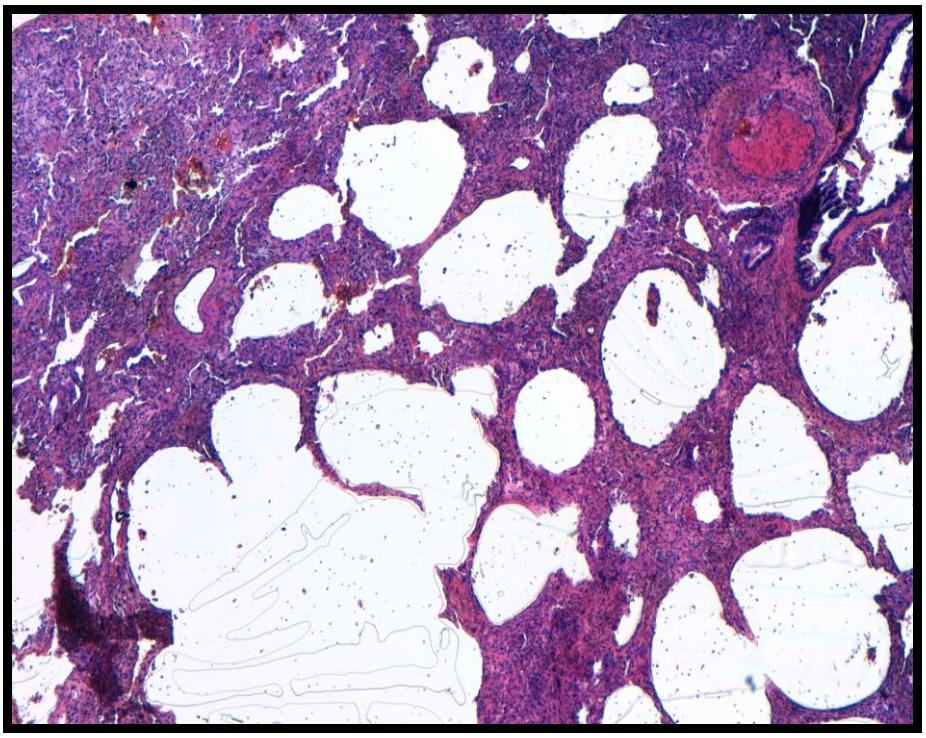

Figure 5 (case 2-lung- HE x4): mild mononuclear inflammatory lesion with DAD that may be part of the COVID-19 infection.

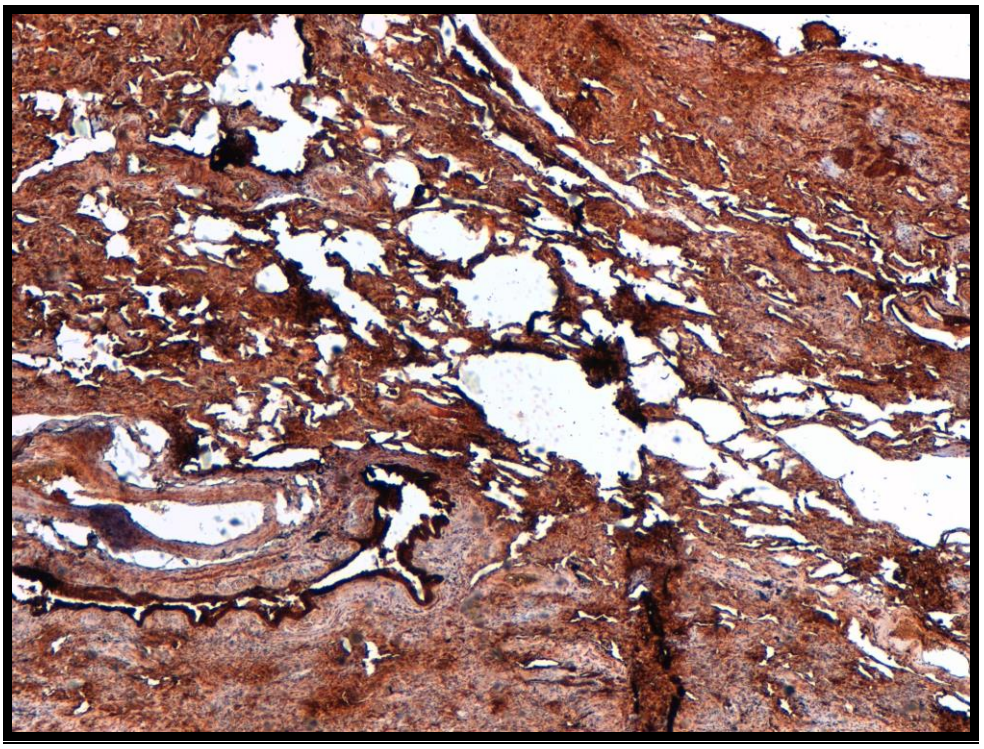

Figure 6 (case 2-lung- ACE 2 IHC x4): intense cytoplasmic expression of endothelial cells, alveoli (pneumocytes), and bronchioles.

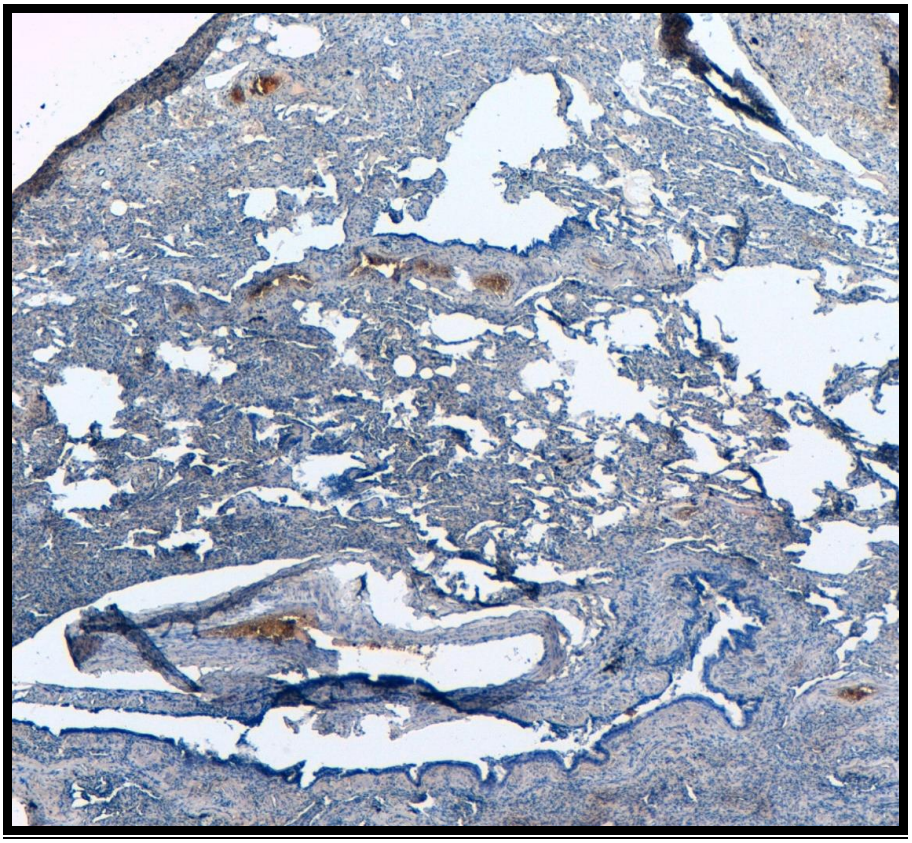

Figure 7 (case 2- lung- SARS IHC x4): absence of expression.

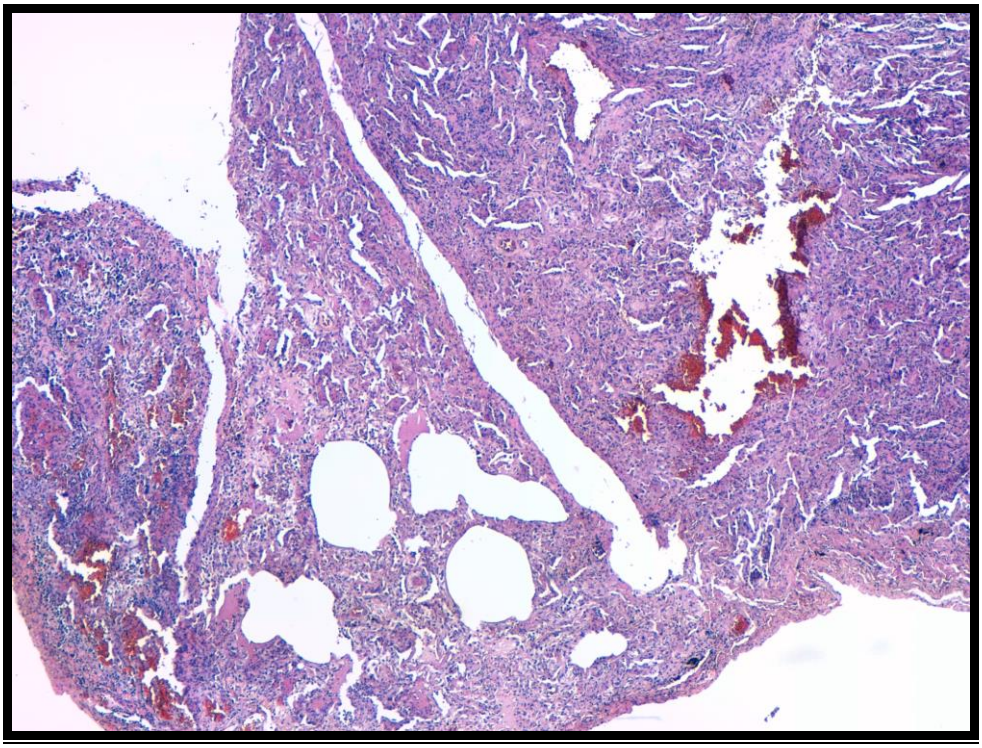

Figure 8 (case 3- lung- HE x4): mild mononuclear inflammatory lesion with DAD that may be part of the COVID-19 infection.

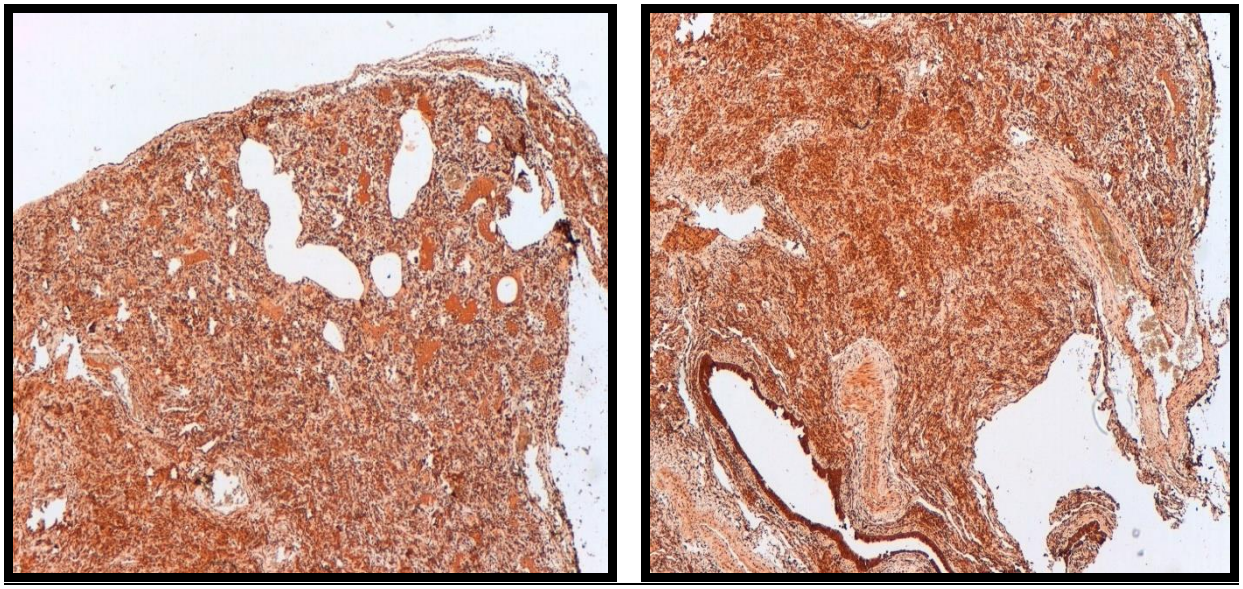

Figure 9 (case 3-lung-ACE2 x4): intense cytoplasmic expression of endothelial cells, alveoli (pneumocytes), bronchioles and mesothelial cells.

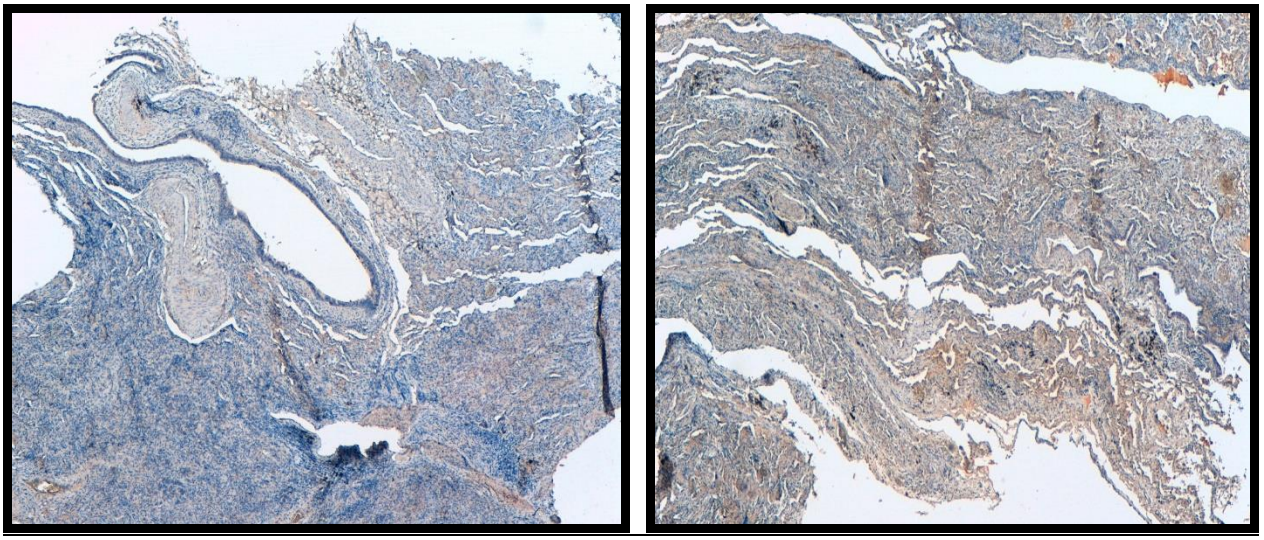

Figure 10 (case3- lung- NP IHC x4): low and diffuse cytoplasmic expression in the respiratory epithelium (bronchi and alveoli).

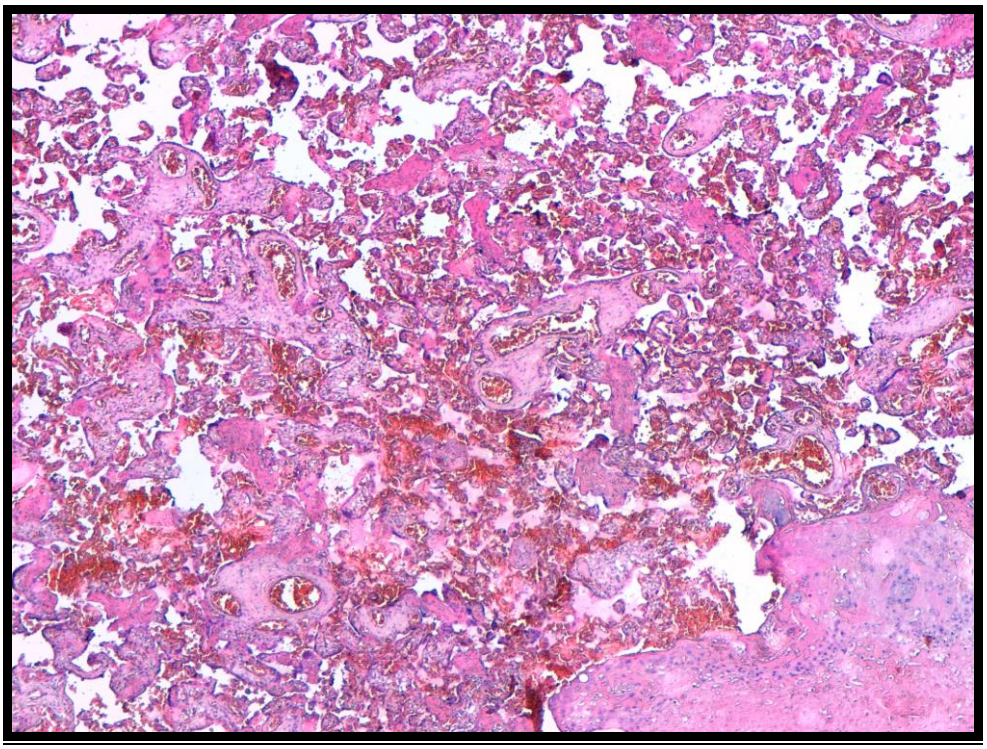

Figure 11 (case 4-placenta- HE x4): placental vascular malperfusion lesions.

- From maternal origin: accelerated villous maturation, intervillous thrombosis
- From fetal origin: dilation of chorioallantoic vessels.
- Hypoxia-ischemia lesions in the villi.

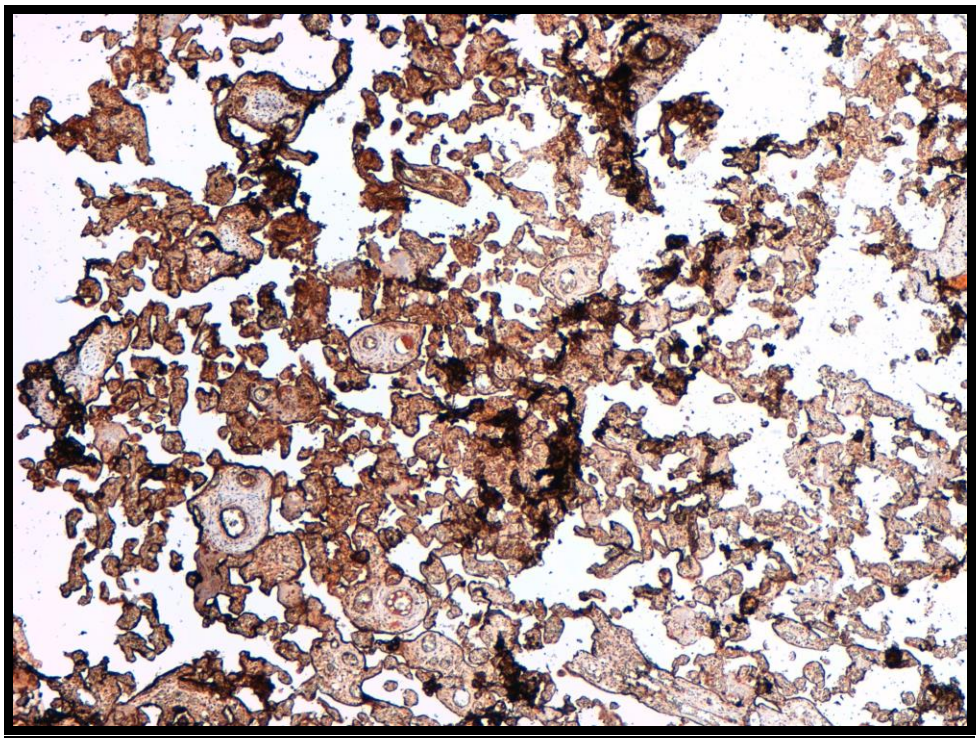

Figure 12 (case 4-placenta-ACE2 IHC x4): intense and diffuse cytoplasmic expression of cytotrophoblast, villus-axis, syncytiotrophoblast and decidual cells.

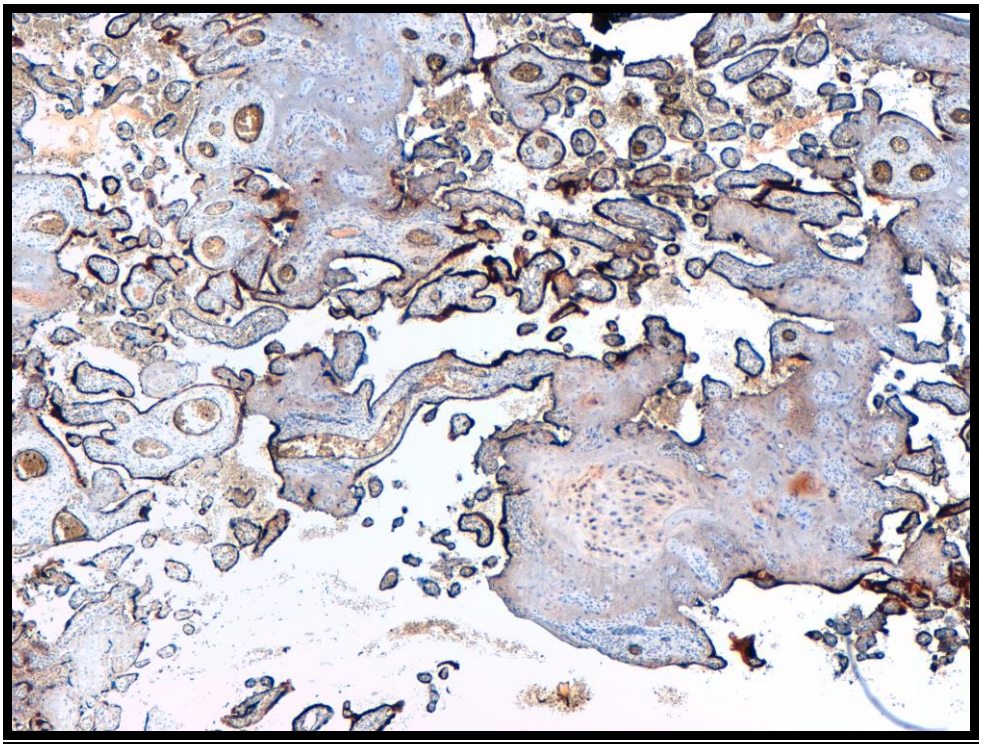

Figure 13 (case 4-placenta- NP IHC x4): intense and diffuse cytoplasmic expression of cytotrophoblastic cells.

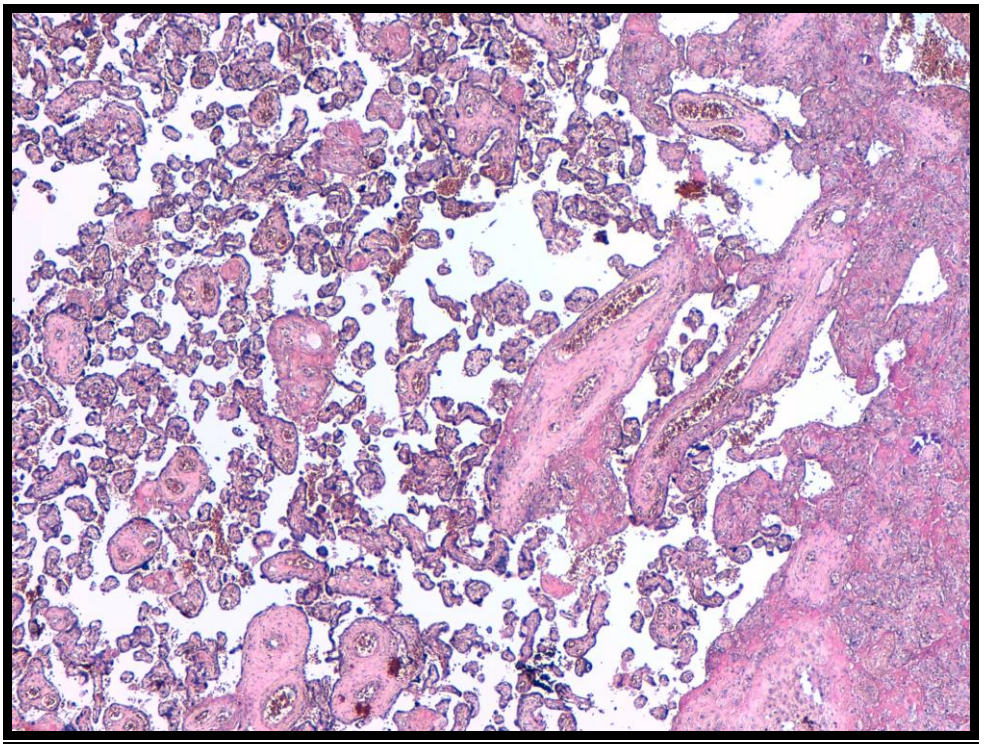

Figure 14 (case 5- placenta- HE x4): placental vascular malperfusion lesions.

- Multiple (6) central and paracentral infarctions measuring from 1.2 cm to 3 cm next to decidual arteriopathy lesions and a basal decidual hematoma extending over 6 cm.
- An intervillous thrombosis measuring 1 cm.
- Hypoxia-ischemia lesions in the villus.

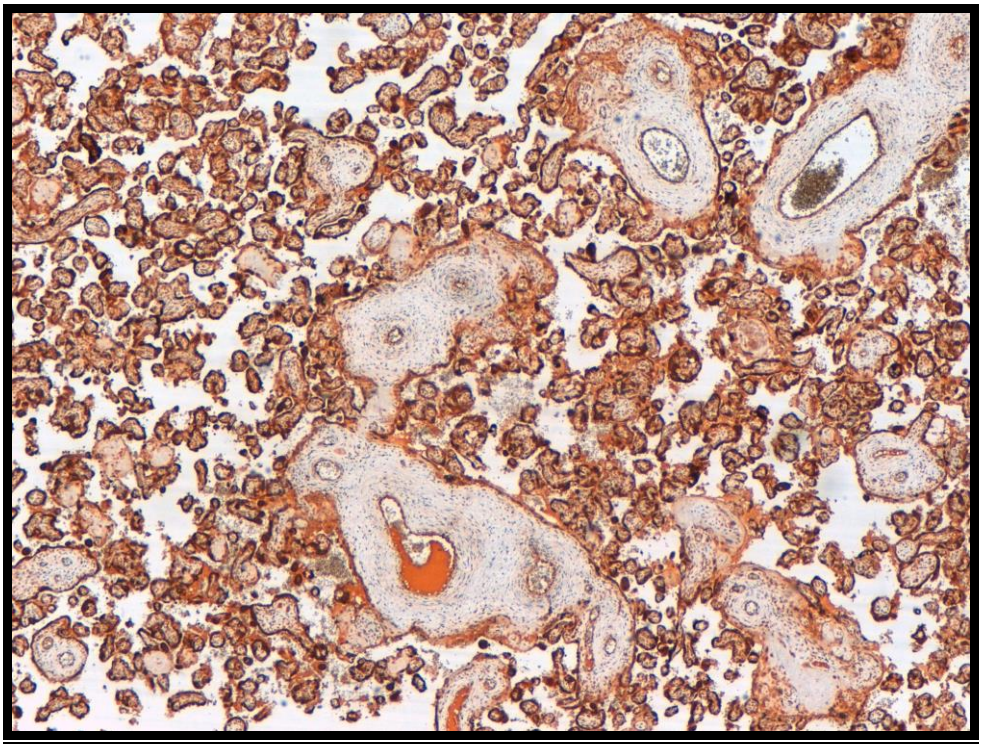

Figure 15 (case 5-placenta- ACE2 IHC x4): intense and diffuse cytoplasmic expression of cytotrophoblast, villus-axis, syncytiotrophoblast and decidual cells.

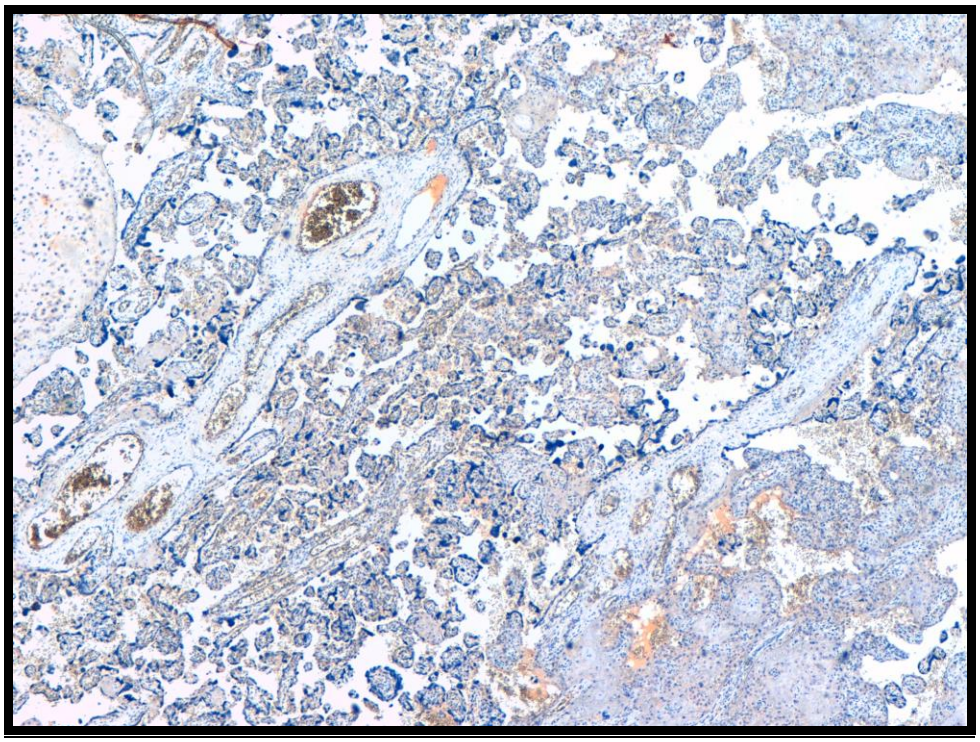

Figure 16 (case 5- placenta- NP IHC x4): absence of expression.

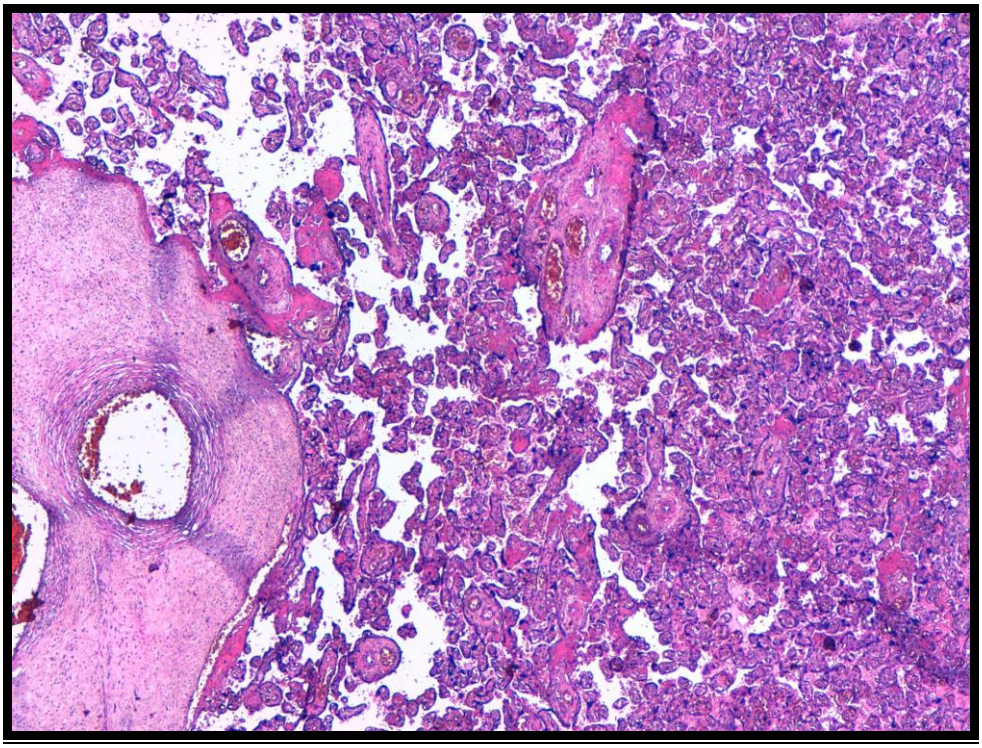

Figure 17 (case 6- placenta-HE x4): placental vascular malperfusion lesions of maternal origin.

- Accelerated villous maturation lesion.
- Hypoxia-ischemia-villous lesions.

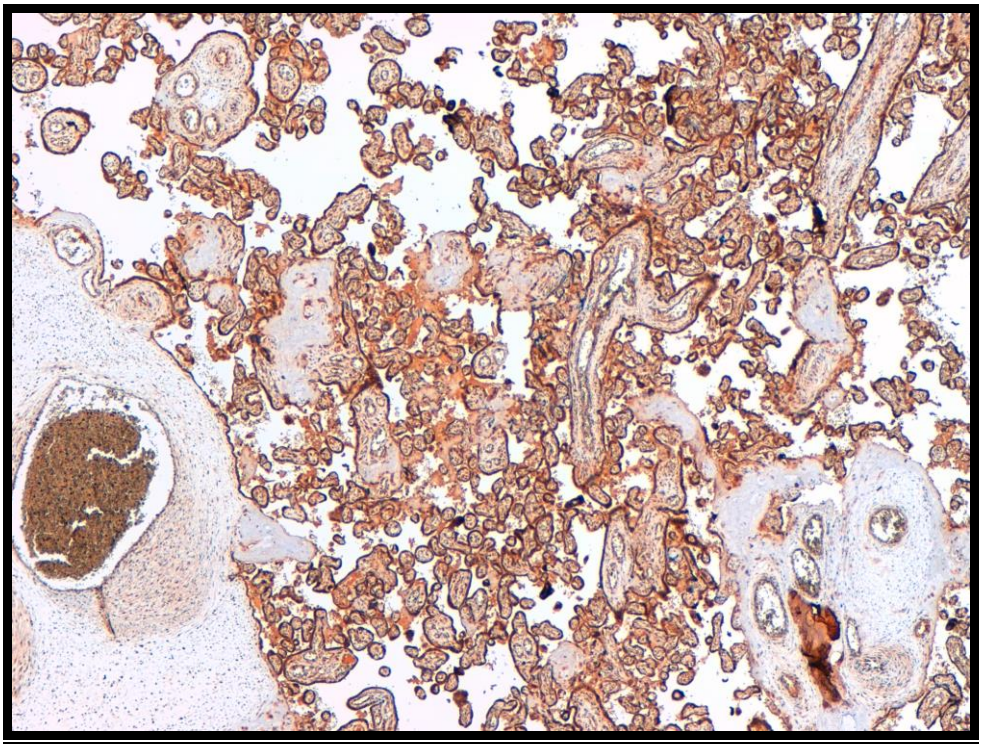

Figure 18 (case 6- placenta-ACE 2 IHC x4): moderate to intense and diffuse cytoplasmic expression of cytotrophoblast, villus-axis, syncytiotrophoblast and decidual cells.

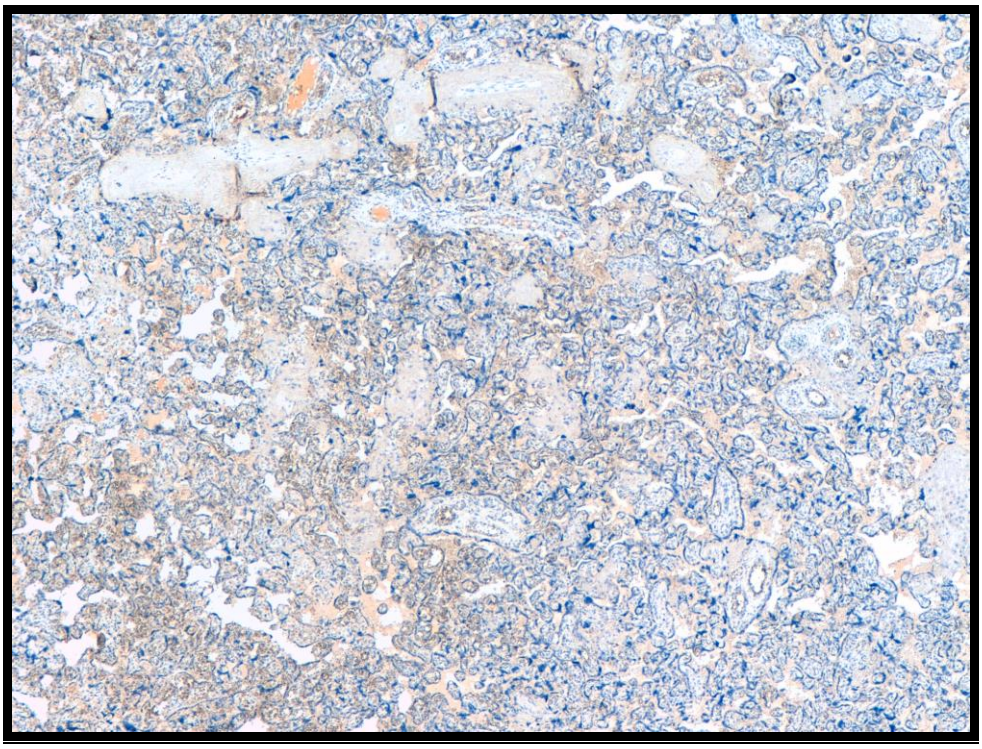

Figure 19 (case 6- placenta-NP IHC x4): absence of expression.

DISCUSSION

I. Recalls:

1. Placenta:

1.1 Physiology of the placenta:

- **The placental barrier overview:**

The placental barrier is defined by the interface between maternal and fetal blood. It has an apical and a basal side, which are structurally and biochemically different, their role is to transfer beneficial molecules and restrict deleterious and biologically active ones. The apical side is a brush border membrane enlarging the contact between the placental barrier and maternal blood. It is also characterized by an abundant presence of transport proteins, both influx transporters (transferring nutrients) and efflux transporters (minimizing the number of undesirable materials). The basal membrane is missing microvilli, however, it features various transporters. The functioning of the placental barrier is reflected in the fetal health, for instance, fetal intrauterine growth restriction is related to aberrations of glucose, fatty acid, and amino acid transporters. The syncytiotrophoblast is the major cell for the placental barrier function, however the other cell types (villous cytotrophoblast, extravillous trophoblasts, trophoblast giant cells, Hofbauer cells, fetal endothelial cell, decidual cells, and proteins found in the basement membrane) participate significantly in the transport. (12)

a. Metabolic function:

- **Transport and metabolism of carbohydrates:**

The major carbohydrate transported through the placenta from mother to fetus is glucose. As it is a fetal energy origin, it undertakes anabolic processes. It is principally resulting from the maternal circulation, with a minor fetal gluconeogenesis participation. A protein-mediated facilitated diffusion is responsible for the glucose diffusion through the placenta with the implication of glucose transporters (GLUTs). It is first absorbed through the microvillous membrane of the syncytiotrophoblast. When inside the syncytiotrophoblast cytoplasm, it can

reach the basement membrane. A glucose conversion into glucose-6- phosphate or placental glycogen occurs in the syncytiotrophoblast. Further, glucose-6- phosphate can be used in the aerobic or anaerobic respiration or via the pentose phosphate pathway. GLUTs cellular distribution differs with gestational evolution, GLUT1 may be crucial for glucose transport all over pregnancy, GLUTs 3,4, and 12 are only important during the first trimester. Human Placentas largely produce lactate which is transported by the placenta. The fetus produces lactate as well, and the placenta eliminates it. (13) Anaerobic glycolysis has been proved in coelomic fluid at 7-11 weeks of pregnancy. Analysis has shown a pH of 7.18, and a lactate concentration of 0.6. At term placentas analysis states a conversion of 22% of the consumed glucose to lactate. (14)

- **Transport and metabolism of amino acids:**

Fetal protein synthesis requires amino acids, which can be metabolized by the fetus. The amino acids transport to the fetus during pregnancy takes place through the microvillous and syncytiotrophoblast basal membranes.(13)Amino acids transport and metabolism vary throughout gestation. In early gestation, intact peptides are transported through the yolk sac, by 12-17 weeks of gestation, the amino acid concentration is more elevated than in the maternal circulation, which indicates the active transport. Around 10-60g of amino acids are transported per day to the fetus for every 1 kg of fetal weight. Most nutrients attend the maximum by the end of gestation, however fetal proteins diminish while fat is dominating. Over 20 amino acids transporters provide the placenta and fetus with amino acids. (15)

- **Transport and metabolism of lipids:**

The fetoplacental development requires maternal lipids since early trophoblast invasion and angiogenesis to the last 10 weeks of pregnancy; at this time 90% of fat is formed. The fetal lipid is provided by maternal essential fatty acids and long-chain polyunsaturated fatty acids. Nonesterified fatty acids are present in maternal circulation to a lesser extent and can cross the syncytiotrophoblast by diffusion. Lipases hydrolyze maternal lipoprotein (triglycerides,

phospholipids) into Nonesterified fatty acids to allow their active transport through the syncytiotrophoblast. Free fatty acids cross the microvillous membrane into the cytoplasm. Transport proteins implicated in this process are: the fatty acid translocase (FAT/CD36), fatty acid transporter proteins 1-6 (FATP1-6), and placenta specific plasma membrane fatty acid binding protein (FABP-pm). (15)

- **Transfer of water, inorganic ions, minerals and vitamins:**

Hydrostatic and osmotic pressure modulate water transfer through the placenta. This transfer is passive, and a water channel forming integral protein expressed in the trophoblast might facilitate it. When sodium and chloride amounts are similar in fetal and maternal blood, potassium, calcium and phosphate are more elevated in fetal blood. A complex system is implicated in the ions transport. Various active ion-transporting systems are involved as Na/K ATPase, Ca ATPase, Na/H exchangers, etc. In addition, proteins like sodium dependent amino acid transporters affect ion transport. Vitamins and many minerals are also transferred to the fetal circulation. (13)

b. Respiratory function

Since fetal lungs are not implicated in gas exchange, oxygen and carbon dioxide transfers depend on the placenta. Passive diffusion is responsible for oxygen transfer, depending on the oxygen partial pressure gradient between maternal blood in the intervillous space and fetal blood in the umbilical arteries. The Bohr effect enhances the oxygen transfer to the fetus. At the maternal-fetal interface, maternal blood receives carbon dioxide, leading to increased acidity. This leads to oxygen liberating to the fetus. The maternal oxyhemoglobin dissociation curve then shifts to the right, favoring the release of oxygen to the fetus. Simultaneously, carbon dioxide is released by fetal blood and is turned to be alkaline. The fetal curve then shifts to the left, leading to oxygen absorption by the fetus. This process is known as the "double Bohr effect". The transfer of oxygen from mother to fetus is enhanced by the presence of fetal hemoglobin, which causes a leftward shift of the fetal oxyhemoglobin dissociation curve to the left. Passive diffusion

is also responsible for carbon dioxide transfer. The transfer from fetus to mother hangs on the partial pressure gradient for carbon dioxide between fetal blood in the umbilical arteries and maternal blood in the intervillous space. The Haldane effect facilitates the carbon dioxide transfer from the fetus to the mother; it means the elevated capacity of deoxygenated blood to carry carbon dioxide in comparison with oxygenated blood. When maternal blood libertate oxygen, it can carry more carbon dioxide as bicarbonate and carbaminohaemoglobin. Simultaneously, when fetal blood receives oxygen and forms oxyhaemoglobin, the affinity for carbon dioxide decreases, then it is released to the mother. These two processes are known as 'Double Haldane Effect'. (16)

c. Immunological function:

Large proteins like immunoglobulin G (IgG) cross the syncytiotrophoblast and fetal capillary endothelium by endocytosis and exocytosis. The microvillous membrane is invaginated to form an intracellular membrane-bound vesicle that englobes extracellular fluid and associated solutes. At acid pH, the internalized protein binds to a FcRn receptor. This binding allows protection from degradation and provides selectivity during transfer. The vesicle release their content by exocytosis at the basal plasma membrane (17)

This transfer process experiences an exponential increase from the early stages of gestation until the third trimester. During the transfer, the fetus might be affected by antibodies included in autoimmune disorders. It also provide passive immunity during the first months of the newborn's life.(16)

d. Drug transfer:

In general drugs will cross the placenta to attain the fetus. Sometimes, this transfer is beneficial for the fetus' treatment in some conditions. Nevertheless, the drugs transfer may be harmful by teratogenicity effects, alteration of fetal growth and development. During the first trimester, when organogenesis occurs, adverse drug effects are more probable. Drugs transfer path goes through the intervillous space and passes through the syncytiotrophoblast, fetal

connective tissue, and the endothelium of fetal capillaries. Four mechanisms are incriminated in this process: simple diffusion (e.g. midazolam and paracetamol), facilitated diffusion (e.g. cephalosporins and glucocorticoids, active transport (e.g. norepinephrine and dopamine), pinocytosis. (16)

e. Endocrine function:

The placenta has a major endocrine function. In early stages of pregnancy, placenta hormones induce a rise in food intake and energy storage, however, around the term, this storage sustains fetal growth and lactation. Placental lactogens (hPL) and placental growth hormone (hGH) are the most important placental hormones. On chromosome 17, a gene cluster encodes five growth hormone-like proteins; hGH-N encoding pituitary growth hormone, hGH-V encoding placental growth hormone and hPL-A, hPL-B and hPL-L encoding placental lactogens. They are all present in the syncytiotrophoblast, hCH-N excepted. As progesterone and hPL stimulates the appetite, they induce an elevation in maternal food intake around the end of the first trimester.

The syncytiotrophoblast secretes leptin, in correlation with maternal serum concentration, that attends its peak by the early stages of the third trimester. It has a local action on placental transporter expression and central action on appetite. Further, insulin resistance is developed, with an elevation of lipolysis and in circulating triglycerides and free fatty acids. Placental growth hormone is secreted by the syncytiotrophoblast, and is implicated in the regulation of insulin-like growth factor 1. It doesn't cross the fetal circulation; it is highly implicated in fetal growth. Birthweight is correlated to maternal concentrations. (14)

1.2 Embryologic development of the placenta:

- **Blastocysts formation:**

When the oocyte is fertilized by a spermatozoid at day 0, we obtain a diploid zygote. Furthermore, at days 2-3, the zygote is cleaved during three rounds, to give blastomeres. The following process is compaction of these cells that gives the morula which, by day 4, makes way for blastocysts. The morula differentiation consists in the transformation of the outer cells into the trophectoderm, and of the fluidic uptake to the blastocoel.

- **Blastocyst apposition and adhesion:**

In parallel with the blastocyst formation, the uterine endometrium is differentiated until its maturity allows the embryo implantation: this is denominated “the implantation window”. Apical glycocalyx is located in the uterus and facilitates tiny molecules diffusion.(18)

“The implantation window” is a temporary phenomenon, however, it enhances the blastocyst apposition and adhesion by stimulating certain molecules. L-selectin possibly binds to stimulated oligosaccharide ligands on uterine luminal epithelium. This would supply a fragile adhesion, then activate signal transduction pathways in order to activate integrin. A stable adhesion is assured by integrins which allows the trophectoderm to migrate and invade into the endometrium.(19)

- **Implantation:**

At day 6-7 post conception, the implantation occurs after a multitude of connections initiated by the trophectoderm since day 4-5 post fertilization. This marks the beginning of the placental development. (20)

After a stable blastocyst apposition and adhesion, the primitive syncytiotrophoblast is formed and is prone to have a major role in the implantation. At day 8, the trophectoderm cells located in the embryonic pole proliferate and differentiate to give the primitive cytotrophoblasts. The cytotrophoblasts take action on cell fusion to give multinuclear cells. Eventual fusion of the

multinuclear cells gives the primitive syncytiotrophoblast, characterized by an inexistant proliferation capacity, but with an exclusive invasion capacity. It permits the invasion in the uterine stroma by entering the epithelium. Schematically, the embryo is in the uterine stroma, bordered by cytotrophoblast, then by the syncytiotrophoblast.(18) (20) At day 9, the primitive syncytiotrophoblast is hollowed out by vacuoles that fuse to form lacunar spaces. By day 10, placental villi start to grow. Then, by day 12-13, discontinuous maternal blood sinusoids are constructed when lacunar spaces penetrate the maternal uterine capillaries. (20)

- **Villous formation**:

The placental villous trees have been determined as the main functional unit of the placenta. The early villous formation phase lasts between day 12-18 and day 28 post-conception, then the villous phase persists up to the completion of pregnancy. After the villous trophoblast differentiation into villous syncytiotrophoblast and cytotrophoblast, cells of the extra-embryonic mesoderm arise from the embryo form a cell layer below the primitive cytotrophoblast. Thereafter, this cell layer leads to the inner stromal placental villi.(18)

Concomitantly, 2 weeks post-conception, chorionic villous trees come from a finger-like shaped trophoblast trabeculae proliferation from the chorionic plate in the intervillous space. Primary villi are made out exclusively from trophoblasts, until their invasion by mesenchyme cells from the embryoblast, around day 21 post conception. At this time, they are reshaped into secondary chorionic villi. Later, by the end of the third week, differentiation of mesenchyme cells into blood cells and small blood vessels occurs and leads to the tertiary chorionic villi constitution. A connective tissue core enclosing fetal blood vessels and macrophages is contained in every tertiary villus.

The free-floating chorionic villi are schematically classified in 5 types (mesenchymal villi, immature intermediate villi, stem chorionic villi, mature intermediate villi, terminal villi). This classification takes into consideration the diameter, stromal features, vessel structure and appearance.(21)

❖ **The maternal-placental circulation:**

The maternal-fetal circulation allows the nutrients to be transported from the mother to the fetus. Its constitution depends upon the trophoblast connection to maternal uterine arteries. Maternal blood is reserved in the uterine vascular network after the trophoblast juxtaposition to the uterine epithelium or the stromal matrix. (14)

Tertiary villi formed at approximately day 17 post-conception, incorporate placental vessels. To begin with, after differentiation from the extraembryonic mesoderm, these vessels start as haemangiogenic foci that evolve afterwards into primitive endothelial tubes. Pericytes are recruited to maintain these tubes and enhance the vascular network. Various parameters taking part in early placental haemangiogenesis and vasculogenesis develop from the extraembryonic mesoderm until the late-first and second trimester. Late in gestation, placental capillaries are longer and form loops that are in close proximity to the syncytiotrophoblast of terminal villi. This results in reducing the maternal-fetal exchange distance, thus increasing oxygen and nutrient transport. (20)

1.3 Morphology of the placenta:

a. Macroscopic morphology:

The placenta is a circular discoid organ. (22) It is true that it is on average round or oval-shaped, however it can be irregular, bilobate, circumvallate, etc. The shape's variation has a significance regarding the placental function. (23)

The macroscopic exploration of the morphology of the normal placenta revealed the average measurements as follows: ~530g wet weight, ~16cm diameter, ~17cm central thickness, ~450cm3 volume, ~18 cotyledons. (24) The placental weight, alongside maternal context, is correlated to the fetus and infant weights. In fact, in low weight placentas, a history of diminished fetal growth has been found in the current pregnancy. The fetal placenta ratio is a measure used to link the placental and birth weights.(23)

The placenta is formed of a maternal and a fetal surface. The maternal one is made out of the basal plate. The fetal one is represented by the chorionic plate which is enveloped by the amnion, and attached to the umbilical cord. (22)

Illustratively, it is disposed in three layers: the maternal surface and anchoring villi, the terminal villous unit, the fetal surface and stem villi. Chorionic arteries and veins drain blood to the umbilical cord. (25)

- **Umbilical cord:**

The umbilical cord is commonly white-colored, darkening with increasing gestational age. As the pregnancy progresses, the fetal growth comes with the expansion of length and diameter of the umbilical cord as for the umbilical vessels.

Regularly, each 10 cm, 1-3 left twists are found. After sectioning, two arteries and one vein are frequently visualized. Eventually, the arteries anastomose on the chorionic plate not far from the umbilical cord positioning.

- **Extraplacental membranes:**

  - **Chorion:** translucent.
  - **Decidua:** velvet pink. (26) It contains decidual stroma cells, and immune cells (natural killers, macrophages, etc.). (22)
  - **Amnion:** Thicker, gelatinous. (26) Its composition can be summed up in one layer of epithelium, and mesenchyme. The amniotic and chorionic mesenchymal tissues are not strongly fixed, thus detachment is simple after delivery. (22)

- **Chorionic plate:**

It is oval-shaped, tan-white in color, covering the villous parenchyma, representing the fetal side. Features may vary with gestation progression. The amnion covering the chorionic plate, the membranous amnion and the umbilical cord are uninterrupted. This continuity begins

in the margin of the disc for the extraplacental membranes, and in an eccentric position for the umbilical cord.

- **Basal plate:**

The basal plate is less developed in the first trimester. Then, in the second and third trimesters, it is developed on the maternal surface. It is gray with round-shaped cotyledons. (26)

It contains fetal extravillous trophoblasts, decidual cells, extracellular matrix, fibrinoid and blood clots. It is divided by placental septa into lobes that reflect the villous trees' disposition. All lobes contain 1-4 lobules, making a full-term placenta containing 60-70 villous trees. (22)

b. Microscopic morphology:

The chorionic plate gives the fetal lobules (villous tree) by a thick villous stem.

The stems branches carry on bifurcating until resulting in freely floating villi in the intervillous space. The freely floating villi is classified into five types of villi:

- -Mesenchymal villi (100–250 mm in diameter): They are present from the earliest weeks of pregnancy until delivery. They are known to be the only villi type present in the developing placenta.
- Immature intermediate villi: (100–400 mm in diameter), large, bulbous, comes from mesenchymal villi differentiation, and are characterized by a highly specific stroma.
- Stem villi: (100–300 mm in diameter) comes from immature intermediate villi differentiation Supports mechanically the villous tree, however, it has a limited participation in the maternal-fetal exchange.
- Mature intermediate villi: (80–120 mm in diameter) comes from mesenchymal villi, and contains a sparse stroma and a few peripheral vessels and capillaries.
- Terminal villi: (about 80 mm in diameter, up to 100 mm in length) It is the last branch of the villous trees, and comes from mature intermediate villi. It is

characterized by high capitalization. Thus, from a physiological point of view, they are the most important part of the villous tree. Further, capillaries will expand in sinusoids that are layered by a vasculo-syncytial membrane. This membrane is made of syncytiotrophoblast, endothelium of the capillary, split up by a joint basement membrane. (22)

1.4 Histological lesions:

The microscopic analysis of the placenta was institution-dependent until the holding of the Amsterdam Placental Workshop Group Consensus Statement. Protocols were proposed for the benefit of pathologists and general practitioners as well. (27) Placental classification (incorporating the 2014 Amsterdam Placental Workshop Group criteria) was described and summed up further in a review (view figure 20) (28) Afterwards, a guide was settled to assist practitioners in the use of the Amsterdam consensus, evoking the four major lesions: maternal vascular malperfusion (MVM), fetal vascular malperfusion (FVM), acute chorioamnionitis (ACA), and chronic villitis (so-called "villitis of unknown etiology", VUE). (29)

TABLE 1

Placental classification (incorporating the 2014 Amsterdam Placental Workshop Group criteria)

1. Placental vascular processes
   - a. Maternal stromal-vascular lesions
     - Developmental
       - Superficial implantation/decidual arteriopathy
       - Increased immature extravillous trophoblast
     - Malperfusion
       - Global/partial
         - Early: distal villous hypoplasia
         - Late: accelerated villous maturation
       - Segmental/complete
         - Villous infarct(s)
     - Loss of integrity
       - Abruptio placenta (arterial)
       - Marginal abruption (venous)
         - Acute
         - Chronic
   - b. Fetal stromal-vascular lesions
     - Developmental
       - Villous capillary lesions
       - Delayed villous maturation (maturation defect)
       - Dysmorphic villi
     - Malperfusion
       - Global/partial
         - Obstructive lesions of umbilical cord
         - Recent intramural fibrin in large fetoplacental vessels
         - Small foci of avascular or karyorhectic villi
       - Segmental/complete
         - Chorionic plate or stem villous thrombi
         - Large foci of avascular or karyorhectic villi
     - Loss of integrity
       - Large vessel rupture (fetal hemorrhage)
       - Small vessel rupture (fetomaternal hemorrhage)
       - Villous edema
2. Placental inflammatory-immune processes
   - a. Infectious inflammatory lesions
     - Acute
       - Maternal inflammatory response: chorioamnionitis, subchorionitis
       - Fetal inflammatory response: chorionic/umbilical vasculitis
     - Chronic
       - Villitis (CMV, others)
       - Intervillositis (malaria, others)
   - b. Immune/idiopathic inflammatory lesions
     - Villitis of unknown etiology and related/associated lesions
       - Chronic villitis
       - Chronic chorioamnionitis
       - Lymphoplasmacytic deciduitis
       - Eosinophil T-cell fetal vasculitis
     - Chronic histiocytic intervillositis
3. Other placental processes
   - Massive perivillous fibrin(oid) deposition (maternal floor infarction)
   - Abnormal placental shape or umbilical insertion site
   - Morbidly adherent placentas (accreta)
   - Meconium-associated changes
   - Increased circulating nucleated red blood cells

CMV, cytomegalovirus.

Redline. Classification of placental lesions. Am J Obstet Gynecol 2015.

Figure 20: placental classification (incorporating the 2014 Amsterdam Placental Workshop Group criteria) (28)

2. Lungs:

2.1 Physiology of the respiratory system:

Diverse systems act in the respiration process: central neural control, sensory input systems, respiratory muscles and lungs. Central neural control and sensory input systems are responsible for ventilation's timing and rate, and air volume intake. Further, a signal is sent to the respiratory muscles and lungs where gases' mechanical exchange occurs.

The upper airway contains soft tissues, muscles, and bony structures regulating respiratory functions. Cortical states, sensory input, drugs, and passive changes in lung volume can influence the upper airway as they regulate reflex activity and provide defense, protection and maintenance of the airway. (30)

The function of the pulmonary system is oxygen extraction and supplying it for aerobic respiration at the cellular level. ATP is then produced, and carbon dioxide is exhaled with other metabolic byproducts. Gases diffusion towards the arterioles happens at the alveoli. The nose, oral cavity, throat, trachea, bronchi are also included in the gas exchange. (31)

2.2 Histological lesions of the lungs:

Small airways disease:

For bronchiolar disorders, there is no consensual classification. Some diseases have specific histological features, on the other hand, others present non-specific features, thus a multidisciplinary analysis is necessary for the final diagnosis. A classification has been proposed for the use of pathologists, when small airways diseases might be implicated.

- **Disorders where bronchioles are primary site of pathology:**
  - **Non-specific features**
    - Cellular changes (acute/acute and chronic/chronic)
    - Fibrotic changes (peribronchiolar/intraluminal/constrictive)

- **Features suggestive of diseases**
  - Follicular bronchiolitis
  - Eosinophilic bronchiolitis
  - Granulomatous bronchiolitis
  - Mineral dust airway disease

❖ **Disease with specific features**

  - Diffuse panbronchiolitis (DPB)
  - Diffuse idiopathic neuroendocrine cell hyperplasia (DIPNECH)
  - Neuroendocrine cell hyperplasia of infancy (NEHI)
  - Other

❖ **Disorders where bronchiolar pathology is secondary to other lung disease**

- **Associated with proximal airway disease**
  - Bronchiectasis
  - Asthma
  - Chronic obstructive pulmonary disease (COPD)
- **Associated with interstitial/diffuse lung disease**
  - Respiratory bronchiolitis
  - Extrinsic allergic alveolitis
  - Organizing pneumonia
  - Sarcoidosis
  - Langerhans cell granulomatosis

- Wegener's granulomatosis

*Airway centred interstitial fibrosis (ACIF), centrilobular fibrosis and idiopathic bronchiolocentric interstitial pneumonia (IBIP), peribronchiolar metaplasia and fibrosis (PBMF): It is controversial if these patterns are a separate clinicopathological entity.

- Other (32)

3. ACE2 receptor:

3.1 ACE2 overview:

The ACE2 gene is positioned on Xp22 and incorporates 18 exons. It is Zn carboxypeptidase with only one catalytic site. This ectoenzyme has an N-terminus and a catalytic site, thus it can process circulating peptides metabolism. (33)

It is implicated in the physiology and pathophysiology of multiple systems. Hypertension, cardiac hypertrophy, and kidney diseases development includes the ACE2. The rate of ACE2 varies from one condition to another. (34)

3.2 ACE2 receptor in the placenta

The detection of Ang II, renin and ACE in the villi, and of the angiotensin II type 1 receptor (AT1R) and AT2R in the trophoblast have supported the presence of a local renin-angiotensin-system (RAS) in the placenta. This system regulates several mechanisms and functions.

Regarding the placenta development, it regulates:

- Angiogenesis: The AT1R is activated in the vascular endothelium and smooth muscle cells by Ang II and regulates the vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) release.
- Proliferation in extravillous cytotrophoblasts.
- Placental perfusion by vascular remodeling of spiral arteries.

Implantation: RAS upregulates blastocyst implantation. Ang II enhances decidual cell differentiation, thus the trophoblast invasion process into the maternal endometrium.

Regarding the placenta function, RAS participates in vasoconstriction, hormone secretion, proteins transport, and immune regulation. (35)

The ACE2 and Ang 1-7 are largely present in the uteroplacental unit, they inhibit Ang II stimulation to prostaglandins and nitric oxide (NO) synthesis. Thus, they sustain the feto-placental vessels vasodilatation. Furthermore, as ACE2 is abundant in the syncytiotrophoblast, which directly interfaces with the maternal blood, it increases levels of maternal circulating Ang 1-7. This results in decreasing maternal systemic vascular resistance. (36)

A study analyzed early pregnancy placentas (6 to 16 weeks) from elective termination of pregnancy, and term placentas (37-41 weeks) from healthy women having incurred elective cesarean sections. Using IHC, the RAS features were analyzed in early pregnancy placentas. The RAS proteins were expressed (REN, ATP6AP2, AGT, ACE, ACE2 and AGTR1 mRNAs) in all samples. All placentas expressed the ACE2 receptor. High positivity was found in the syncytiotrophoblast and villous stroma, it was also expressed in the cytotrophoblast but with less intensity. This distribution in syncytiotrophoblast supports the implication of ACE2 in the regulation of maternal circulation. In fact, the Ang 1-7 has a vasodilator role. Moreover, it is more intense in early than term pregnancies. (37)

3.3 ACE2 receptor in the lungs:

More than 20 years have passed since ACE2 was repaired in organs for the first time. The respiratory system localization has been proved in the epithelial respiratory system including the trachea, epithelial cells, and alveolar epithelial cells. (38) It is firstly dominating in type II pneumocytes and macrophages in the respiratory system, however it is also found in lung fibroblasts, bronchial and tracheal epithelial cells, and macrophages. The high expression of ACE2 in different organs allows its implication in various pathophysiologies, for instance, fibrosis, inflammation, oxidative stress, vasoconstriction. (39)

Similar to the cardiovascular system, ACE2 has a direct protective action on lung alveolar epithelial cells. Among the various physiological functions of ACE2 in the lungs, the majority protects against lung injury. As in the endothelial location, ACE2 removes a single amino acid from the C-terminal end of the octapeptide Ag II, to form the heptapeptide Ang1-7. Ang II that has a vasoconstrictive and proapoptotic function is degraded and its actions are inhibited after Ang 1-7 binding to the MAS receptor. Pharmaceutical preparations of recombinant ACE2 have been experimentally given to animals and have demonstrated protection against lung cell death, inhibition of acute lung injury and prevention of lung fibrosis prevention following chronic lung injury. (8)

Moreover, besides the RAS pulmonary system, lungs are considered as the major site for Ang I conversion to Ang II, which explains the ACE2 protection from AngII-AT1R induced inflammation. (33)

II. Results discussion:

1. Lung samples study:

1.1 ACE2 expression in the lungs:

COVID-19 infection occurs after SARS-CoV-2 entry in the respiratory tract through the ACE2 receptor that has been certainly characterized as the functional host receptor of the virus.(40) An IHC study was the first to prove that the virus entry in airways is synergistically moderated by ACE2 and TMPRSS2 proteins. (41) Hence, we studied the ACE2 receptor presence in the lungs. The three lung samples included in our study showed an intense cytoplasmic expression in the endothelial cells, alveoli (pneumocytes), and bronchioles. In addition to these sites, one sample expressed the receptor also in mesothelial cells. Some investigations show common findings with our study (40) ,(41)In addition, a study proves common expressions to ours, with supplementary findings.(42)**(table IV)** In our samples, one expressed the receptor also in mesothelial cells which we didn't find in literature.

1.2 NP expression in the lungs:

The direct evidence of SARS-CoV-2 infection is the presence of the viral particle in the infected organ since the infection occurs when the virion spike protein binds to the ACE2 receptor in the host cell. (43) NP, as a structural protein of the virus, has a relevant role in the infection. In the infected tissue, it is abundant and influences the interactions. (44) Thus, we studied the presence of the virus in the lungs and the placenta through the NP.

In the lungs, two out of our three samples presented low and diffuse cytoplasmic expression of SARS-COV-2 NP in the respiratory epithelium (bronchi and alveoli), one sample showed no expression of the NP. One study shows common spots for the receptor expression (43) , while others have common sites with supplementary findings.(45) ,(46) **(table IV)**

1.3 The infection severity and the receptors expression:

Regarding the virus aggressiveness, all of our three patients had a severe form of COVID-19 and presented intense expression of the ACE2 receptor in the IHC technique. This led us to think of a possible correlation between the severity related to SARS-CoV-2 and the receptor intensity. Though, publications are controversial in this regard. Intense expression reflects high ARDS severity, while it has been proved that ACE2 has a protective outcome on ARDS since the receptor deficiency has been related to high occurrence of morbidities. (42) Further investigations using IHC for ACE2 found different results, thus dissimilar conclusions. A severe pneumonia case showed no expression, and a moderate pneumonia case showed a weak expression in cells of the epithelium and endothelium. They conclude an association between the receptor level and the infection phase: the ACE2 is negative in the exudative phase of acute inflammation, and expands in the proliferative phase and fibrosis. (47)

Concerning the progression of the infection and its continuous effects, researchers are still debating. One Spike protein IHC study claims a persistent and continuous damage, while another one with the NP antibody claims that damage is a transitory phenomenon. Also, as the immune system eradicates the virus, tissues will lose their positivity over time. (48)

As ACE2 intensity is positively correlated to the case severity, this allows the virus entrance. The viral particle is present on the tissue, however it decreases and disappears with the infection evolution. We concluded that the low level of NP in our lung samples might be related to the phase of the infection. Thus, a low level is still significant and attests that the infection had occurred. Regarding the sample with no expression, it might be related to a late phase of the infection since the patient is at day 13th in the reanimation department, the latest among our lung samples.

2. Placenta samples study:

2.1 ACE2 expression in the placenta:

In our study, the three included placentas showed diffuse intense and moderate to intense expression of the ACE2 receptor. Reports from the literature demonstrated that ACE2 is expressed in the human endometrium and placenta among non-pregnant and pregnant women. (49) During pregnancy, it is intensively expressed in the reproductive organs, placenta, uterus and maternal-fetal interface, with the placenta revealing the highest expression. (50) Hence, the increased expression during pregnancy may induce hemodynamic changes. (51) While some scientists stand by the ACE2 mRNA and protein levels decrease among pregnant COVID-19 positive women, others defend the idea of the increase in the placental levels among at term pregnant women. (49)

The three placentas in our study showed a common expression of the ACE2 receptor in the cytotrophoblast, villus-axis, syncytiotrophoblast and decidual cells. A literature review manifested studies with similar results (52), others with similar and supplementary findings (50), or common and supplementary findings.(49) **(table IV)**

All through gestation, the ACE2 expression is diffuse in the uteroplacental unit which strengthens the hypothesis of placental vulnerability to SARS-CoV-2, eventually causing vertical transmission.(36)

2.2 NP expression in the placenta:

In our placenta samples, one out of three showed intense diffuse and focal cytoplasmic expression of cytotrophoblastic cells. The other samples showed no expression. A question that arises is how the virus can cross the placenta and cause direct effects on the fetus as it has been noticed.

Perinatal SARS-CoV-2 transmission is conditioned by proofs regarding mother's infection, exposure of the fetus, and a persistent infection in the fetus or neonate. A review from 2021 attests that intrauterine transmission has been rarely reported and tends to be uncommon. The viral particle should traverse the placenta to allow this transmission, which is not the case for SARS-CoV-2 as it doesn't involve high levels of viremia. (53) Another review from 2021 reveals the presence of the virus mRNA or virions in syncytiotrophoblasts and suggests the transplacental transmission, however knowing the low levels of viremia in COVID-19 patients, the probability is low. (54)

Our study, along with other publications, is proving maternal fetal transmission. Indeed, we had evidence regarding tests sampling from a woman in our study. The newborn, stool and placenta tested positive, and the receptor expression is intense and diffuse. One IHC research with SARS-CoV-2 NP antibody assessed the virus presence in the placenta of 9 COVID-19 patients, they found similar results to us with supplementary findings. (55) Another study performed IHC on placentas from different patients, one positive sample shows a trophoblastic expression, but different to ours, with other sites. (56)(table IV)

2.3 The infection severity and the receptors expression:

Since our samples were from different gestational ages, we got interested in the ACE2 receptor variation with the pregnancy evolution. In healthy pregnancies, ACE2 and Ang 1-7 are regulated by the estrogens release and increase in the plasma of pregnant women. Though, in COVID-19 pregnant women, it has been noticed that ACE2 levels are decreased and cases of preeclampsia-like syndrome where levels are low have been described. (36) However, in our study, the receptor expression was intense or moderate to intense in the three samples from 32

weeks and 2 days, 39 weeks and 41 weeks of pregnancy. Besides, the woman at 32 weeks and 2 days had a severe preeclampsia, still she showed an intense and diffuse expression for the receptor. Indeed, it has been proved that COVID-19 increases the probability of gestational hypertension and preeclampsia occurrence. (36) Recent studies have indicated that the high expression in the placenta has been observed among severe COVID-19 forms. (57) This is compatible with our results. In our cases, ⅔ cases were severe and showed intense and moderate to intense expression, however one non severe case showed an intense expression. Thus, we couldn't correlate between the severity and the intensity. Most especially, ACE2 and Ang-(1-7) tend to be a regulator that adapts with pregnancy cases varying from a low to a high level depending on the situation. (51) For instance, a study points out that cellular senescence causes ACE2 positive regulation. (52) In this optic, we highlight that the three of our samples had placental vascular malperfusion lesions in the histological analysis.

ACE2 was intense in all cases including severe and non-severe patients, in women from different gestational ages, moderate to intense at term. Thus, the virus intensity is not correlated to the severity nor the term. This high intensity allows the viral particle to cross the placenta. As this presence is translated by the NP positivity. NP was lowly positive in ⅓, and negative in ⅔ cases, probably due to low viremia. As immunity increases, NP levels decrease and the virus is eradicated (48), hence, cases might be from patients with chronic infection.

Regarding the vertical transmission, since other studies besides ours have proved that the virus entry through ACE2 in the placenta is possible (58), and samplings of the placenta, stool and newborn from a woman in our study tested positive to SARS-CoV-2, our hypothesis of the vertical materno-fetal transmission remains tenable. Plus, the fact that SARS-COV-2 infection is related to ACE2 high expression in the respiratory tract supports the conjecture that it is also related to ACE2 high expression in the placenta.

Table IV: ACE2 and NP receptors' expression in lung and placenta specimens among COVID-19 patients in studies by different authors

Reference	The expression's location
ACE2 receptor's expression in lung specimens	
(40) A. R. Bourgonje *et al., 2020*	Abundant expression on the surface of alveolar type II pneumocytes. Attention has been given to the capillary endothelial cells in close proximity since they have the same basement membrane and highly express ACE2. Accordingly, type II pneumocytes along with the close capillary endothelium are prone to allow primary entry.
(41) S. Damiani *et al.,2021*	ACE2 and TMPRSS2 are both expressed in the alveolar type II pneumocytes, in shed alveolar cells where DAD had occurred, and in recently formed interstitial capillaries. ACE2 is expressed in addition to this in endothelial cells.
(42) S. Alabsi et al.,2023	The receptor is abundantly expressed in type II pneumocytes and macrophages, in addition to other locations counting the lung fibroblasts and bronchial and tracheal epithelial cells.
NP receptor's expression in lung specimens	
(43) A. Nalwa *et al.,2022*	A study proves through IHC for NP that the virus has a cytoplasmic location in alveolar epithelial cells; large airway epithelium was not included in the samples.
(45) K. Skok *et al., 2021*	Lung autopsy realized by a study on 19 COVID-19 patients and IHC for a mouse monoclonal antibody against the SARS-CoV-2 NP shows a positive staining in the pneumocytes, bronchial epithelium, bronchial mucosal glands.
(46) L. R. Massoth *et al., 2021*	A comparison has been established between RNA in situ hybridization and IHC for SARS-CoV-2 NP on 8 autopsies from COVID-19 patients in the lungs and other tissues. Both techniques detected respectively the viral RNA and the NP in pneumocytes, bronchial epithelial cells, hyaline membranes and other uncertain locations.
ACE2 receptor's expression in placenta specimens	
(52) C. M. Zelop and E. A. Bonney, 2022	A research shows that it is expressed in decidua including stromal and perivascular cells, and in villous cells including cytotrophoblast and syncytiotrophoblast.
(50) A.Dhaundiyal *et al.,2021*	ACE2 expression in the placenta is diffuse and is located mainly in the syncytiotrophoblast, cytotrophoblast, endothelium and vascular smooth muscle of primary and secondary villi. In addition to maternal stroma in the invading and intravascular trophoblast and decidual cells. Moreover, arterial and venous endothelium and smooth muscle of the umbilical cord express the receptor

(49) K. G. Pringle and L. K. Philp, 2023	Another IHC study proves the presence in cytotrophoblasts, syncytiotrophoblasts, and invading and intravascular trophoblasts.
NP receptor's expression in the placenta specimens	
(55) D. Rakheja *et al., 2022*	One IHC research with SARS-CoV-2 NP antibody assessed the virus presence in the placenta of 9 COVID-19 patients. All samples show intense focal, multifocal or diffuse intracytoplasmic trophoblastic expression. In addition, they have considered trophoblastic necrosis and extracytoplasmic granular to chunky staining
(56) F. Facchetti *et al., 2020*	Another study performed IHC on placentas from different patients but we got interested in the 15 placentas from COVID-19 patients. SARS-CoV-2 Spike protein and NP antibodies were used. One placenta stained positively; the common results show intense cytoplasmic syncytiotrophoblastic expression. Concerning NP, it is particularly stained in rare intervillous macrophages and Hofbauer cells and its distribution is intense and homogenous in the parenchyma.

Conclusion :

To sum up, literature has well studied the SARS-CoV-2 structure and biology, and the infection process through the ACE2 receptor in host cells. Evidence is various and certain regarding the virus entry through the respiratory tract and its presence in lung tissue, however researchers are still debating the particle's presence in the placenta and the vertical transmission of COVID-19. In our retrospective IHC study, we proved the virus presence in the lungs and placenta, and support the maternal-fetal transmission hypothesis.

ABSTRACT

Abstract

Introduction: coronavirus disease of 2019 (COVID-19) is an initially respiratory and occasionally multisystemic infectious disease. It is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It is a highly pathogenic and remarkably transmissible virus whose two known receptors are angiotensin converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2). A series of autopsy cases and tissue samples have been presented in the literature using immunohistochemical and in situ hybridization assays which are the most promising for the tissue identification of the virus and the evaluation of its distribution in different tissue types. We proposed then the detection of viral particles and the evaluation of the distribution of receptor proteins in the different tissue samples recruited in our department; fixed and included in paraffin and this since the detection of the first cases in our city.

Methods: we carried out an immunohistochemical detection of viral particles and receptor proteins on viral SARS-CoV-2 nucleocapsid and ACE2 receptor protein antigens. It was performed on different fixed and paraffin-embedded tissue samples from our pathology department at the Mohammed 6th university hospital of Marrakech involving 6 cases positive to SARS-CoV-2.

Results:

-ACE2 expression in lungs: three cases showed intense cytoplasmic expression of endothelial cells, alveoli (pneumocytes), and bronchioles, one case expressed also mesothelial cells.

-NP expression in lungs: two cases showed low and diffuse cytoplasmic expression in the respiratory epithelium (bronchi and alveoli), one case showed no expression.

-ACE2 expression in placenta: two cases showed intense and diffuse cytoplasmic expression of cytotrophoblast,villus-axis, syncytiotrophoblast and decidual cells. One case showed a moderate to intense expression with the same features.

-NP expression in placenta: one case showed intense and diffuse cytoplasmic expression of cytotrophoblastic cells, two cases showed no expression.

Conclusion: evidence is various and certain regarding the virus entry through the respiratory tract and its presence in lung tissue, however researchers are still debating the particle's presence in the placenta and the vertical transmission of COVID-19, a hypothesis that we defend.

Résumé

Contexte : la maladie à coronavirus 2019 (COVID-19) est une maladie infectieuse initialement respiratoire et occasionnellement multisystémique. Elle est causée par le severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Il s'agit d'un virus hautement pathogène et remarquablement transmissible dont les deux récepteurs connus sont l'enzyme de conversion de l'angiotensine 2 (ACE2) et la protéase transmembranaire sérine 2 (TMPRSS2). Une série de cas d'autopsie et d'échantillons de tissus ont été présentés dans la littérature en utilisant des tests immunohistochimiques et d'hybridation in situ qui sont les plus prometteurs pour l'identification tissulaire du virus et l'évaluation de sa répartition entre les différents types de tissus. Nous proposons alors la détection des particules virales et l'évaluation de la distribution des protéines réceptrices dans les différents prélèvements tissulaires recrutés dans notre service; fixés et inclus en paraffine et ce depuis la détection des premiers cas dans notre ville.

Méthodes : on a procédé à une détection immunohistochimique des particules virales et des protéines réceptrices sur les antigènes nucléocapside virale SARS-CoV-2 et la protéine réceptrice ACE2. Elle a été réalisée sur différents échantillons de tissus fixés et inclus en paraffine provenant de notre service d'anatomie pathologique au Centre Hospitalier Universitaire Mohammed 6 de Marrakech, impliquant 6 cas positifs au SARS-CoV-2.

Résultats:

-Expression de l'ACE2 dans les poumons : trois cas ont montré une expression cytoplasmique intense des cellules endothéliales, des alvéoles (pneumocytes) et des bronchioles, un cas a également exprimé les cellules mésothéliales.

-Expression de NP dans les poumons : deux cas ont montré une expression cytoplasmique faible et diffuse du revêtement bronchique et du revêtement pneumocytaire.), un cas n'a pas montré d'expression.

-Expression de l'ACE2 dans le placenta : deux cas ont montré une expression cytoplasmique intense et diffuse dans cytotrophoblaste, de l'axe des villosités, du syncytiotrophoblaste et des cellules de la caduque. Un cas présentait une expression modérée à intense avec les mêmes caractéristiques.

-Expression de NP dans le placenta : un cas a montré une expression cytoplasmique intense et diffuse des cellules cytotrophoblastiques, deux cas n'ont pas montré d'expression.

Conclusion : les preuves sont diverses et certaines en ce qui concerne l'entrée du virus par les voies respiratoires et sa présence dans le tissu pulmonaire, mais les chercheurs débattent encore de la présence de particules dans le placenta et de la transmission verticale de COVID-19, une hypothèse que nous défendons.

ملخص

المقدمة: إن مرض فيروس كورونا 2019 (COVID-19) هو مرض معدٍ يبدأ عادةً كمرض تنفسي وقد يكون أحيانًا متعدد الأجهزة. يتسبب فيه فيروس المتلازمة التنفسية الحادة الوخيمة كورونا 2 (SARS-CoV-2). يعد هذا الفيروس شديد الشراسة وقابلًا للانتقال بشكل ملحوظ، حيث يمتلك مستقبلين معروفين وهما إنزيم تحويل الأنجيوتنسين 2 (ACE2) والبروتياز السيرين عبر الغشائي 2 (TMPRSS2). تم تقديم سلسلة من حالات تشريح الجثث وعينات الأنسجة باستخدام فحوصات الكيمياء النسيجية المناعية والتهجين الموضعي التي تعتبر واعدة لتحديد الفيروس على الأنسجة وتقييم توزيعه في أنواع الأنسجة المختلفة. نقترح بعد ذلك الكشف عن الجسيمات الفيروسية وتقييم توزيع البروتينات المستقبلة في مختلف العينات النسيجية المأخوذة من قسمنا؛ المثبتة والمضمنة في البارافين منذ الكشف عن الحالات الأولى في مدينتنا.

المنهجية: تم الكشف عن الجسيمات الفيروسية والبروتينات المستقبلة عن طريق الكشف االمناعي الكيميائي لمستضدين النوكليوكابسيدا الفيروسي SARS-CoV-2 والبروتين المستقبل ACE2. تم إجراء ذلك على عينات نسيجية مختلفة مثبتة ومضمنة في البارافين بقسم التشريح المرضي بالمركز الاستشفائي الجامعي محمد السادس بمراكش، حيث شمل ذلك ست حالات إيجابية لفيروس SARS-CoV-2.

النتائج:

تعبير ACE2 في الرئتين: أظهرت ثلاث حالات تعبيراً سيتوبلازمياً مكثفاً في الخلايا البطانية والحويصلات الهوائية (الخلايا الرئوية) والقصيبات الهوائية، وأظهرت حالة واحدة تعبيراً في الخلايا الظهارية أيضا.

تعبير NP في الرئتين: أظهرت حالتان تعبيرًا سيتوبلازميًا منخفضًا ومنتشرًا في طلاء الجهاز التنفسي (القصبات الهوائية والحويصلات الهوائية)، ولم تظهر حالة واحدة أي تعبير.

تعبير ACE2 في المشيمة: أظهرت حالتان تعبيراً سيتوبلازمياً مكثفاً ومنتشراً في خلايا الأرومة الغاذية الخلوية والخلايا المحورية الزغبية وخلايا الأرومة الغازية المخلوية والخلايا الخلايا الساقطية. أظهرت حالة واحدة تعبيراً متوسطاً إلى شديد بنفس السمات.

تعبير NP في المشيمة: أظهرت حالة واحدة تعبيرًا سيتوبلازميًا شديدًا وموزعًا في خلايا الأرومة الغاذية الخلوية. لم تظهر حالتان أي تعبير.

الخاتمة: الأدلة متنوعة ومؤكدة فيما يتعلق بدخول الفيروس عبر الطرق التنفسية ووجوده في الأنسجة الرئوية، لكن ما زال الباحثون يناقشون وجود الجسيمات في المشيمة وانتقال COVID-19 من الأم إلى الجنين عموديًا، وهي فرضية ندافع عنها.

Appendices:

Data Sheet

- **Identity:**

-Test number:

-Sexe:

-Age:

- **Specimen site:**

□ Lung □ Placenta

- **Specimen type:**

□ Biopsy □ Surgical specimen

- **Clinical information:**

-Past medical history:

-History of present illness:

-Clinical signs:

-CT scan:

-PCR:

-Treatment:

- **Histological findings:**
- **Immunohistochemistry results:**

-ACE2 expression: □ Present □ Absent;

□ Intense □ Moderate □ Weak;

□ Diffuse □ Focal;

Location:

-NP expression: □ Present □ Absent;

□ Intense □ Moderate □ Weak;

□ Diffuse □ Focal;

Location:

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قسم الطبيب

أقْسِم بِالله العَظِيم

أن أراقبَ الله في مِهْنَتِي.

وأن أصُونَ حياة الإنسان في كآفّةِ أطوَارهَا في كل الظروف والأحَوال باذلا وسْعِي في انقاذها مِن الهَلاكِ والمرَضِ والألَم والقَلَق.

وأن أحفَظَ لِلنَاسِ كرَامَتهُم، وأسْتر عَوْرَتهُم، وأكتمَ سِرَّهُمْ.

وأن أكونَ عَلى الدوَام من وسائِل رحمة الله، باذلا رعايتي الطبية للقريب والبعيد، للصالح والطالح، والصديق والعدو.

وأن أثابر على طلب العلم، وأسَخِّرَه لِنَفْعِ الإنْسَان لا لأذَاه.

وأن أُوَقّرَ مَن عَلَّمَني، وأُعَلّمَ مَن يَصْغرَني، وأكون أخا لِكُلِّ زَميلٍ في المِهنَةِ الطِّبِّيَة مُتعَاونِينَ عَلى البرِّ والتقوى.

وأن تكون حياتي مِصْدَاق إيمَاني في سِرّي وَعَلانيَتي، نَقِيَّة مِمّا يُشينهَا تجَاهَ الله وَرَسُولِهِ وَالمؤمِنين.

والله على ما أقول شهيد

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MARRAKECH
جامعة القاضي عياض
UNIVERSITÉ CADI AYYAD

السنة 2024 الاطروحة رقم 400

الكشف عن جزيئات سارس-كوف-2 وتقييم توزيع البروتين المستقبل ACE2 في عينات أنسجة مختلفة بقسم التشريح المرضي بالمستشفى الجامعي محمد السادس بمراكش.

الأطروحة

قدمت ونوقشت علانية يوم 2024/10/22

من طرف

السيدة إحسان الزاهري

المزداد في 25/04/1999 بمدينة آسفي

لنيل شهادة الدكتوراه في الطب

الكلمات المفتاحية

مرض فيروس كورونا 2019 (كوفيد-19) - فيروس المتلازمة التنفسية الحادة الوخيمة كورونا 2 (SARS-CoV-2) - الكشف المناعي الكيميائي- بروتين النوكليوكابسيد (NP)-إنزيم تحويل الأنجيوتنسين 2 (ACE2) -الرئتين-المشيمة- الحمل- الانتقال العمودي- العدوى عبر المشيمة.

اللجنة

السيدة	ح. رايس أستاذة التشريح المرضي.	الرئيسة
السيدة	ف. هزميري أستاذة في علم الأنسجة وعلم الأجنة وعلم الخلية.	المشرفة
السيدة	ب. فخير أستاذة في طب النساء والتوليد.	الحكام
السيد	ع. هاشمي أستاذ في الإنعاش الطبي.	
السيد	أ. فخري أستاذ في علم الأنسجة وعلم الأجنة وعلم الخلية	