The effect of COVID-19 on the cytokine profile, placental function and morphology during pregnancy

By

CHANEL HEERALALL

214528607

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A Thesis submitted to

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In fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Discipline of Clinical Anatomy

Supervisor: Prof Irene Mackraj

Co-supervisor: Prof Lelika Lazarus

Preface

The experimental and research work described in this thesis was conducted by the candidate at the University of KwaZulu-Natal (Durban, South Africa) and Groote Schuur Hospital, University of Cape Town (Cape Town, South Africa) from March 2021 to December 2024, under the supervision of Professor Irene Mackraj and co-supervision of Professor Lelika Lazarus for the award of Doctor of Philosophy Degree in Clinical Anatomy and has not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it has been duly acknowledged in the text in the form of references. The results reported are from investigations by the candidate.

[redacted] 27 February 2025

Signed: C Heeralall Date:

[redacted] 28 February 2025

Signed: Professor Irene Mackraj (Supervisor) Date:

[redacted] 23 February 2025

Signed: Professor Lelika Lazarus (Co-supervisor) Date:

Declaration

I, Chanel Heeralall, declare as follows:

1. The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work.
2. This dissertation has not been submitted in part or in full for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.
4. This dissertation does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    i. Their words have been re-written, but the general information attributed to them has been referenced.
    ii. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced.

5. This dissertation does not contain text, graphics, or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and the references sections.

The PhD candidate performed experimental work described in this thesis, where others have made contributions, it is duly acknowledged in the text. The candidate drafted this publication in full and it has been reviewed by co-authors.

27 February 2025

Signed: C Heeralall

Date:

Publications and Presentations

Peer-reviewed publications contributing to this thesis:

Heeralall C, Ibrahim U H, Lazarus L, Gathiram P, & Mackraj I. (2023). The effects of COVID-19 on placental morphology. Placenta, 138, 88-96.

https://doi.org/10.1016/j.placenta.2023.05.009 **(Chapter Two of this thesis)**

Part of the findings observed in this study was presented at the following symposium:

Heeralall C, Ibrahim U H, Jenneker M, Singh S, Mackraj I. The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile.

Presented at the University of KwaZulu-Natal, College of Health Science CHS Annual Research Symposium 2024, 27-28th August in Durban, South Africa **(Appendix 6.6)**

Abstract **published** in the UKZN CHS Symposium 2024 Book of Abstracts- **First Prize for Oral PhD Presentation**

Statement

The following publications have been included as chapters in this thesis (i.e., Chapter Two to Four):

Published:

Chapter Two: The effects of COVID-19 on placental morphology. (Published: Placenta, 2023, pages 88-96)

In Revision:

Chapter Three: The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile. **(Submitted to:** American Journal of Reproductive Immunology, manuscript ID- AJRI-09-24-296**)**. Peer review and revision has been done. Revised manuscript is under consideration for publication **(Appendix 6.4)**.

Chapter Four: The effect of COVID-19 on placental functioning in South African pregnancies: Investigation of kisspeptin expression, vascular and inflammatory alterations. **(Submitted to:** Histochemistry and Cell Biology Journal, submission ID- 69490395-7d17-4fb8-bec8-5986ae1f6c13**)**. Peer review has been complete, in revision for publication **(Appendix 6.5)**.

The PhD candidate performed all the experimental work described in this publication, where others have made contributions, this is duly acknowledged in the text. The candidate drafted this publication in full and it has been reviewed by co-authors.

Funding

This project was funded by the College of Health Sciences at the University of KwaZulu-Natal and the National Research Foundation of South Africa (Grant No MND210518602191).

[redacted] 27 February 2025

Signed: C Heeralall Date:

[redacted] 28 February 2025

Signed: Prof I Mackraj Date:

[redacted] 23 February 2025

Signed: Prof L Lazarus Date:

Dedication

To the women globally who doubt their worth and are robbed of opportunities and education due to their gender. To the women who doubt their ability to make their dreams a reality. To the women who have a greater vision that many may not understand or support, this is a sign you can do it. Therefore, I dedicate this dissertation to women, those in science, those fighting for equality, education and an opportunity to make their dream a reality.

Remember, "There is no limit to what we, as women, can accomplish." — Michelle Obama

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My postgraduate journey has been long and strenuous. It is through God that I have managed to reach this finish line. Over the last twelve years, God has granted me the strength, courage, wisdom, and perseverance to continue in this pursuit even when times were bleak, and I considered giving up. Hence, words cannot express the gratitude I possess for my higher power and the universe which gave me the support and tools to complete this journey. The greatest tool the universe has gifted me with, has been my support structure, without which I would not be here today. The author wishes to convey her gratitude to the following:

- To my supervisors, Prof Irene Mackraj and Prof Lelika Lazarus- I am enthralled to have witnessed the strides you have both made as women in science, and I sincerely thank you for taking me under your wing. You both are certainly role models to me, and I aim to use your motivation and guidance to keep pushing as a woman in science.
- To my family, my parents Roshini and Harold Heeralall- you have not only given me life but have consistently supported my dreams and endeavours to help me find my purpose. For this, thank you is not enough. Your sacrifices, strength, and motivation have moulded me into the woman I am. You both are my greatest blessings. I could never forget my sunshine on the darkest days, my Dog, Wrinkles Heeralall. The happiness I received from your presence was immeasurable. You parted with me in December 2024, but your memory and support will never be forgotten. Rest in peace, my angel.
- To my best friend Roushka Bhagwan Valjee- we have walked this road together, and it has not been an easy journey. These past years were filled with much pain, tears, and struggles. But through it all, you were next to me, and I could not have received a greater blessing. As we approach the end of our journey, we embark on a new road to hopefully make a difference in the world, and with you by my side, nothing is impossible; the world is our oyster!
- To postdoctoral fellow Dr. Usri Hassan Ibrahim- You have guided me, kept me motivated, and never failed to assist me; for that, I thank you. I would not have been at the finish line without your constant support.
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- To my collaborators, Dr. Marwah Jenneker, Prof Mushi Matjila, and Dr. Shoohana Singh, words cannot express my gratitude to thank you enough for all the assistance, guidance, and support you have provided me. My research would not have been possible without you all.
- To my family and friends, I thank you all for your constant motivation and support. In particular, the Naidoo family and my sister Denata Naidoo. Your constant support has been my pillar of strength.
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TABLE OF CONTENTS

List of Figures

Manuscript One

Manuscript Two

Manuscript Three

List of Abbreviations

ACE-2 - Angiotensin-converting enzyme 2

ARDS - Acute respiratory distress syndrome

COVID-19 - Coronavirus disease

CRS - Cytokine release syndrome

DCs - Dendritic cells

ER - Endoplasmic reticulum

ERGIC - Endoplasmic reticulum-Golgi intermediate compartment

EVs - Extracellular vesicles

FVM - Foetal Vascular Malperfusion

HGF - Hepatocyte Growth Factor

HGFR - Hepatocyte Growth Factor Receptor

IFN-γ - Interferon gamma

IL-1β - Interleukin-1 beta

IL-6 - Interleukin-6

IL-8 - Interleukin 8

IUGR - intrauterine growth restriction

KISS - Kisspeptin

MVM - Maternal Vascular Malperfusion

MCP-3 - Monocyte chemotactic protein-3

MIP-1α/CCL3 - Macrophage Inflammatory Protein-1 Alpha

NK - Natural Killer

RAS - Renin-angiotensin system

RAAS - Renin Angiotensin Aldosterone System

RTC - Replication-transcription complex

SARS-CoV-2 - Severe acute respiratory syndrome coronavirus 2

TNF-α - Tissue necrosis factor-alpha

TMPRSS2 - Transmembrane serine protease 2

WHO - World Health Organisation

Abstract

The outbreak of COVID-19 in 2019 affected the world globally with a particularly detrimental impact on the healthcare sector. It has now passed; however, the long-term consequences of the infection are yet to be fully elucidated. Inflammation, which is of great concern, has been linked to COVID-19 infections, commonly referred to as the 'cytokine storm.' This storm poses a significant risk to mothers and neonates due to its association with gestational complications, along with its role in predisposing infants to disorders. These alterations due to COVID-19, together with its hypoxic nature, have further resulted in concerns for proper placental functioning in these pregnancies, which are yet to be investigated in the Black South African cohort. Thus, for the first time, the present study focuses on the cytokine profile, placental function and morphology in South African pregnancies.

These concerns prompted the evaluation of the cytokine profile in the plasma and extracellular vesicles (EVs) of women with these pregnancies, along with assessing the kisspeptin expression and morphology in the placentae from these pregnancies. An altered cytokine profile was identified, suggestive of hyperinflammation in the plasma and EVs from COVID-19 pregnancies in the South African cohort. Further histopathological analysis revealed that the placentae from these pregnancies presented with severe signs of inflammation and malperfusion, which were considered to be linked to the altered kisspeptin expression we observed in these placentae, thereby suggesting altered placental functioning in COVID-19 pregnancies from the South African cohort.

It is clinically evident that pregnant women of South Africa, who already face challenges due to an increased risk of HIV and other gestational complications, were severely negatively impacted during this pandemic. These findings reveal an increased prevalence of complications like preeclampsia, preterm birth, intrauterine growth restriction (IUGR), and stillbirth during pregnancy. In addition to a host of complications in foetal development, these alterations could predispose these neonates to anomalies. Importantly, this study has identified that the cytokine profile was altered in COVID-19 pregnancies. This alteration could possibly have had an impact on the kisspeptin signalling, which would then affect optimal placental functioning, thus suggesting a probable cause for the severe dysfunction observed in the placentae. In addition, this environment did not support ideal foetal development and growth.

Therefore, it is believed that the findings from this study warrant monitoring and evaluation of neonates from COVID-19 pregnancies, especially from mothers in the South African cohort, as anomalies or neurodevelopmental disorders can arise due to these alterations observed.

1.0 CHAPTER ONE

1.1 Introduction

The novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) identified in Wuhan, China (2019) impacted every aspect of life, especially globalization, health care, and travel, thereby changing the course of history (Shrestha *et al.*, 2020, Yuki *et al.*, 2020). As a result, a worldwide pandemic ensued, resulting in an increased death toll, which peaked at 6.9 million in October 2023 (Yuki *et al.*, 2020, WHO, 2023). SARS-CoV-2 was discovered to be transmitted by respiratory droplets that entered the body by attaching themselves to the angiotensin-converting enzyme 2 (Kumar *et al.*, 2021a, Kumar *et al.*, 2021b, Jackson *et al.*, 2022). Consequently, resulting in a cascade of reactions including dysregulation of the immune system (Kumar *et al.*, 2021a, Kumar *et al.*, 2021b, Jackson *et al.*, 2022).

According to reports, this virus takes five days to incubate (range: two to fourteen days), causing symptoms such as headaches, fever, diarrhoea, myalgia, and coughing (Rasmussen *et al.*, 2020). Based on the severity of infection severe respiratory illness and death could occur (Rasmussen *et al.*, 2020). The accelerated global spread and lack of a robust therapeutic strategy during the pandemic prompted global lockdowns and measures such as social distancing (Dashraath *et al.*, 2020, Yuki *et al.*, 2020). While investigations have concentrated on the disease within diverse groups, it holds particular relevance in the field of reproductive health, where the repercussions may continue to materialize in the developing foetus, postnatally.

Specifically, pregnant women's immunological, pulmonary, cardiovascular, and haematological systems are significantly impacted by the mechanical and physiological changes that take place during pregnancy (Dashraath *et al.*, 2020, Wastnedge *et al.*, 2021, Simbar *et al.*, 2023). This affects their susceptibility to infections, making expectant mothers and their developing foetuses a high-risk population during this pandemic (Dashraath *et al.*, 2020, Wastnedge *et al.*, 2021, Simbar *et al.*, 2023). In addition, they are more susceptible to respiratory pathogens with a low tolerance for inflammation, which has been linked to several gestational conditions such as pregnancy loss, premature birth, and death (Vesce, 2021). Hence, this resulted in significant concerns for expectant mothers throughout the pandemic due to the ‘infective-inflammatory’ nature of the coronavirus disease (COVID-19) (Cavezzi *et al*., 2020, Fajgenbaum and June, 2020, Rahman *et al*., 2021).

Such an environment, of hyperinflammation is known for altering placental homeostasis, raising concerns about placental sufficiency in COVID-19 pregnancies (Shanes *et al.*, 2020, Fatih *et al.*, 2022). This excessive inflammation resulted in the 'cytokine storm', which has been further linked to placentitis, thrombosis, and other adverse outcomes (Corn *et al.*, 2023, Holland *et al.*, 2023). Kisspeptin (KISS) is critical in decidualisation and implantation; however, no extensive research has yet been conducted on how this regulator may be affected by COVID-19 or by inflammation as a result of this virus (Rosario *et al.*, 2008, Tsoutsouki *et al.*, 2022). Assessing this in pregnancy is essential as any alterations could affect placental functioning and foetal health, thereby posing very high risks. This study aim to shed light on these risks.

1.1.1 Purpose of the study

Studies have investigated how COVID-19 has impacted pregnancies with alarming evidence of thrombotic, inflammatory, and vascular alterations (Prochaska *et al.*, 2020, Wastnedge *et al.*, 2021). It is apparent that placentae from COVID-19 positive pregnancies exhibit signs of inflammation and malperfusion (Baergen and Heller, 2020, Hecht *et al.*, 2020, Shanes *et al.*, 2020). However, the mechanism through which COVID-19 results in these alterations has not yet been elucidated. Thus, attention has been drawn towards the cytokine storm in pregnant women, as evidence suggested that COVID-19 results in an exaggerated immune response. Importantly, Wang *et al.* (2020) noted there was a gap in the literature regarding this response in pregnant women, especially in terms of the role that EVs play in these pregnancies. EVs have been reported to be secreted in cases of poor placentation and to promote an inflammatory state. However, this is yet to be explored in COVID-19 pregnancies (Matsubara *et al.*, 2021). Importantly, the placental development and functioning in these pregnancies have not been fully assessed, and should be investigated to establish potential mechanisms.

Furthermore, in South Africa, pregnant women have been found to be the most vulnerable in this pandemic due to the lack of access to proper healthcare facilities, increased pregnancy complications, and other social disparities that exist in the developing world (Lone and Ahmad, 2020b, Stratton *et al.*, 2021). This cohort is yet to be studied to determine the effects that COVID-19 could have had on these vulnerable mothers and their neonates. This is essential for their future health and well-being. Hence, a global effort is considered essential in determining the consequences of COVID-19 infection during pregnancy on the cytokine profile, placental functioning, maternal health, and foetal development (Wastnedge *et al.*, 2021).

Consequently, this study aims to investigate how COVID-19 has impacted South African pregnant women by analysing the cytokine profile in the plasma and EVs of these women. This was done together with assessing the expression of kisspeptin as a marker of proper placental development and thereafter assessing the morphology of these placentas to determine functionality. Thus, this research can be utilised to begin to establish any adverse maternal or foetal outcomes that may occur as a result of COVID-19, to ensure monitoring of the future health of women and infants from COVID-19-positive pregnancies post-pandemic.

1.1.2 Broad Aims

The aims of this study were as follows:

AIM 1: To critically review the literature pertaining to placental morphological alterations **(Chapter 2).**

AIM 2: To investigate how COVID-19 impacts EVs and the pro-inflammatory cytokine profile in the plasma and EVs of South African pregnancies **(Chapter 3)**.

AIM 3: To assess placental morphology and histopathological changes in COVID-19 pregnancies of South African women **(Chapter 4)**.

1.1.3 Specific Objectives

The objectives of this study were as follows:

- To investigate how COVID-19 impacts the placenta, its morphology, and functional ability.
- To assess how COVID-19, other pregnancy complications, and therapeutics are related.
- To isolate EVs from plasma using the Invitrogen™ Total Exosome Isolation Kit.
- To characterize these EVs using Nanoparticle tracking analysis and Transmission electron microscopy.
- To measure the plasma and EVs levels of IFN gamma, IL-6, MIP-1 alpha and TNF alpha in COVID-19 positive and negative pregnancies.
- To fix and stain central and peripheral pieces of placental tissue, using Haematoxylin and Eosin (H&E) and Masson's Trichrome (MT) staining for identification of any morphological alterations.
- To use immunohistochemistry to evaluate the expression of kisspeptin in this placental tissue from COVID-19-positive and COVID-19-negative pregnancies.

- To morphometrically analyse this kisspeptin expression using colour deconvolution on Fiji ImageJ software.

The outcomes from this study are novel and will contribute to further knowledge on the pathophysiology of pregnancy-related COVID-19 infection in the Black South African population, which may assist in understanding and treating pregnancy during future viral pandemics.

1.2 Literature review

1.2.1 SARS-CoV-2 pandemic

December 2019 proved to be a pivotal point in history with the discovery of COVID-19, which originated in Wuhan, in China's Huanan Seafood Wholesale Market (Ciotti *et al.*, 2020). This highly contagious virus resulted in a worldwide crisis emerging through rapid global transmission, resulting in millions of deaths (Ciotti *et al.*, 2020, Lone and Ahmad, 2020a, WHO, 2023). This single-stranded RNA virus, responsible for dire consequences worldwide through transmission *via* aerosols and respiratory droplets, was officially identified as SARS-CoV-2 in January 2020 (He *et al.*, 2020, Parasher, 2020). On 11th March 2020, the World Health Organisation (WHO) declared a worldwide pandemic due to this viral outbreak, with 2.1 million cases being confirmed in just one month (Lone and Ahmad, 2020a). As a result, turmoil in the health sector ensued with a great need for precautionary measures such as national lockdowns and quarantines to be implemented (Lone and Ahmad, 2020a). Bats, hosts of SARS-CoV-like and MERS-CoV-like viruses from the Coronaviridae family, have been implicated in the development of SARS-CoV-2 (Lone and Ahmad, 2020a, Pollard *et al.*, 2020). Unfortunately, no one was immune to this virus, with SARS-CoV-2 possessing the ability to transfer not only from surfaces but even across the placenta (Singhal, 2020, Vivanti *et al.*, 2020). Posing notable risk to vulnerable populations including pregnant women and their unborn foetuses. It became critical for scientists to investigate and acquire more knowledge about this virus (Singhal, 2020, Vivanti *et al.*, 2020). The respiratory system had to endure the greatest impact from this virus as it could result in pneumonia to hypoxia, as depicted in Figure 1, with Acute Respiratory Distress Syndrome (ARDS) and even dyspnoea (He *et al.*, 2020, Yuki *et al.*, 2020). Therefore, mechanical ventilation was a critical requirement as severe cases could even experience respiratory failure and septic shock whilst milder symptoms included coughs, fevers and diarrhoea (He *et al.*, 2020, Yuki *et al.*, 2020).

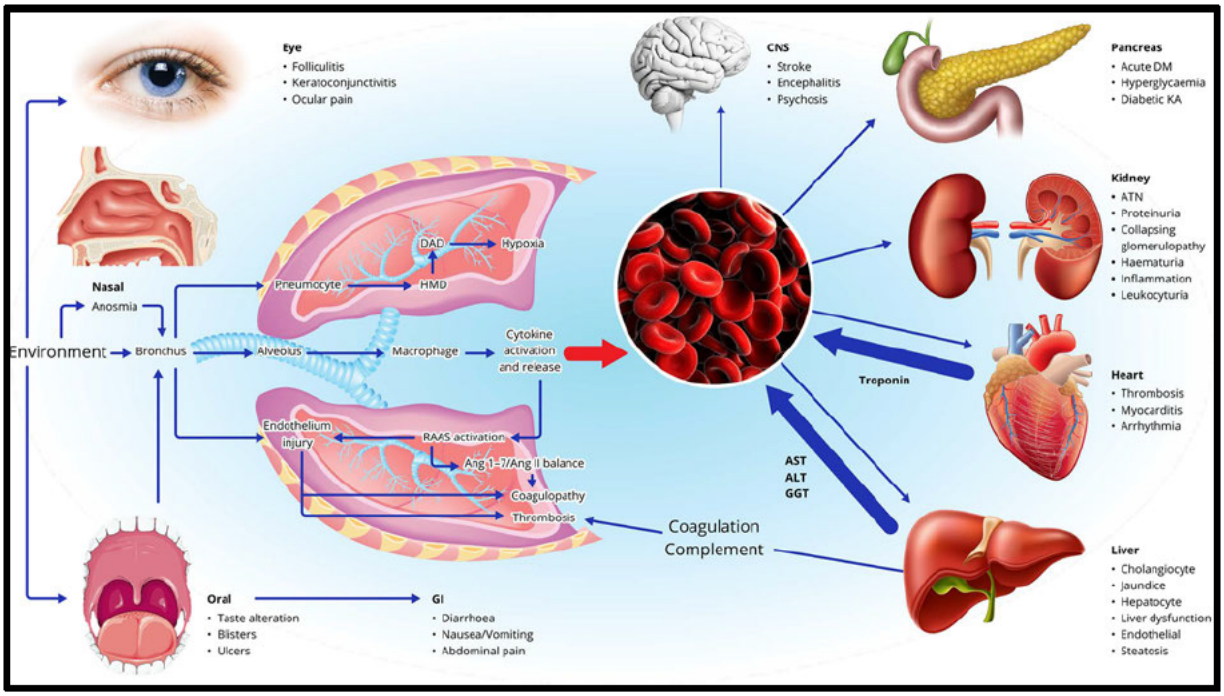

Figure 1: Diagrammatic representation depicting histological alterations due to coronavirus 2 infection in severe acute respiratory syndrome.

Illustrating the three primary entrances into the respiratory system, namely the eye, nasal cavity, and oral route. When the pneumocytes in the respiratory tract are infected, a hyaline membrane is formed, after which diffuse alveolar damage and hypoxia occur. When bronchiolar epithelial cells are damaged and macrophages are stimulated, cytokines are released into the bloodstream and alveolar spaces. As a result of these alterations, the renin-angiotensin-aldosterone system is activated, which promotes a pro-thrombotic state. In addition, organs like the heart, brain, pancreas, liver, and kidney can be damaged via viraemia or cytokinaemia. This damage is activated through the increase in liver enzymes or troponin, in addition to the factors released from the pro-thrombotic state. [Adopted from: (Sridhar and Nicholls, 2021); Copyright obtained from RightsLink (Appendix 6.8)]

1.2.2 Pathophysiology: Mechanism of SARS-CoV-2 invasion into host cells

Clinically, the coronavirus has three stages of infection in humans (Mason, 2020). These include the asymptomatic phase- which represents the initial infection; the respiratory phase- whereby the infection progresses along the respiratory tract; and lastly, the alveolar phase- in which the virus impacts the alveolar cells responsible for gaseous exchange (Mason, 2020).

SARS-CoV-2 utilises the transmembrane serine protease 2 (TMPRSS2) along with the ACE-2 receptor as the entry activator and receptor, respectively (Shang *et al.*, 2020).

1.2.2.1 ACE-2 receptors and their role

SARS-CoV-2 effortlessly targets and enters the lungs, immune system, and vasculature via binding to the surface of respiratory cells, thereby entering the host using the ACE-2 receptor (Das, 2020). The ACE-2 receptor, which is found in type 2 alveolar cells and is strongly expressed in the gastrointestinal tract, makes the upper respiratory tract the virus's main entry point (Galanopoulos *et al.*, 2020, Triggle *et al.*, 2021). The renin-angiotensin system (RAS) depends on this receptor, a crucial peptide that breaks down angiotensin II (Ang II), which is involved in significant processes such as hypertension, inflammation, and oxidative stress (Das, 2020). Infection due to SARS-CoV-2 causes a decrease in ACE-2 levels, thereby disrupting the Renin Angiotensin Aldosterone System (RAAS), which results in impaired circulatory and inflammatory functioning (Guo *et al.*, 2020a, Triggle *et al.*, 2021).

1.2.3 Viral life cycle and host cell invasion

1.2.3.1 Mechanism of viral entry into host cells

The life cycle of SARS-CoV-2 comprises viral attachment, penetration via membrane fusion or endocytosis, biosynthesis, maturation, and release, as shown in Figure 2 (Yuki *et al.*, 2020).

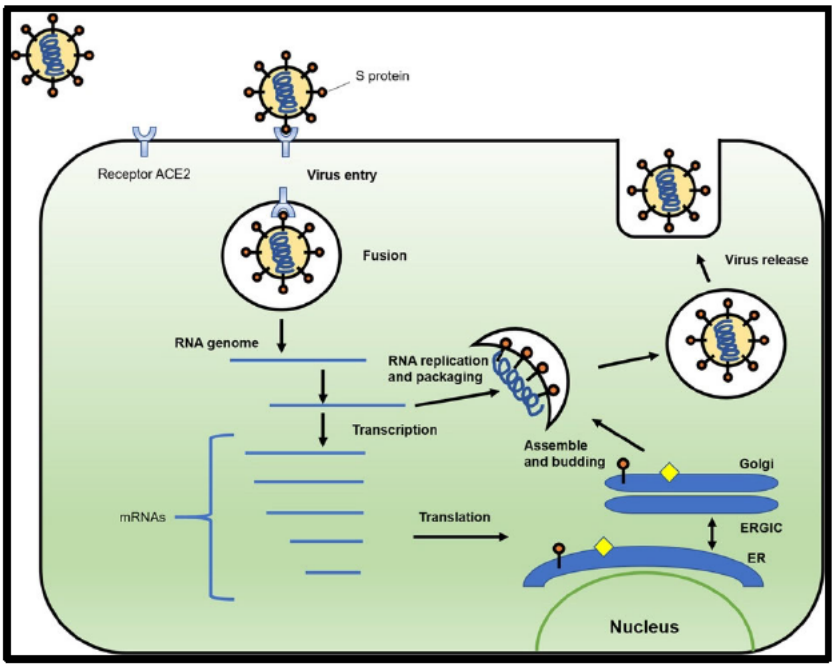

Figure 2: Schematic model of SARS-CoV-2 life cycle.

The S protein binds to the ACE-2 receptor, which enables viral entry. Thereafter, fusion of the membranes, viral replication, and transcription occurs. As a result, proteins are manufactured and assembled along with new viral RNA genome in the Golgi and ER. This is followed by the release of additional virions and budding into the ERGIC lumen. ACE-2, angiotensin-converting enzyme 2; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum-Golgi intermediate compartment.[Adopted from: (He et al., 2020); CC BY 4.0- https://creativecommons.org/licenses/by/4.0/]

Coronaviruses are made up of four structural proteins, viz. the membrane (M), envelope (E), nucleocapsid (N), and spike (S) proteins (Parasher, 2020). The virus' S protein attaches itself to the ACE-2 receptor on the host cell, thereafter, undergoing conformational alteration to generate 2 subunits *via* proteolysis through the TMPRSS2 (Das, 2020). The subunits S1 and S2 that are the products of this cleavage are then respectively responsible for receptor binding and membrane fusion to the host cell (He *et al.*, 2020, Parasher, 2020, Jackson *et al.*, 2022). The ACE-2 receptor is utilised in humans to release its viral content (He *et al.*, 2020, Parasher, 2020, Jackson *et al.*, 2022). This could then manifest clinically as both symptomatic and even asymptomatic, thereby posing a greater risk (He *et al.*, 2020, Parasher, 2020, Jackson *et al.*, 2022). The distinctive furin cleavage site that exists between these subunits, along with the D614G mutation present in SARS-CoV-2, is considered to be unique and possibly intensifies transmission (Harrison *et al.*, 2020). After virion attachment and fusion, which is facilitated by this cleavage, the SARS-CoV-2 RNA takes over the host cell to induce polypeptide chain synthesis and replication of the viral genome (Kumar and Al Khodor, 2020). These steps are necessary to form the replication-transcription complex (RTC), which is required to manufacture proteins (nucleocapsid and envelope) and sub-genomic RNA (Kumar and Al Khodor, 2020). This envelope is then critical in the release and pathogenesis of the virus (Schoeman and Fielding, 2019).

1.2.3.2 Viral Replication and Spread

Once the lipid bilayer of SARS-CoV2 is disintegrated by the host cell's lysosomal enzymes, the virus then translates mRNA into polyproteins using the host cell's ribosomes (Hussain *et al.*, 2020, Kumar *et al.*, 2021b). The viral load in host cells is elevated *via* these polyproteins which also utilise proteinases to form structural components (nucleocapsid, spike proteins, etc.) (Guo *et al.*, 2020b, Hussain *et al.*, 2020, Kumar *et al.*, 2021b). Consequently, a SARS-CoV2 viral molecule is formed together with the replicated ssRNA, which bud off the pneumocytes

to exit the host cell via exocytosis to infect other cells (Guo *et al.*, 2020b, Hussain *et al.*, 2020, Kumar *et al.*, 2021b).

1.2.4 Cytokine Storm and Inflammation

Inflammatory mediators are generated when SARS-CoV-2 buds off the host cells' type II pneumocytes, which are subsequently killed (Hussain *et al.*, 2020). This triggers macrophages which causes cytokines such as interleukin-6 (IL-6) and tissue necrosis factor-alpha (TNF-α) to be released (Hussain *et al.*, 2020). These cytokines are essential in ensuring that the immune system operates optimally, due to their involvement in coagulation, tissue repair and inflammation. However, excess production causes dysfunction, thereby resulting in systemic hyperinflammation (Zanza *et al.*, 2022). This cytokine storm has been linked to ARDS, and tissue damage and can therefore lead to organ failure and even death (Ragab *et al.*, 2020). In recent years, greater concern regarding this storm has emerged due to its association with the deadly COVID-19. As studies documented that COVID-19 is accompanied by an upregulation of pro-inflammatory cytokines based on the severity of the infection (Ragab *et al.*, 2020, Tang *et al.*, 2020, Yuki *et al.*, 2020). This production of pro-inflammatory cytokines is thought to be one of the ways that SARS-CoV-2 causes coagulopathy (Darif *et al.*, 2021). The exacerbated inflammatory response in COVID-19, referred to as cytokine release syndrome (CRS) (Figure 3), has been documented by several studies which noted elevations in IL-6, TNF-α, Interleukin 8 (IL-8), Interferon gamma (IFN-γ), Monocyte chemotactic protein-3 (MCP-3), Macrophage Inflammatory Protein-1 Alpha (MIP-1α, CCL3) and Interleukin-1 beta (IL-1β) (Chen *et al.*, 2020a, Del Valle *et al.*, 2020, Huang *et al.*, 2020, Jamilloux *et al.*, 2020, Yang *et al.*, 2020b, Chang *et al.*, 2021, Darif *et al.*, 2021).

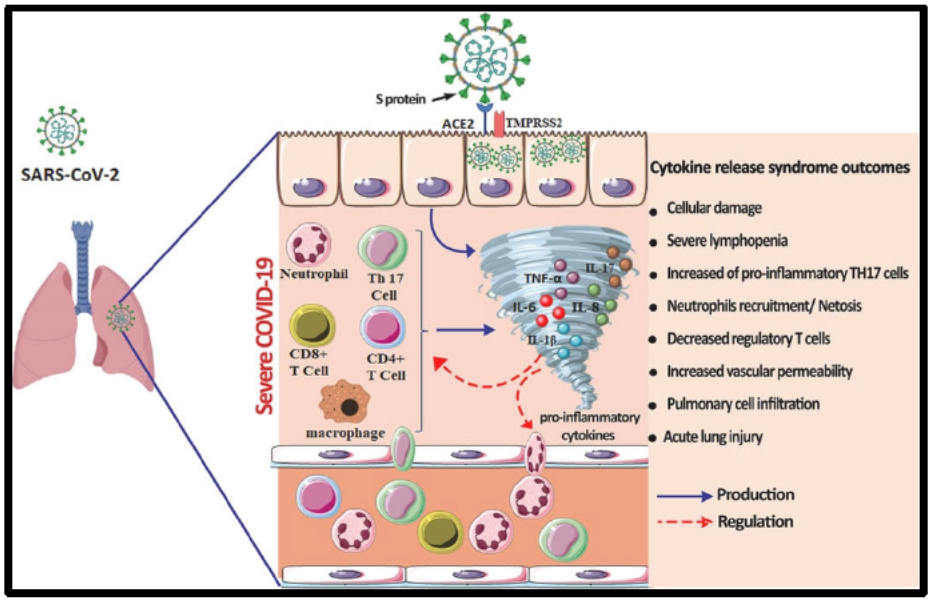

Figure 3: Clinical outcomes and the cytokine release syndrome in the pathophysiology of COVID-19.

Several pro-inflammatory cytokines including IL-6, 1L-1β, IL-17 and TNF-α are involved in the onset of cytokine release syndrome. Cells including neutrophils, macrophages, T-lymphocytes and epithelial cells secrete these cytokines, thereby exacerbating SARS-CoV-2 infection. This excessive release of cytokines results in dire consequences including cellular damage, lymphopenia, lung injuries and an altered microbiota.[Adopted from: (Darif et al., 2021); Copyright obtained from RightsLink (Appendix 6.9)]

IL-6 has been associated with regulating the immune system, inflammation, and haematopoiesis, and is considered to be the eye of the storm with great relevance in COVID-19 due to its direct correlation with COVID-19 severity (Chen *et al.*, 2020b, Fara *et al.*, 2020, Herold *et al.*, 2020, Darif *et al.*, 2021). Another key player in CRS is TNF-α, which also stimulates the production of other cytokines including IL-6 and has been associated with severe COVID-19 cases in several studies (Chen *et al.*, 2020a, Huang *et al.*, 2020, Qin *et al.*, 2020, Darif *et al.*, 2021). Studies suggest that the S protein of the SARS-CoV-2 virus is responsible for increasing activity in the NF-κβ pathway which has been further associated with the release of cytokines including including TNF-α and IL-6 (Wang *et al.*, 2007, Ma *et al.*, 2020). Viral infections are also known to result in the release of macrophages which produce Type II interferon (IFN-γ) (Fara *et al.*, 2020). These alterations are considered to be a reliable measure of the severity and development of COVID-19 (Fara *et al.*, 2020). Chemokines are critical during immune responses as they are inflammatory mediators. However, when produced in excess they also participate to the cytokine storm as seen in COVID-19, where MIP-1α was associated with case severity as well as ICU admissions during the pandemic (Khalil *et al.*, 2021, Hamza *et al.*, 2022). MIP-1α is produced by interferon, released by dendritic cells (DCs), which are triggered by viruses (Khalil *et al.*, 2021). This subsequently results in the recruitment of Natural Killer (NK) cells which in turn triggers the production of other cytokines (Khalil *et al.*, 2021). These NK cells, along with neutrophils, DCs, and macrophages, are also involved in a controlled immune response during pregnancy to assist with implantation amongst many other adaptations to ensure a safe pregnancy (Obuchowska *et al.*, 2021). An immunosuppressive state results in pregnant women becoming more vulnerable to pathogens (Obuchowska *et al.*, 2021).

1.2.4.1 Inflammation in pregnancy

Similar to COVID-19, pregnancy's first and third trimesters are pro-inflammatory, increasing the likelihood that the virus would further trigger an abnormal hyperinflammatory response in the mother that could put her and the foetus at risk (Wegmann *et al.*, 1993, Phoswa and Khaliq, 2020). Inflammation has been the principal cause of pregnancy loss, abortions, premature delivery and consequently is the leading factor in cerebral and pulmonary foetal syndromes (Vesce *et al.*, 2022). As a result, studies have monitored pregnant COVID-19-infected women who presented with significantly increased cytokine levels, risk of preeclampsia and pregnancy complication rates (Fenizia *et al.*, 2020, Tanacan *et al.*, 2021, Rosen *et al.*, 2022, Forrest *et al.*, 2023). In addition, the role of Extracellular vesicles (EVs) in inflammatory responses has been explored in recent years as these vesicles are considered to be mediators in inflammation and even contribute to inflammatory disease development (Chan *et al.*, 2019). However, their role in COVID-19 is yet to be fully elucidated.

1.2.4.2 Extracellular vesicles in inflammation

EVs are a broad category of entities surrounded by membranes that are secreted by cells to aid in vital physiological functions including maternal-foetal communication (Chan *et al.*, 2019, Ghafourian *et al.*, 2022, Buzas, 2023). EVs are further classified according to their size and biogenesis, as large EV's (≥1,000 nm) such as apoptotic bodies, medium EV's (~200–800 nm) including microvesicles and exosomes which are classified as small EV's with a size ranging from ~50–150 nm (Buzas, 2023). EVs are responsible for carrying cargo including lipids, proteins and RNAs, which thereby exacerbates their risk of being utilised by viruses and bacteria in the transmission of virulence factors (Figure 4) (Console *et al.*, 2019, Herrmann *et al.*, 2021).

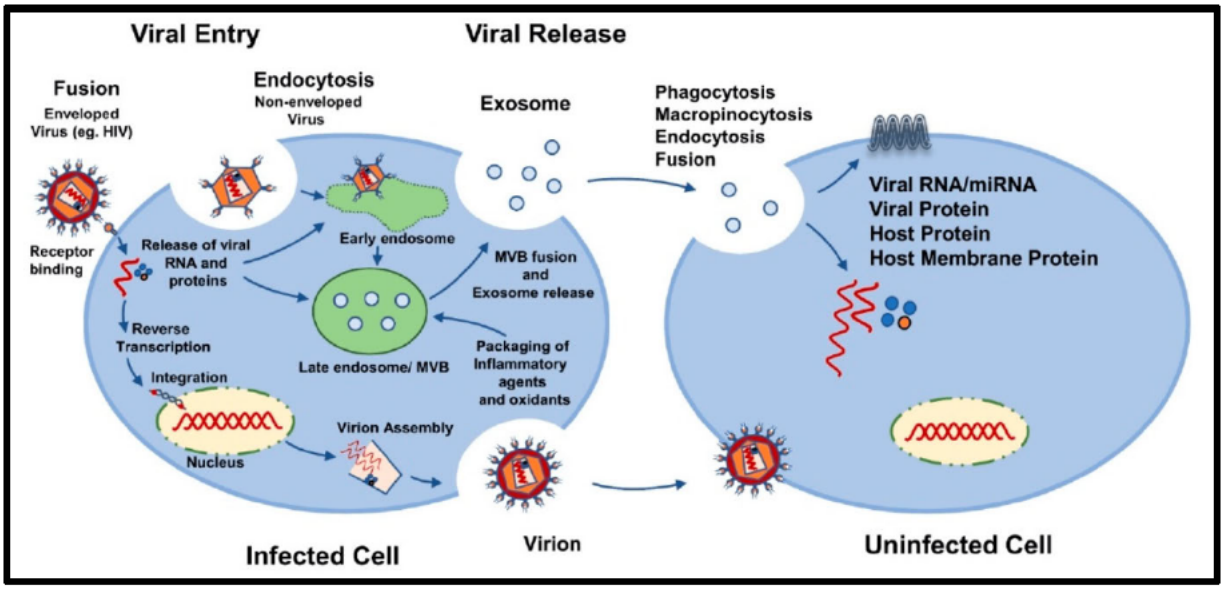

Figure 4: Potential extracellular vesicle-based viral dissemination mechanisms.
Virus-infected cells produce EVs, which are considered to carry viral material that could facilitate infection. This can occur via assisting viral evasion of the immune system, transfer of chemokine co-receptors or cell surface proteins to cells without endogenous viral co-receptors, or via transferring components of the virus, including RNA and proteins, which results in cytotoxic consequences and apoptosis, thus causing a cumulative loss in immune cells. [Adopted from: (Kumar et al., 2020); CC BY]

EVs have also been reported to be released from immune cells, with the ability to assist with inflammatory processes. Alterations in the nature or expression of the exosomal cargo are therefore critical in inflammatory diseases (Chan *et al.*, 2019, Console *et al.*, 2019). Furthermore, EVs possess the ability to release their contents which can induce inflammation, making them even more significant in inflammatory diseases, like COVID-19 (Tian *et al.*, 2022). A recent study found that exosomes extracted from COVID-19 plasma can initiate an inflammatory response in distant organs by triggering the endothelial cell's NLRP3 inflammasome (Sur *et al.*, 2021). Moreover, EVs carry cell-free DNA that is considered to trigger an inflammatory pathway and is thereby hypothesized to be linked to the pathogenesis of pregnancy complications (Konečná *et al.*, 2019). Hence, EVs are of considerable importance in COVID-19 pregnancies, as they have been linked to increased levels of cytokines in other viruses (Babaei *et al.*, 2022). However, the effects of COVID-19 on these vesicles are still unclear; hence, the second manuscript of this study aims to clarify this.

In addition, these EVs are of great relevance in pregnancies as they are involved in implantation and angiogenesis with further links to gestational complications such as foetal growth restriction, preterm birth, diabetes, and hypertension (Ghafourian *et al.*, 2022). MiRNAs found in trophoblast cells are abundant in EVs, and their expression is responsive to changes in the ideal placental environment, such as hypoxia and inflammation (Yang et al., 2019). Oxygen tension has been documented to alter the cargo contained in EVs that originate from extravillous trophoblasts (EVTs), which controls inflammation (Rosenfeld, 2024). Studies have shown that EVs in such conditions undergo alterations, can promote inflammatory conditions through secretion of cytokines and have now been linked to serious adverse pregnancy outcomes (Atay et al., 2011, Donker et al., 2012, Sheller-Miller et al., 2019, Mitchell et al., 2024). Therefore, assessing inflammation and its impact on EVs in COVID-19 pregnancies will provide significant understanding on adverse pregnancy complications and outcomes as a result of infection.

1.2.5 COVID-19 in pregnancy

1.2.5.1 Effects of COVID-19 on expectant mothers and their neonates

In recent years, the COVID-19 pandemic has had a significant influence on the health of expectant mothers and their developing foetuses. Comorbid pregnant women, such as those with HIV, high blood pressure, and diabetes mellitus, have also been reported to exhibit a significantly increased probability of developing severe COVID-19 (Smith *et al.*, 2023, Wali *et al.*, 2024). Contracting SARS-CoV-2 during pregnancy posed further challenges as it was found to elevate the risk of adverse gestational complications such as preterm birth, caesarean delivery, acute respiratory distress syndrome, preeclampsia, stillbirth, sepsis, adverse renal/cardiac outcomes, placental abruption, death and vertical transmission (Khalil *et al.*, 2020, Ko *et al.*, 2021, Fallach *et al.*, 2022, Marchand *et al.*, 2022, Seif *et al.*, 2023, Wali *et al.*, 2024). Unfortunately, the newborns from these pregnancies also faced challenges as a result of COVID-19 infection, by exhibiting a higher risk of respiratory distress syndrome, impaired intrauterine growth, low birth weight, neonatal sepsis, pneumonia, death and transmission of COVID-19 (Fenizia *et al.*, 2020, Khan *et al.*, 2020, Marchand *et al.*, 2022, Seif *et al.*, 2023, Celik *et al.*, 2024). These findings are of foremost importance as the conditions under which foetal growth is required to occur are compromised as a result of inflammation, hypoxia, and placental insufficiency due to COVID-19 (Brum and Vain, 2023). This poses a great threat to proper neurodevelopment as depicted in Figure 5 below.

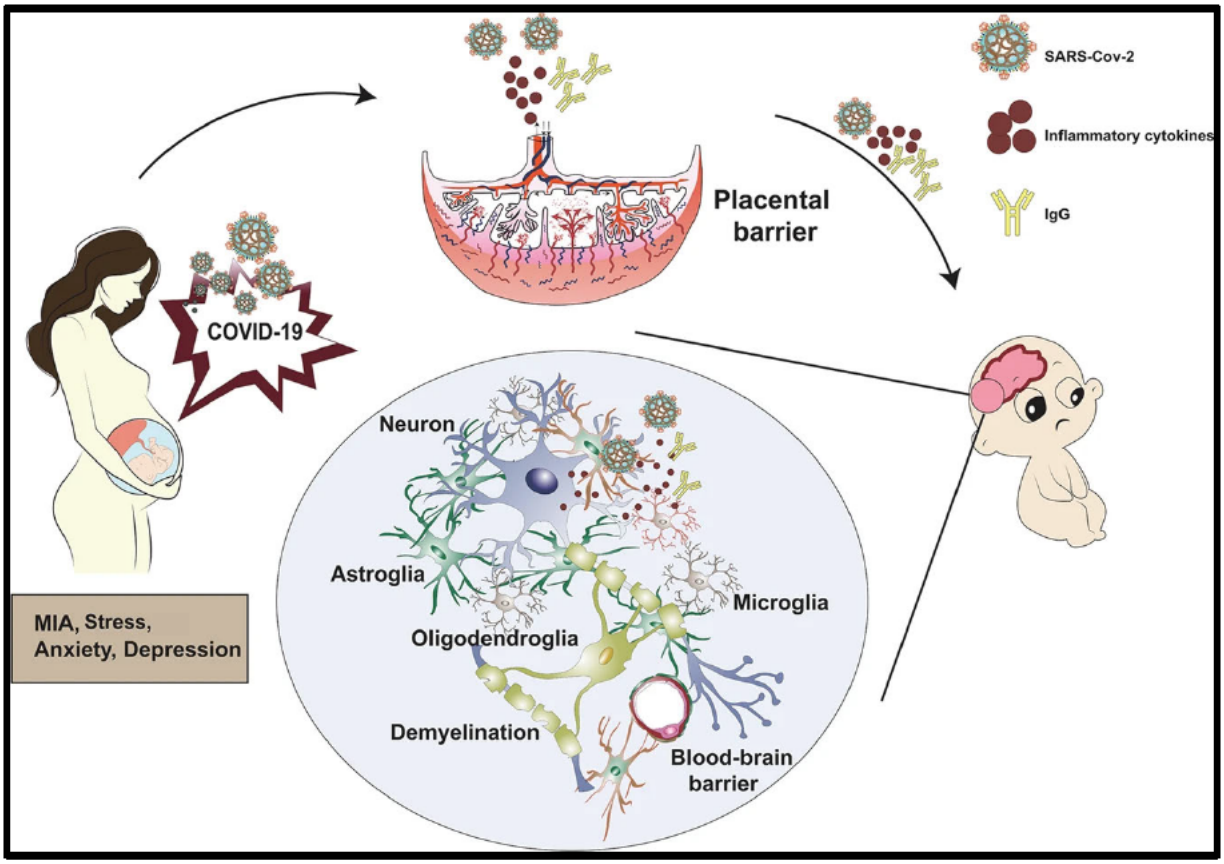

Figure 5: Possible consequences of a pregnancy-related SARS-Cov-2 infection on the neonate's neurological system.

The neurodevelopment in infants from COVID-19 pregnancies has been impacted through maternal immune activation (MIA), which could lead to inflammatory cytokines and even SARS-CoV-2 passing through the placental barrier to affect the foetus. [Adopted from: (Wang et al., 2022); CC BY]

The immunological response observed in SARS-CoV-2 affects the foetal brain *via* dysregulation in neurotransmitter signalling, increased oxidative stress, and the proinflammatory state (Shook *et al.*, 2022). Studies have now identified detrimental neurodevelopment effects in infants exposed to COVID-19, whereby the motor, cognitive, and verbal performances of these infants are impacted (Deoni *et al.*, 2021, Huang *et al.*, 2021, Shuffrey *et al.*, 2022, Ayesa-Arriola *et al.*, 2023, Yangin Ergon *et al.*, 2024). This has raised significant concern and the urgent need for scientists to delve into this area of research. Along with these concerning results, SARS-CoV-2 has now been found in the umbilical cord plasma, vaginal mucosa, and newborn milk (Fenizia *et al.*, 2020, Knight *et al.*, 2020, Vivanti *et al.*, 2020). Although it has been reported that organisms (such as the SARS-CoV-2 virus) rarely pass through the placenta, the possibility of maternal antibodies, passing through, is of

significance (Gupta and Pushkala, 2021). Furthermore, the entry receptor for this virus is ACE-2, which is also present in the placenta validating the potential that SARS-CoV-2 could enter the feto-maternal circulation (Vivanti *et al.*, 2020, Saadaoui *et al.*, 2021, Motwani *et al.*, 2022). As a result this poses a significant risk to pregnant mothers and foetus (Vivanti *et al.*, 2020, Saadaoui *et al.*, 2021, Motwani *et al.*, 2022).

1.2.5.2 The role of the placenta in pregnancy

The embryo depends on the placenta for survival. This is critical in embryonic development as it serves as communication with the mother, protects and sustains the foetus during the intrauterine period (Cross *et al.*, 1994, Burton and Jauniaux, 2015). During foetal development, the placenta is essential in supplying nutrients and oxygen whilst eliminating waste products and carbon dioxide from the foetus (Gude *et al.*, 2004, Burton and Jauniaux, 2015). Furthermore, it is known to produce essential pregnancy hormones which regulate and maintain processes including implantation, development of the foetus, angiogenesis and immune tolerance, as well as protecting the foetus from illnesses in the mother, infections, and xenobiotics (Gude *et al.*, 2004, Costa, 2016). Since its primary function is to ensure a healthy pregnancy, research into the effects of infection on the placenta is crucial, particularly since the organ's blood flow accounts for a significant portion of cardiac output (Mourad *et al.*, 2021).

1.2.5.3 Placental Structure

The placenta, a 15-22 cm disc-shaped organ weighing approximately 500g, is composed of primary structures, termed chorionic villi, with two surfaces viz. the basal plate is located close to the maternal endometrium, and the chorionic plate contacts the foetus (Griffiths and Campbell, 2014, Burton and Fowden, 2015). Briefly, the endometrium transforms in early pregnancy to form the maternal part of the placenta, the decidua, while the chorionic villi receive oxygen in the intervillous space from the spiral arteries which originate from the radial artery. This is illustrated in Figure 6 (Griffiths and Campbell, 2014, Burton and Jauniaux, 2015, Slator *et al.*, 2018). These spiral arteries undergo remodelling, whereby the decidua is invaded by trophoblast cells to permit the mother's blood to enter the intervillous area; hence, the lining, the endothelium, and the size of the spiral arteries are altered to allow for this flow of blood, which is crucial for proper oxygenation of the foetus (Burton *et al.*, 2009, Jensen and Chernyavsky, 2019).

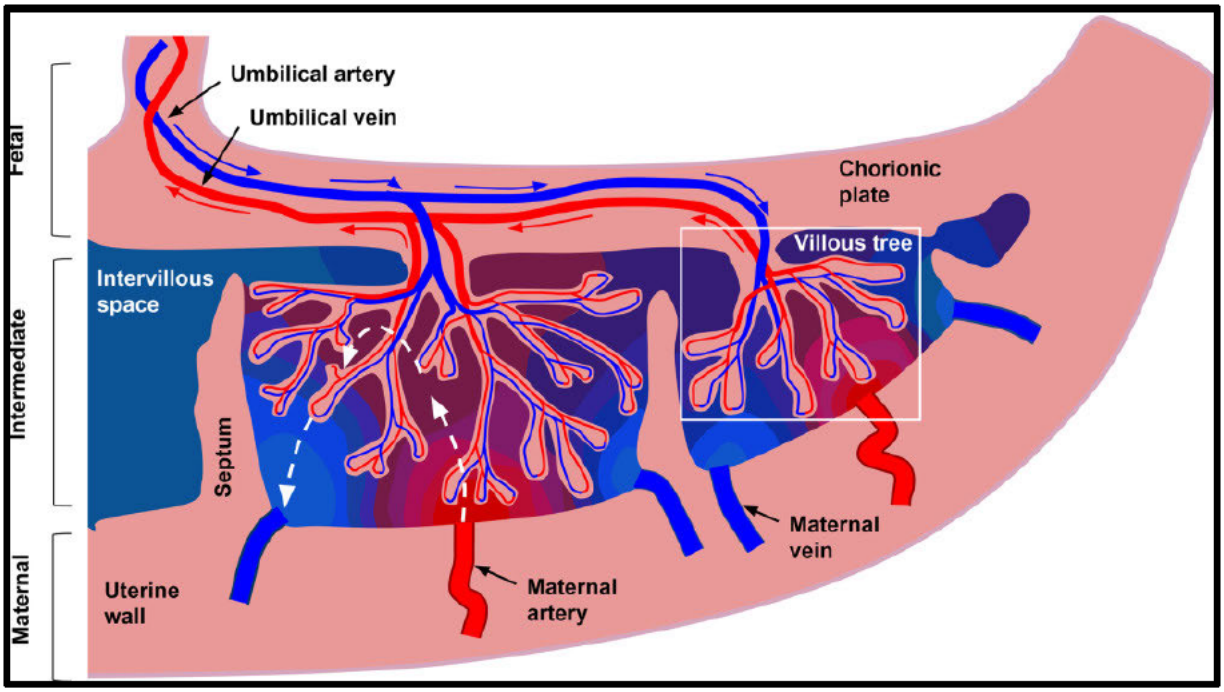

Figure 6: An illustration of blood flow via the placenta and surrounding tissue.

The flow directions of oxygenated (red) and deoxygenated (blue) foetal blood via the placental vasculature are indicated by blue and red arrows. Idealized flow lines across the intervillous area for maternal blood are depicted by dashed white arrows. The colour gradient from red to blue represents idealized oxygenation conditions. [Adopted from: (Slator et al., 2018); CC BY 4.0-https://creativecommons.org/licenses/by/4.0/]

The developed placenta is also made up of an elaborate villous network of foetal blood vessels which make contact with maternal blood (Lahti-Pulkkinen *et al.*, 2018). These villi in the placenta are composed of two layers, through which water, substrates and gases must pass in order to reach the foetal circulation (Brett *et al.*, 2014). The multinucleated syncytiotrophoblasts make contact with the maternal circulation, as they are the outermost layer lining the villi (Brett *et al.*, 2014, Prochaska *et al.*, 2020). Whilst the inner mononuclear cytotrophoblast layer, performs a crucial part in autophagy and viral infection resistance (Prochaska *et al.*, 2020). The placental foetal unit forms a selective barrier that stops infections from moving from the circulation of the mother to the foetus (Hoo *et al.*, 2020, Hosier *et al.*, 2020, Wong *et al.*, 2022, Benny *et al.*, 2023). therefore, proper placentation is essential. In the COVID-19 pandemic vertical transmission of SARS-CoV-2 to the placenta has been associated with malperfusion and triggering an inflammatory response (Hoo *et al.*, 2020, Hosier *et al.*, 2020, Wong *et al.*, 2022, Benny *et al.*, 2023).

1.2.5.4 The effect of SARS-CoV-2 on the placental morphology

The repercussions of COVID-19 on the morphology and functioning of the placenta have been severe. In the previous years, a substantial body of evidence has been established (Hosier *et al.*, 2020, Prochaska *et al.*, 2020, Wastnedge *et al.*, 2021). Consequently supporting the fact that COVID-19 results in altered functioning of the placenta with signs of thrombo-embolic complications, vascular and inflammatory alterations (Hosier *et al.*, 2020, Prochaska *et al.*, 2020, Wastnedge *et al.*, 2021). A multitude of studies have reported that placentae from COVID-19 pregnancies, presenting with altered vascular functioning through the presence of malperfusion, thrombosis, infarction, changes in fibrin deposition and the villi (Algarroba *et al.*, 2020, Vivanti *et al.*, 2020, Ikhtiyarova *et al.*, 2021, Patberg *et al.*, 2021, Ramphal *et al.*, 2022, Garg *et al.*, 2023, Kummer *et al.*, 2024, Ryan *et al.*, 2024). Further signs of inflammation such as villitis, villous edema and placentitis have also been observed in COVID-19 pregnancies (Shanes *et al.*, 2020, Patberg *et al.*, 2021, Kato *et al.*, 2022, Schwartz *et al.*, 2022, Garg *et al.*, 2023, Kummer *et al.*, 2024, Ryan *et al.*, 2024). These COVID-19-related changes to the placental morphology have been elucidated in detail in the form of a review which forms part of Manuscript One in this thesis (Heeralall *et al.*, 2023). Malperfusion and other changes in uteroplacental circulation were associated with shock and hypoxia, with hypoxic conditions noted as a feature of inflammatory sites (Jahani *et al.*, 2020, Jang *et al.*, 2021). The ample presence of alterations observed in the placental morphology from COVID-19 pregnancies indicates that sufficient placentation did not occur, thus making this a critical focal point for research on COVID-19 pregnancies. Therefore, it is essential that further investigations are needed on placental dysfunction as presented in these COVID-19 pregnancies. Trophoblast invasion is a key starting point, as shallow trophoblast invasion results in placental deficiencies already previously reported (O'Tierney-Ginn and Lash, 2014, Harmon *et al.*, 2016). Kisspeptins are essential in this regard as they have been documented to inhibit and regulate trophoblast invasion but are yet to be elucidated in COVID-19 pregnancies (Hiden *et al.*, 2007, Francis *et al.*, 2014).

1.2.5.5 Placental development and functioning

Optimal placental function is critical, with proper placentation playing a pivotal role in ensuring that a pregnancy is successful, as gestational disorders, miscarriages, and unsuccessful implantation have all been linked to improper placentation (Fisher, 2015, Boss *et al.*, 2018, Herrick and Bordoni, 2019, Ortega *et al.*, 2022). Upon fertilization, a blastocyst is formed, which then differentiates into the trophectoderm (placenta) and inner cell mass (embryo) that

attaches to the endometrium. This is known as implantation and consequently initiates placentation (Kim and Kim, 2017, Ortega *et al.*, 2022). Placental development and the maintenance of a full-term pregnancy depends on trophoblast migration, proliferation, and invasion into the maternal decidua and myometrium in the early phases of pregnancy (Reynolds and Redmer, 2001, Boss *et al.*, 2018). This is vital for the correct formation of the placenta to ensure adequate development of the morphology and network of blood vessels that are responsible for supplying the foetus (Reynolds and Redmer, 2001, Boss *et al.*, 2018). The cytotrophoblasts originating from the chorionic villi are responsible for the invasion of the uterus into the myometrium (Fisher, 2015, Staud and Karahoda, 2018). The myometrium thereafter further invades and remodels the spiral arteries in healthy pregnancies (Fisher, 2015, Staud and Karahoda, 2018). Proper placental development is necessary for the ideal uterine environment to be established so that the foetal and maternal metabolic needs can be met (Reynolds and Redmer, 2001, Fisher, 2015, Lawless *et al.*, 2023). However, in cases such as preeclampsia, abnormal placental development occurs whereby there is shallow cytotrophoblast invasion, which consequently results in a maternal inflammatory response (Reynolds and Redmer, 2001, Fisher, 2015, Lawless *et al.*, 2023). Kisspeptin (Metastin) has been shown to be crucial for placental growth and functioning due to its function in cell migration (Vodneva *et al.*, 2014, Hu *et al.*, 2019b, Ibanoglu *et al.*, 2022, Tsoutsouki *et al.*, 2022).As a result it influencing trophoblast invasion; therefore, alterations in this peptide have also been linked to unfavourable pregnancy outcomes, such as preeclampsia, miscarriage, and preterm birth (Vodneva *et al.*, 2014, Hu *et al.*, 2019b, Ibanoglu *et al.*, 2022, Tsoutsouki *et al.*, 2022). Studies have reported these alterations in kisspeptin to be an indicator of placental dysfunction due to its role in inhibiting placental invasion (Bilban *et al.*, 2004, Vodneva *et al.*, 2014, Matjila *et al.*, 2016, Al-Kaabi *et al.*, 2020, Ibanoglu *et al.*, 2022). However, there remains a huge gap regarding kisspeptin, placental development and functioning in COVID-19, which should be under focus, due to the alterations observed in placentae from COVID-19-positive pregnancies which depict placental dysfunction. Therefore, this study aims to form a foundation to show how COVID-19 affects placental development and functioning through the analysis of kisspeptin in pregnancies.

1.3 Methodology Overview

1.3.1 Ethical approval

Regulatory ethical and Institutional approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004591/2022), South Africa.

1.3.2 Study population

Black South African women over 34 weeks of gestation from Inkosi Albert Luthuli Central Hospital, who were confirmed either COVID-19 positive or negative for SARS-CoV-2 RNA via real-time polymerase chain reaction (RT-PCR) on nasopharyngeal swabs were considered for this observational study. Black South African women below the age of 40 who were over 34 weeks of gestation, with confirmed singleton pregnancies, and who were confirmed either COVID-19 positive or negative through PCR testing, were considered for this study. Only those who could provide full consent and did not present with other major co-morbidities such as hypertension, pre-eclampsia, IUGR or other gestational complications were then recruited.

1.3.3 Sample collection

Immediately after PCR test results and consent were obtained, a doctor from the hospital obtained 5ml of whole blood via venepuncture from which plasma was isolated through centrifugation (n=10 per group). Additionally, placental samples were taken from women who had elective caesarean sections. Within 10 minutes of delivery placental tissue was dissected from central (n=6 per group) and peripheral regions (n=6 per group) of the placenta. Within ten minutes of delivery, the placental tissue was removed, washed with PBS (1X, pH 7.5), and preserved in 10% phosphate-buffered formalin.

1.3.4 Tissue processing and analysis

The plasma acquired was utilised to extract EVs using the Invitrogen™ Total Exosome Isolation Kit according to the manufacturer's instructions. The isolated EVs were characterized using Transmission electron microscopy and Nanoparticle Tracking Analysis (NTA). The levels of IFN gamma, IL-6, MIP-1 alpha, and TNF alpha were then analysed in the EVs and plasma using a ProcartaPlex 4 Plex **(Chapter Three- Manuscript Two)**.

Following standard laboratory protocol, placental samples (n = 12 per group) were embedded into paraffin wax blocks and analysed using the Haematoxylin and Eosin (H&E), and Masson's Trichrome (MT) stains. Placental samples were further analyzed through immunohistochemistry to determine the kisspeptin levels in COVID-19-positive and negative placentae **(Chapter Four- Manuscript Three)**. Kisspeptin levels were analyzed through microscopic analysis and quantified using colour deconvolution on Fiji ImageJ software (Madison, WI).

1.3.5 Statistical analysis

All data analyses and graphical representations were produced using an unpaired t-test on GraphPad Prism, with p-values < 0.05 considered significant.

1.4 Overview of the Thesis

This dissertation is written and submitted in manuscript format as per the College of Health Science (University of KwaZulu-Natal) guidelines. It consists of Five (5) Chapters.

Introduction: Chapter One introduces the COVID-19 pandemic, the pathophysiology of SARS-CoV-2, and the repercussions of infection. It further explores the effects of COVID-19 infection in pregnant women, specifically focusing on inflammation, placental development and functioning. This chapter also provides information on the aims and objectives as well as an overview of the methodology.

Manuscript One: The effects of COVID-19 on placental morphology- Published: Placenta, 138, 88-96. This review paper elucidates the effects of COVID-19 on the functioning and morphology of the placenta.

Formatting, tables/figures, and referencing are done in accordance with the journal requirements.

Manuscript Two: The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile- In revision for publication in the: American Journal of Reproductive Immunology. This experimental paper focuses on how COVID-19 infection during pregnancy impacts the cytokine profile in the plasma and EVs in the South African cohort.

Formatting and referencing are done in accordance with the journal requirements.

Manuscript Three: The effect of COVID-19 on placental functioning in South African pregnancies: Investigation of kisspeptin expression, vascular and inflammatory alterations- In revision for publication in the: Histochemistry and Cell Biology Journal. This experimental paper elucidates histopathology and kisspeptin expression in the placentae from COVID-19-positive and negative pregnancies in South Africa.

Formatting and referencing are done in accordance with the journal requirements.

Synthesis: Chapter Five provides an in-depth synthesis of Chapters Two, Three, and Four by highlighting the main findings and the link between these Chapters. Thereafter, a conclusion

and summary of the main findings are provided, along with future recommendations and limitations of this study.

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BRIDGING TEXT

FROM CHAPTER ONE TO TWO

The introduction and the literature review from the previous chapter examined COVID-19 and its impact globally. This publication critically reviews the effects of COVID-19 on pregnant women. The pathophysiology of COVID-19, pathological changes, and observed outcomes have been discussed. Moreover, a connection between preeclampsia and COVID-19 has been identified and discussed, with thoughts and recommendations for future studies to consider. Finally, this study investigated how the COVID-19 vaccination and treatments might affect expectant mothers and their unborn children, along with highlighting the future research prospects for therapeutics. This review could be of great interest to a broad audience of scientists in the fields of medicine, physiology, anatomy, and pharmaceutics, as well as biomedical scientists and medical practitioners engaged in treating pregnant women, developing and implementing novel diagnostic and therapeutic agents for the effective treatment of COVID-19.

2.0 CHAPTER TWO

MANUSCRIPT ONE

The effects of COVID-19 on placental morphology

C Heeralall [1], U H Ibrahim[2#], L Lazarus[1], P Gathiram[3], I Mackraj[2#]

[1]Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

[2] Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

[3]Discipline of Family Medicine, School of Public Health and Nursing, University of KwaZulu-Natal, Durban, South Africa.

[#] **Corresponding authors: Professor Irene Mackraj and Dr Usri Ibrahim**

Address: Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville Campus, Private Bag X54001, Durban, South Africa.

Email address: Mackraji@ukzn.ac.za, IbrahimU@ukzn.ac.za

Tel: +27 729085646, +27 651489693

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The effects of COVID-19 on placental morphology

C. Heeralall [a], U.H. Ibrahim [b,**], L. Lazarus [a], P. Gathiram [c], I. Mackraj [b,*]

[a] Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[b] Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[c] Discipline of Family Medicine, School of Public Health and Nursing, University of KwaZulu-Natal, Durban, South Africa

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ABSTRACT

The impact of the COVID-19 infection, caused by Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), during the pandemic has been considerably more severe in pregnant women than non-pregnant women. Therefore, a review detailing the morphological alterations and physiological changes associated with COVID-19 during pregnancy and the effect that these changes have on the feto-placental unit is of high priority. This knowledge is crucial for these mothers, their babies and clinicians to ensure a healthy life post-pandemic. Hence, we review the placental morphological changes due to COVID-19 to enhance the general understanding of how pregnant mothers, their placentas and unborn children may have been affected by this pandemic. Based on current literature, we deduced that COVID-19 pregnancies were oxygen deficient, which could further result in other pregnancy-related complications like preeclampsia and IUGR. Therefore, we present an up-to-date review of the COVID-19 pathophysiological implications on the placenta, covering the function of the placenta in COVID-19, the effects of this virus on the placenta, its functions and its link to other gestational complications. Furthermore, we highlight the possible effects of COVID-19 therapeutic interventions on pregnant mothers and their unborn children. Based on the literature, we strongly suggest that consistent surveillance for the mothers and infants from COVID-19 pregnancies be prioritised in the future. Though the pandemic is now in the past, its effects are long-term, necessitating the monitoring of clinical manifestations in the near future.

1. Introduction

The novel corona virus, SARS-CoV-2, discovered in Wuhan, China (2019), led to the calamitous pandemic, which left a massive death toll at its peak, resulting in several health-related repercussions which impacted healthcare, including maternal and foetal outcomes [1]. The virus, with an incubation period of ~5 days (range, 2–14 days), results in symptoms including headaches, fever, diarrhoea, myalgia, cough, severe respiratory illness and death depending on its severity [1]. Notably, pregnant women and their unborn children are considered high-risk populations, as pregnancy-related infections correlate with a greater risk of morbidity and death [2,3]. In 2020, a total of 3,613,647 births were recorded in the United States, with 225,225 women delivering during the pandemic and approximately 6.9% of these births being affected by COVID-19 [4,5]. This was alarming for the healthcare system, as the effects of COVID-19 on pregnancies are still to be fully determined [6].

Mechanical and physiological alterations during normal pregnancies can significantly affect the immune system, respiratory system, susceptibility to infections, cardiovascular function and coagulation [2,7]. In addition, studies in pregnant women have shown that COVID-19 can result in haematological changes, inflammation that can or may result in a 'cytokine storm' and hypoxia which have all been linked to high mortality [6–8]. Furthermore, once infection triggers the maternal immune response, this will impact on the development of the foetal immune and nervous system, which could potentially result in neural impairments of the unborn baby [9,10]. In addition, mothers who contracted COVID-19 have been found to have a greater risk of preterm labour and pre-eclampsia [11]. On the other hand, there have been reports of the placental unit and foetus being unaffected by COVID-19 [12,13]. Hence, further research is needed on the placenta and its role in COVID-19 pregnancies since impairment to the placental function is central to a successful pregnancy.

A meta-analysis conducted by Wei et al. (2021) documented that the outcomes of a COVID-19 pregnancy, which can range from preterm birth, preeclampsia, chorioamnionitis, diabetes, lymphopenia, stillbirth,

* Corresponding author.
** Corresponding author.
E-mail addresses: IbrahimU@ukzn.ac.za (U.H. Ibrahim), Mackraji@ukzn.ac.za (I. Mackraj).

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low birth weight and even neonatal death. Elsaddig and Khalil (2021) noted that pregnant women with COVID-19 are at a greater risk of adverse maternal outcomes, with many in their third trimester even requiring intensive care [14]. The exact factors underlying the link between COVID-19 and preeclampsia remains unclear. However, common pathways are expected, given their mutual impact on angiogenic pathways and vascular alterations [15]. Furthermore, foetal vascular malperfusion were documented in numerous pregnancies, which could possibly contribute to preterm birth, stillbirth and affect foetal growth [15,16].

The placenta has numerous critical functions, including protecting the foetus from infections, xenobiotic molecules and maternal diseases [17,18]. Its key role in a successful pregnancy necessitates an interrogation of the impact of infection especially given that the blood flow to this organ forms a substantive part of cardiac output [19]. The placental foetal unit plays a key role in protection through forming a selective barrier which prevents the movement of pathogens from the maternal to foetal circulation, with a mononuclear inner layer of cytotrophoblasts which play an essential role in autophagy and resistance for viral infections (Fig. 1) [16,20,21].

The design of the villi allows it to innately play a role in the defence within the placenta, with a syncytium that is selective to pathogen entry through different receptors which are able to recognize different pathogens [23–25].

Importantly, the surge in new-borns who were found to be COVID-19 positive has resulted in transplacental transmission becoming the focal point in COVD-19 transmission [26–29]. Furthermore, the possibility of antibodies from the mothers circulating blood passing through is of significance [30]. Indeed, a study conducted in the UK cohort reported that two of the five babies that died could have been due to COVID-19 complications, with one in 20 babies testing positive for COVID-19 in this study [12]. A case study conducted on 17 pregnant women in 2021, indicated that two neonates contracted COVID-19 and concluded that the SARS-CoV-2 infection could potentially result in preterm delivery and neonatal pneumonia [31]. Alarmingly, 25.5% of births were noted to be preterm in women who presented with COVID-19 [32]. Furthermore, a study conducted in Italy detected two cases of neonates who presented with COVID-19 as well as the SARS-CoV-2 genome in 2 placentas, 1 milk specimen, vaginal mucosa and the umbilical cord plasma indicating that mother-to-child transmission is possible [33]. Hence evidently COVID-19 can be transmitted through the placenta, thereby possibly affecting its structure and function [34,35].

Therefore, this review sheds light on the effects of COVID-19 in pregnancies, with its impact on the placenta being the focal point. It includes a background that covers the role of the placenta in COVID-19 pregnancies. Furthermore, the effects of COVID-19 on the placenta have been discussed, in addition to highlighting the link between COVID-19 and other gestational complications. Finally, the vaccine and therapeutic implications of COVID-19 on the placenta have been included in this review together with recommendations for life post pandemic.

2. The effect of COVID-19 on pregnancies

The pathophysiology of COVID-19 in general has been extensively described [36]. The angiotensin converting enzyme 2 (ACE2) receptor (a component of the Renin-Angiotensin system-RAS) is the entry point for SARS-CoV-2 in the human body and has multiple implications in terms of physiological responses where RAS is the main protagonist [37,38]. Active replication of this virus results in, amongst other effects, an increase in inflammatory responses, and the dysregulation of the RAS including the downregulation of ACE2 which increases vascular permeability, inflammation and vasoconstriction [39]. Interestingly, Fenizia et al. (2020) postulated that modulation of ACE2 levels could possibly be associated with susceptibility to the SARS-CoV-2 infection in the placenta.

The SARS-CoV-2 virus is thought to be transmitted through the placenta by infecting the syncytiotrophoblasts of the villi resulting in an inflammatory response, or via the maternal blood through the uterine artery which will cross the interstitial space to enter the foetal circulation [40]. Furthermore, initially the virus infects the immune cells of the mother, thereafter transferring to the extravillous proximal trophoblast cells which allows it to be transmitted further to the core the villus and vasculature of the foetus [40].

Moreover, recent studies on COVID-19 pregnancies have noted several placental pathological changes, which include vascular and inflammatory alterations, placental infiltration, thrombo-embolic complications, necrosis and ischemia [7,41,42].

Various vascular pathological changes in placenta have been associated with the COVID-19 infection during pregnancy. These changes mainly include thrombosis, malperfusion and vasculopathy in both maternal and foetal circulations as summarized in Table 1. Vascular changes may adversely affect the health of pregnant women and their unborn babies and cause severe health consequences [43,44].

Placental vascular changes due to COVID-19 infections during the third trimester have been extensively studied. Studies documented signs of maternal vascular malperfusion which included the presence of infarcts, thrombosis, increased syncytial knots, increased fibrin deposition, villous agglutination and accelerated villous maturation [44,47–54,56–60,62–65,68,71,73,74]. Furthermore, subsequent studies that reported multi case investigations of placentae from COVID-19 positive mothers which presented different features of foetal vascular malperfusion including avascular villi, karyorrhexis, mural fibrin deposition, villous hypoplasia and chorangiosis [44,46,48,49,51,54,56,59,62,63,65,66,69,73]. Patberg et al. (2021) concluded that COVID-19 pregnancies exhibited an increase in histopathological abnormalities of the placenta, namely villitis of unknown aetiology and foetal vascular malperfusion. Infarction together with chorionic haemangioma in the placenta have also been documented in these past few years [61,75].

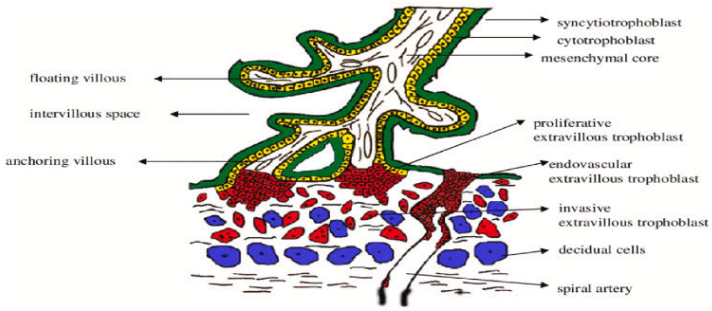

Fig. 1. Shows the schematic representation of the human chorionic villi highlighting the structure. The trophoblast differentiates into the extravillous trophoblast and villous. The villous is composed up of a monolayer of cytotrophoblastic cells from the floating villi (in the intervillous space) which are still attached the villous basement membrane. When these cells differentiate and proliferate, they form the external covering of the villus, the syncytiocytotrophoblast layer. Adapted from Evain-Brion and Malassiné [22].

Table 1
Vascular alterations reported in the placentas of COVID-19 pregnancies.

Placental pathological variations	Type of Study	Number of COVID-19 cases	Reference
- Decidual vasculopathy on the maternal surface	-Case Study	-1 Case	[45]
- Foetal vascular malperfusion.	-Case Study	-20 Cases	[46]
- Foetal vascular thrombosis			
- Avascular villi			
- Villous stromal-vascular karyorrhexis			
- Focal increase in perivillous fibrin deposition			
- Increased intervillous fibrin deposition	-Case study	-1 Case	[47]
- Maternal vessels thrombosis of capsularis decidua	-Case control study	- 15 COVID + Cases - 34 COVID - Cases	[48]
- Intervillous hematoma			
- Maternal decidual vasculopathy			
- Borderline massive perivillous fibrin deposition			
- Avascular and fibrotic villi and stroma-vascular karyorrhexis			
- Infarct			
- Chorionic plate infarct			
- Maternal vascular malperfusion	-Case study	-19 Cases	[49]
- Foetal vascular malperfusion			
- Intervillous thrombus			
- Decidual arteriopathy			
- Increased perivillous fibrin			
- Focal placental infarct	-Case study	-1 Case	[41]
- Possible elements of infarction	-Case study	-1 Case	[50]
- Increased perivillous fibrin deposition			
- Foetal vascular malperfusion	-Case study	-5 Cases	[51]
- Thrombosis in larger vessels			
- Villous stromal-vascular karyorrhexis			
- Avascular villi			
- Ischemic necrosis	-Case study	-1 Case	[52]
- Extensive intervillous fibrin depositions			
- Findings suggestive of ischemia	-Case study	-5 Cases	[53]
- Massive deposition of fibrin			
- Maternal vascular malperfusion.	-Case control study	-16 Cases	[54]
- Intervillous thrombi			
- Decidual arteriopathy			
- Villous agglutination			
- Central villous infarction			
- Peripheral villous infarction			
- Atherosis and fibrinoid necrosis			
- Clustered avascular villi			
- Increased perivillous fibrin			
- Necrosis	-Case study	-1 Case	[55]
- Foetal vascular malperfusion	-Case control study	-51 COVID + Cases -25 COVID - Cases	[56]
- Maternal vascular malperfusion			
- Subchorionic thrombi			
- Infarction	-Case study	-1 Case	[57]
- Peri-villous fibrin deposition			
- Maternal vascular malperfusion	-Case study	-8 Cases	[58]
- Increased syncytial knots			
- Increased focal perivillous fibrin depositions			
- Maternal vascular underperfusion	-Case study	-5 Cases	[59]
- Foetal vascular underperfusion			
- Intervillous thrombi			
- Avascular villi			
- Maternal vascular malperfusion	-Case study	-1 Case	[60]
- Subchorionic laminar necrosis			
- Multifocal infarction	-Case study	-19 Cases	[61]
- Different degree of fibrin deposition			
- Maternal vascular malperfusion	-Case control study	-27 COVID + Cases	[62]
- Fibrinoid necrosis			
- Retroplacental hematomas			
- Increased perivillous fibrin deposition		-27 COVID - Cases	
- Foetal vascular malperfusion			
- Thrombosis of the foetal chorionic plate			
- Maternal vascular malperfusion	-Case study	-7 Cases	[63]
- Excessive villous infarction			
- Increased syncytial knots			
- Intervillous thrombosis			
- Increased fibrin deposition			
- Accelerated villous maturation			
- Foetal vascular malperfusion			
- Avascular villi			
- Maternal Vascular Malperfusion	-Case control study	-31 COVID + Cases -67 COVID - Cases	[64]
- Increased intervillous thrombus			
- Increased syncytial knots			
- Decidual arteriopathy			
- Villous infarction			
- Increased intervillous fibrin			
- Maternal malperfusion	-Case study	-5 Cases	[65]
- foetal malperfusion			
- Foetal vascular malperfusion,	-Case control study	-77 COVID-19 + Cases -56 COVID-19 - Cases	[66]
- Avascular villi			
- Mural fibrin deposition			
- Preplacental hypoxia	-Case control study	-23 COVID + Cases -7 COVID - Cases	[67]
- Maternal vascular malperfusion	-Case study	-11 Cases	[68]
- Necrosis			
- Perivillous fibrin deposition	-Case study	-7 Cases	[69]
- Trophoblast necrosis			
- Foetal vascular malperfusion.			
- Maternal vascular malperfusion	-Case study	-50 Cases	[44]
- Extensive villous trophoblast necrosis			
- Foetal vascular malperfusion			
- Thrombohematomas	-Case study	-40 Cases	[70]
- Intervillous thrombus	-Case study	-1 Case	[71]
- Maternal arteriole with atherosis			
- Extensive trophoblast necrosis			
- Perivillous fibrin deposition			
- Subchorionic and intervillous hemorrhages	-Case study	-1 Case	[72]
- Intervillous fibrin deposition			
- Massive perivillous fibrin deposition			
- Thrombi	-Case study	-4 Cases	[73]
- Necrosis			
- Perivillous fibrin deposition			
- Foetal malperfusion			
- Mural hypertrophy			
- Ectasis			
- Syncytial knots			
- Villous trophoblast necrosis	-Case study	-68 Cases	[74]
- Increased fibrin deposition			

Alterations in the uteroplacental circulation like malperfusion were attributed to hypoxia and shock [63]. Last year in South Africa Ramphal et al. (2022) documented vascular maladaptation, substantial fibrin deposition, an increase in villitis and vascular malperfusion. In addition to vascular alterations, pathological features indicative of inflammatory changes including chorioamnionitis/subchorionitis, intervillositis, chronic villitis and villous edema have also been identified in COVID-19 placentas as summarized in Table 2 [41,44,45,48–50,52–55,57–60,63,65,66,68–74].

Edlow et al. (2020) noted that a decrease in the expression of transmembrane serine protease 2 and angiotensin-converting enzyme 2 in the placenta can possibly protect the foetus against vertical transmission. However, aggregates of cytotoxic T lymphocytes as well as

Table 2
Shows alterations reported in the placentas of COVID-19 pregnancies which are indicative of inflammation.

Placental pathological variations	Type of Study	Number of cases	Reference
- Focal villous edema - Histiocytic-neutrophilic intervillositis - Chorionic vasculitis	- Case study - Case control study	- 1 Case - 15 COVID + Cases - 34 COVID - Cases	[45] [48]
- Villitis - Histiocytic intervillositis - Villous edema	- Case study	- 19 Cases	[49]
- Intervillositis	- Case study	- 1 Case	[41]
- Intervillositis	- Case study	- 1 Case	[50]
- Chronic intervillositis	- Case study	- 1 Case	[52]
- Mixed intervillitis/villitis	- Case study	- 5 Cases	[53]
- Chronic villitis - Villous edema	- Case control study	- 16 Cases	[54]
- Histiocytic intervillositis - Villitis	- Case study	- 1 Case	[55]
- Chronic intervillositis	- Case study	- 1 Case	[57]
- Mild acute intervillositis, - Edema	- Case study	- 8 Cases	[58]
- Chronic villitis - Chorioamnionitis	- Case Study	- 5 Cases	[59]
- Focal lympho-histiocytic inflammation (chronic villitis)	- Case study	- 1 Cases	[60]
- Chronic villitis - Chorioamnionitis/ subchorionitis	- Case study	- 7 Cases	[63]
- Lymphohistiocytic villitis - intervillositis	- Case study	- 5 Cases	[65]
- Villitis	- Case control study	- 77 COVID-19 + Cases - 56 COVID-19 - Cases	[66]
- Chronic histiocytic intervillositis	- Case study	- 11 Cases	[68]
- Histiocytic intervillositis	- Case study	- 7 Cases	[69]
- Focal intervillositis	- Case study	- 50 Cases	[44]
- Placentitis	- Case study	- 40 Cases	[70]
- Intervillositis	- Case study	- 1 Case	[71]
- Chronic histiocytic intervillositis	- Case study	- 1 Case	[72]
- Villitis	- Case study	- 4 Cases	[73]
- Chronic histiocytic intervillositis	- Case study	- 68 Cases	[74]

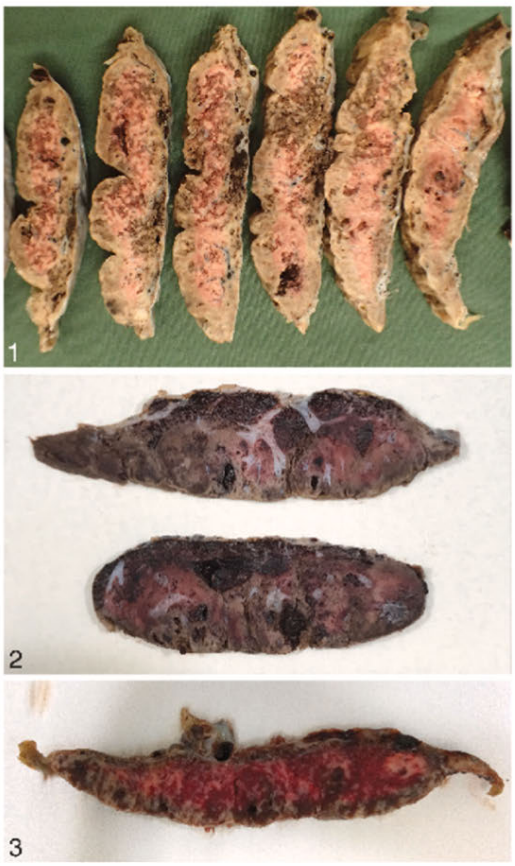

Fig. 2. Shows sections of placental samples that have been affected by SARS-CoV-2. Image 1 shows serially sectioned placenta from a case showing appearance of SARS-CoV-2 placentitis. Microscopic examination showed massive perivillous fibrin deposition, chronic histiocytic intervillositis, and trophoblast necrosis. The extent of pathology resulting from these destructive lesions was greater than 90% and led to placental insufficiency and stillbirth. Image 2 shows gross pathological appearance of massive perivillous fibrin deposition that occurred with SARS-CoV-2 placentitis from a stillborn foetus. Intervillous thrombohematomas can be seen. Image 3 shows sectioned placental specimen from a case illustrating SARS-CoV-2 placentitis. There was 70% involvement of placental tissue with this destructive process [Adapted from Schwartz et al. (2022)].

histiocytes have been detected in the intervillous space and further confirmed through CD8, CD68 and CD3 immunohistochemical staining which support and suggest the detection of chronic intervillositis in COVID-19 placentas [52]. Chronic intervillositis as a result of COVID-19 has been reported to be indicative of the virus in the syncytiotrophoblast [76]. Furthermore, Schwartz et al. (2021) documented that the presence of both syncytiotrophoblast necrosis and chronic histiocytic intervillositis together can result in the increased risk for transplacental foetal infection. Transplacental transmission of COVID-19 was documented in a case where the neonate was born with neurological complications and upon further investigation perivillous fibrin deposition together with intervillositis and infarction were detected in the placenta [57]. Sadly, there have been stillbirth cases which have observed the combined presence of massive perivillous fibrin deposition, trophoblast necrosis and chronic histiocytic intervillositis in the placenta which have been identified as SARS-CoV-2 placentitis (Fig. 2) [69,74,77].

Placentitis results in destructive events within the placenta that can affect >75% of it, thereby impacting its function to provide oxygen to the foetus consequently causing malperfusion and neonatal death [77]. Increased subchorionic and intervillous fibrin in placentas attributed to maternal hypoxia, have been documented in a past study [78]. Also a case of intrauterine foetal death was recently attributed to coagulopathy and hypoxia as a result of placental dysfunction due SARS-CoV-2 placentitis [71]. Hence vascular changes together with inflammatory alterations caused by COVID-19 in the placenta can result in dire consequences.

Furthermore, this virus often results in severe hypoxemia in pregnancy therefore altering the oxygen distribution to the placenta, as it is dependent on the uterine blood flow, fetoplacental system and maternal oxygen saturation [79]. Hypoxia and ischemia can be identified in the placenta through the increase in syncytial knots, whilst foetal hypoxia can be identified in the circulation through the increase in erythroblasts and nuclear debris [52,58,63,64,67,73]. Hypoxia induced by COVID-19 can result in altering the development of blood vessels, the blood supply as well as the development of the placenta which can have a devastating impact on the growing foetus [80]. This alteration in the blood supply and oxygenation due to COVID-19 is of critical interest, as in pregnancy, the demand for oxygen increases significantly, therefore with this

compounding effect, there is bound to be dysregulation in oxygen supply to the placenta [81,82]. This then leads to the assumption that these alterations in the blood supply to the placenta can be mechanistically responsible for the morphological changes observed in the past years. A previous study conducted on severe acute respiratory syndrome (SARS) observed similar morphological alterations in the placenta and deduced these may be as a result of the changes in the blood flow [78]. Furthermore, a study in rats documented reduced levels of oxygen in pregnancy, resulting in a surge of oxidative stress markers which are associated with malperfusion [83].

Alterations in the placental structure due to hypoxia were noted to be adaptive changes that occur in order to enhance placental function, however some changes may be suggestive of ineffective placental development and damage [84]. In addition to the morphological changes mentioned above in the placenta as a result of COVID-19, a study conducted last year found an abnormality in the umbilical cord of COVID-19 pregnancies to be high, whereby it attached to the margin of the placenta resulting in altered functioning and blood flow [82]. In addition, vascular remodelling in the arteries of the placenta in COVID-19 pregnancies have now been identified through histological examinations and documented [85]. Therefore, it can be speculated that in COVID-19 pregnancies the oxygen demand increases, however this demand is not met.

3. COVID-19 and other gestational complications

In the previous section, the hypoxic conditions experienced during COVID-19 pregnancies were highlighted. The presence of such conditions are known to put a pregnant individual under risk for other gestational complications such as preeclampsia and intrauterine growth restriction (IUGR) which can impact the development of the foetus [86,87]. This poses great concern as it leads to the question of whether COVID-19 and its effects have the ability to predispose and cause further gestational complications. This raises further concerns for women who are already diagnosed with gestational complications and then contract COVID-19.

Jamieson and Rasmussen (2021) documented that COVID-19 pregnancies are related to unfavourable consequences like premature births and preeclampsia [88]. Furthermore, in the USA, it was found that women with COVID-19 were 1.2 times more likely to develop preeclampsia [89]. SARS-CoV-2 was more likely to present in the placentas of preeclamptic women and could trigger hypertensive disorders in pregnant woman [90]. IUGR was also documented in a case where the foetus presented with this abnormality at approximately 36 weeks of gestation, following the mother contracting COVID-19 in the third trimester [91]. Furthermore, COVID-19 has been documented to be associated with increasing the risk of IUGR [92]. Villar et al. (2021) documented a link between elevated preeclampsia occurrence and COVID-19 however this association is yet to be confirmed, as COVID-19 and preeclampsia may result in similar pathological alterations [93].

Placental hypoxia has been noted to be a contributing factor in both preeclampsia and IUGR [86]. Interestingly we propose that the alterations observed in the placenta as a result of COVID-19, preeclampsia and IUGR are as a result of hypoxia.

Recent studies have found that changes seen in COVID-19 pregnancies mimic those that are seen in preeclampsia and preeclamptic women should therefore be considered high risk if they contract COVID-19 [94–96]. Hence we propose that COVID-19 alters the blood flow in pregnancy resulting in placental hypoxia which has been documented to result in preeclampsia and IUGR, and therefore COVID-19 can predispose one of these gestational complications with similar clinical manifestations [97,98].

4. COVID-19 vaccine implications in pregnancies

It is known that vaccines signify important public health-advancement, through saving approximately 2–3 million lives yearly by providing adaptive immunity through generating antibodies upon exposure to a pathogen [99]. There are licensed vaccines available for 26 human pathogens and with the rapid rise in the number of vaccines becoming available, hesitancy towards vaccines have arisen with refusal differing across continents and cultures due to many concerns [100]. These concerns and hesitancy were further intensified with the implications of COVID-19 infections pertaining to maternal and foetal health [101]. In particular, vaccination during pregnancy protecting both the mother and unborn child from infection via the transfer of antibodies through the placental circulation (Immunoglobulin G- IgG) and mucosa (IgM, IgA, IgG) which releases antibodies into milk and colostrum to protect the neonate after birth [102]. However concerns around the COVID-19 vaccine only worsened with rumours of it eliciting antibodies that could attack the placenta thereby creating fear and anxiety in pregnant women, preventing them from considering the importance of this vaccine [103]. Furthermore, there has been a history of rumours about vaccines causing infertility which had also been circulated with the COVID-19 vaccine, where claims of cross reactivity between the human placental protein syncytin 1 and antibodies that recognize the SARS-CoV-2 spike protein emerged, resulting in many women declining this vaccine [103]. This despite the fact that, one should weigh the risk of a disease vs the risk of side effects of a vaccine when making a decision, as all types of medical treatment can pose adverse effects [99,100]. In keeping with this, the benefits of the COVID-19 vaccine outweighed the risks in pregnant women, as it posed minimal risk like side effects ranging from nausea to fever and myalgia [104]. Furthermore, studies have found that antibodies generated from the COVID-19 vaccine were able to be transferred through the placenta to foetuses providing passive immunity postpartum [105–107]. Thus far several studies have documented that the COVID-19 vaccines have elicited maternal responses as they were able to document the presence of maternal IgG as well as foetal IgM antibodies for SARS-CoV-2, as presented in Fig. 3 [105,106,108–110]. In addition, the presence of SARS-CoV-2 protein receptor binding domain (RBD) and spike (S) antibodies were detected in umbilical cords as well as infants [105,111]. Wang et al. (2021) observed that the IgG levels for the SARS-CoV-2 antibodies decreased drastically postpartum. Global data on the

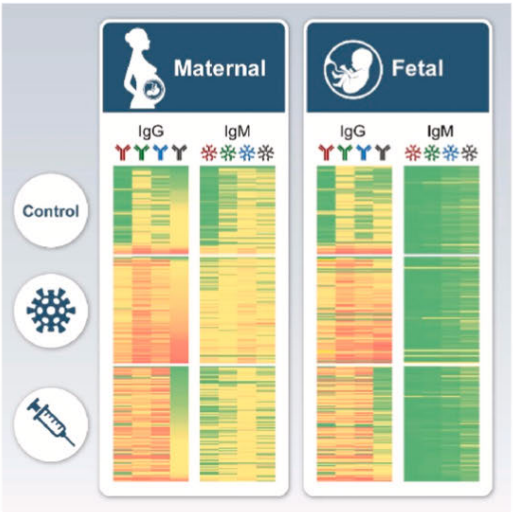

Fig. 3. A representative schematic diagram illustrated the maternal and foetal antibodies found after COVID-19 infection and vaccination [108].

uptake of the COVID-19 vaccine in pregnancies, infection rates and outcomes still need to be determined [112]. More importantly, the long-term effects of these vaccines still need to be established with critical focus on clinical manifestations that may arise due to these interventions.

5. Therapeutic implications

Therapeutic interventions during pregnancy, may pose threat to maternal and foetal health if not properly validated for safety. In this regard COVID-19 put an extra load by jeopardizing the ability to maintain a healthy pregnancy hence a host of new interventions were introduced [113,114].

Therapeutic interventions to treat COVID-19 included using various antiviral drugs, convalescent plasma (Passive immunotherapy) including using antiviral antibodies; nutritional supplements (folic acid, vitamin C, vitamin D); and miscellaneous treatments (Telbivudine, Azithromycin, Cobicistat) [114,115]. Significant efforts have been made to repurpose FDA-approved therapeutic drugs with known safety profiles during pregnancy in order to safely treat COVID-19 infection, which helped reduce the effects of COVID-19 therapeutic interventions [116]. However, some of the interventions mentioned above can have profound effects on pregnancy by affecting the placenta. Therefore, monitoring the impact of these drugs through investigating the physiological changes on the placenta would be beneficial in controlling future undesired side effects.

Pregnant women have been included in very few clinical trials for COVID-19 infection management (eg, SOLIDARITY trial [117], RECOVERY trial [117]). The severity of the mother's condition, underlying risk factors, gestational age, any potential maternal benefits, the likelihood of placental transfer, potential mechanisms for foetal harm, and the lack of knowledge regarding foetal and new-born risks should all be taken into account when deciding whether to use COVID-19-specific therapies during pregnancy. Patients being treated in hospitals may take the following medications presented in Table 3.

Several studies have observed pathophysiological changes in the placentas of COVID-19 positive patients [41,45,46,48,54,56,65,126]. Majority of these studies were able to report the pathological effects of COVID-19 but lacked a pharmacological and therapeutic perspective. However, a recent study was able to reveal that COVID-19 treatment with antivirals, antibiotics, low molecular weight heparins and chloroquine increased the weight and efficiency of the placenta compared to untreated group [127].

Further investigation into the effects of COVID-19 therapeutics and vaccines on the placenta and pregnancy in general are recommended to improve the health/safety of the mother and infant in the future.

6. Life after COVID-19

After exploring COVID-19 and the severity of its effects in pregnancy, it is critical to monitor the health of these mothers and new-borns to establish if there are any clinical manifestations as a result of the virus or its interventions. Many of the studies in this review reported no maternal deaths, illness or death in the new-borns as a result of COVID-19 [54,58]. However there have also been reports of maternal death as a result of COVID-19 and new-borns presenting with infection after birth [128,129]. Hence it is essential that these mothers and their new-borns from these COVID-19 pregnancies are monitored post-delivery and even after they recover as one is uncertain if the effects of COVID-19 will manifest clinically in the future. A study conducted by Liu et al. (2021) followed up with these infants for 9 months, where they observed transient early fine motor abnormalities in these babies born from COVID-19 pregnancies. However, we are still unaware of the long-term effects that COVID-19 and its therapeutic interventions may have, which will only manifest in the years to come, hence consistent surveillance on the mothers and new-borns from COVID-19 pregnancies need to be made a priority. It is in the best interest of these mothers and infants to be screened for potential aftereffects.

Table 3
Therapeutic agents recommended for COVID-19 Pregnant patients.

Therapeutic agent	Indications	Dosing, Precautions and other considerations
Heparin	Venous thromboembolism prophylaxis in hospitalized patients.	- Prophylactic or intermediate dose (5000–10000 units twice daily (BID), sub cutaneous (SC)) of unfractionated heparin for patients who might soon give birth [118]. - Prophylactic or intermediate dose (5000or 40 mg OD, SC) of low molecular weight heparin for patients who are unlikely to be delivered within a few days [118].
Dexamethasone	- Patients who are on supplemental oxygen or ventilatory support. - Management of refractory shock in critically ill patients.	- 6 mg orally or intravenously (IV) daily for 10 days After the initial four doses (6 mg, BID, IV) [119]. - Glucose monitoring and switching to other glucocorticoid should be considered as per WHO and the Society for Maternal-Foetal Medicine guidelines [120].
Nonsteroidal anti-inflammatory drugs (NSIAD) and Paracetamol	- When clinically indicated. - Low-dose aspirin for prevention of preeclampsia.	- The lowest effective dose should be used. - Paracetamol is the drug of choice for antipyretic and analgesic effects [121].
Remdesivir	- Antiviral activity	- The potential for human placental transfer is unknown [121]. - No reported foetal toxicity during pregnancy [122]
Baricitinib, Tofacitinib	- Anti-inflammatory activity - Antiviral activity	- The potential for human placental transfer is expected based on its molecular weight [123]. - Embryo-foetal toxicity have been observed in animal studies [123]. - Tofacitinib pregnancy outcomes were comparable to those in the general population.
Tocilizumab, Sarilumab, Siltuximab, Anakinra	- Anti-inflammatory activity	- Tocilizumab did not reveal clear serious safety signals during pregnancy [124]. - Less information is available about use of sarilumab, siltuximab, and anakinra in pregnancy [125].

7. Conclusion

This review has highlighted the impact that COVID-19 had on maternal and foetal health. The effects of COVID-19 observed in the placenta were concerning as it suggested that there were alterations in the blood flow which resulted in hypoxic conditions as the placenta and consequently the foetus were not receiving adequate blood supply. Furthermore, we found that this could result in predisposing mothers to

preeclampsia and IUGR resulting in further complications. Therefore, understanding the effects of the virus is imperative in determining therapeutic interventions to overcome current and even future adverse effects in both mother and baby. But most importantly, mothers and children from these pregnancies need to be monitored for any clinical manifestations that may arise in the years to come as a result of the alterations caused by COVID-19.

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Declaration of competing interest

The authors declare that there is no conflict of interest.

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BRIDGING TEXT

FROM CHAPTER TWO TO THREE

One of the main concerns during pregnancy is inflammation due to COVID-19 infection, as highlighted in manuscript one **(Chapter Two)**, where placental inflammation markers were widespread. Therefore, this manuscript focuses on how COVID-19 alters the cytokine profile in pregnancy in both the plasma and EVs from the South African cohort, taking into account the emerging role of EVs as signalling entities that can alter the outcome of pregnancies. This was determined by assessing the cytokine profile in both the plasma and EVs from South African pregnancies, through the analysis of IFN gamma, IL-6, MIP-1 alpha and TNF alpha levels. The results have outlined alterations in the cytokine profile as a result of COVID-19, further postulated mechanisms for the changes identified, related clinical manifestations and relevant recommendations to future studies as well as clinicians in South Africa treating mothers and infants from these pregnancies to ensure their future wellbeing.

3.0 CHAPTER THREE

MANUSCRIPT TWO

The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile

C Heeralall [1], U H Ibrahim[2#], M Jenneker[3], S Singh[4], L Lazarus[1], I Mackraj[2#]

[1] Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

[2] Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

[3] Discipline of Obstetrics and Gynaecology, School of Clinical Medicine, University of KwaZulu-Natal, Durban, South Africa.

[4] Optics & Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban 4041, South Africa.

[#] Corresponding authors: Professor Irene Mackraj and Dr Usri Ibrahim

Address: Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville Campus, Private Bag X54001, Durban, South Africa.

Email address: Mackraji@ukzn.ac.za, usrihasan@yahoo.com

Tel: +27 729085646, +27 651489693

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Purpose: The Coronavirus disease (COVID-19) has impacted pregnant women significantly, with increased mortality and morbidity. The implications of this virus are linked to extracellular vesicles (EVs) and maternal inflammation due to the cytokine storm. Hence, this study aims to investigate the impact of COVID-19 on the pro-inflammatory cytokine profile in both plasma and EVs of South African pregnant women.

Methods: Plasma samples were obtained from pregnant women in the third trimester, from which EVs were extracted using the Invitrogen™ Total Exosome Isolation Kit. These plasma-derived EVs were characterized using transmission electron microscopy and nanoparticle tracking analysis.

Results: COVID-19 infection in pregnancy did not significantly affect the average particle size and concentration of isolated EVs. The levels of IFN gamma, IL-6, MIP-1 alpha and TNF alpha were analysed in the plasma and circulating EVs through a multiplex assay. Compared to control group, a significant increase in IL-6, IFN-γ, TNF-α and MIP-1α levels were observed in both plasma and EVs content of COVID-19 pregnancies.

Conclusion: These findings suggest that COVID-19 infection impacts the pro-inflammatory cytokine profile in the plasma and EVs of South African pregnant women.

Keywords: COVID-19; Pregnancy; Inflammation; Cytokine storm; Extracellular vesicles

1. Introduction

In 2019, the catastrophic COVID-19 pandemic affected over 181 countries in just 5 months, with 1197405 confirmed cases (Hoseinpour Dehkordi *et al.*, 2020). However, the long-term consequences of this virus are yet to be determined, particularly its impact on COVID-19-positive pregnancies and their associated defects. During pregnancy, COVID-19 infection has been connected to several cases of foetal distress, demise, and preterm delivery due to the cytokine storm and maternal inflammation (Joma *et al.*, 2021). Inflammation is of great concern in pregnancy, as inflammatory cytokines can pass to the foetus through the blood, resulting in systemic inflammation (Joma *et al.*, 2021). Therefore, improvement in knowledge of the pathogenesis of COVID-19 infection during pregnancy and its associated inflammatory responses could facilitate ongoing efforts to reduce the harmful long-term effects of COVID-19 in mothers and their foetuses. Together with enabling a better understanding of how to approach future viral outbreaks to improve the management for pregnant women.

Elevated pro-inflammatory cytokine levels have been documented in COVID-19 patients; hence, the 'cytokine storm' is central to COVID-19 pathogenesis (McGonagle *et al.*, 2020, Yang *et al.*, 2020a, Darif *et al.*, 2021). This elevated expression in cytokines is considered to be as a result of the decreased expression in ACE2 and an increase in angiotensin 2 (Ang-II), which can result in a multitude of adverse consequences including septic shock (Hirano and Murakami, 2020, Ntounis *et al.*, 2022, Ruan *et al.*, 2020). Among other cohorts, pregnant women have been extensively impacted by this increase in pro-inflammatory cytokines, however this profile is yet to be fully understood in the South African cohort (Rosen *et al.*, 2022, Tanacan *et al.*, 2021). These inflammatory cytokines can pass through the placenta, posing substantial risk to infants and their neural development (Figueiredo *et al.*, 2021, Shuffrey *et al.*, 2022, Kurokawa *et al.*, 2023, Van Steenwinckel *et al.*, 2014, Basheer *et al.*, 2022, Redline, 2004). Therefore, elucidating the cytokine profile in the South African

population will allow us to physiologically evaluate what occurred in pregnancies impacted by COVID-19 and determine any long-term effects as a result, which could be beneficial for neonates from these pregnancies.

This pro-inflammatory state observed in patients from the COVID-19 pandemic has further been linked to affecting extracellular vesicles (EVs) and their protein secretion (Gurunathan *et al.*, 2021). EVs can release their cytokine cargo along with various other functions, including intercellular communication through transporting the cargo for transfer between cells (Jung *et al.*, 2020, Benjamin-Davalos *et al.*, 2021). In addition, viral infections have been reported to utilize EVs as entry points for transmitting viral proteins, evading the immune system, and thereby resulting in tissue damage (Babaei *et al.*, 2022). Furthermore, EVs are known to play a role in pregnancy; hence, great interest in investigating their role and effect on pregnant women emerged as alterations in the level or cargo of these vesicles have been linked to pre-eclampsia and preterm delivery (Tannetta *et al.*, 2014, Konadu *et al.*, 2015). Pre-eclampsia has been linked to elevated EV levels, which results in increased maternal immune activation, endothelial dysfunction and vascular impairments, that has also been observed in carcinogenesis (Konadu *et al.*, 2015, Matsubara *et al.*, 2021, Pillay *et al.*, 2016). While pregnant women who recovered from COVID-19 showed a significant decrease in EVs and suggested that EVs could control inflammation (Cao *et al.*, 2022), however, the role of EVs in inflammation of COVID-19 pregnancies is yet to be completely elucidated and understood. This dysregulated cytokine response has been identified in critical cases of COVID-19 in the Sub-Saharan African cohort; however, the effect of COVID-19 on the cytokine profile in pregnant women of the South African cohort is yet to be fully determined (Shaw *et al.*, 2023).

Therefore, this study aims to investigate the effect of COVID-19 on the cytokine profile in the plasma and EVs of pregnant South African women. The results obtained from the isolation and characterization of EVs from the plasma of COVID-19-positive and negative pregnant women

(≥34 weeks), as well as levels of most important cytokines (IFN gamma, IL-6, MIP-1 alpha and TNF alpha) in both circulating EVs and plasma, are herein reported.

2. Materials and Methods

2.1. Ethics Approval and consent to participate

Regulatory ethical and Institutional approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004591/2022), South Africa. Patients were recruited based on the inclusion and exclusion criteria. Once they were identified for this study, its purpose and requirements were explained to them. Upon agreeing to participate, all participants signed a consent form.

2.2. Study Population

The Inkosi Albert Luthuli Central Hospital was under the triage system for COVID-19 diagnoses, and therefore upon arrival, all patients were tested prior to admission. Black South African women over 34 weeks of gestation who were confirmed either COVID-19 positive (n=10) and negative (n=10) for SARS-CoV-2 RNA via real-time polymerase chain reaction (RT-PCR) on nasopharyngeal swabs were considered for this study. Gestational matching was performed in this study. Immediately after consent was obtained, a doctor from the hospital obtained 5ml of whole blood via venepuncture using plasma tubes (BD Vacutainer Tubes (EDTA), Becton Dickinson and Company, South Africa). The blood tubes were gently shaken and left to stand for 20 minutes at room temperature. Vacutainers containing blood were centrifuged within one hour (3500rpm for 15 minutes at 4^{o}C). Following centrifugation, plasma was separated from the blood and aliquoted into Eppendorf tubes. All samples were stored at -80°C until used. Samples were processed according to the accepted guidelines pertaining to EVs (Chatterjee *et al.*, 2015).

2.2.1. Inclusion and exclusion criteria

Black South African women below the age of 40 who were over 34 weeks of gestation, with confirmed singleton pregnancies, who were confirmed either COVID-19 positive or negative through PCR testing were considered for this study. Only those who could provide full consent

and did not present with other major co-morbidities such as hypertension, pre-eclampsia, IUGR or other gestational complications were then recruited.

2.3. EV isolation

EVs were isolated using the Invitrogen™ Total Exosome Isolation Kit from plasma (Life Technologies, CA, United States) according to the manufacturer's instructions. Briefly, 0.6 ml of plasma sample was centrifuged at 2000 × g for 20 minutes at room temperature to remove cells and debris. After that, the supernatant was transferred and centrifuged at 10,000 × g for 20 minutes at room temperature. 250 µl of PBS was then added to the clarified plasma and vortexed for 10 seconds. Subsequently, 150 µl of precipitation reagent was added, vortexed for 10 seconds and then incubated for 10 minutes at room temperature. Centrifugation was then repeated at 10,000 × g for 5 minutes at room temperature, after which the supernatant was discarded, and the EVs were resuspended in 250 µl of PBS and stored.

2.4. Transmission electron microscopy

After being diluted in 1X PBS to a 1:20 dilution ratio, isolated EVs were put on a continuous nickel grid. 2% uranyl acetate was used to negatively stain the samples. A JEOL JEM 1400 transmission electron microscope (JEOL, Peabody, MA, USA) was used to analyse the particle morphology at a 20k magnification. Pictures were taken at 50 and 100 nm. Images were obtained using Gatan Capture software (AMETEK. Inc., USA).

2.5. Nanoparticle tracking analysis (NTA)

Using the NanoSight 500, which has a 405 nm laser and a sCMOS camera, NTA examined the particle size distribution, average particle size, and concentration of the plasma-derived EVs (NanoSight NTA 3.2 Nanoparticle Tracking and Analysis Release, Version Build 0069, Malvern Panalytic, Malvern, UK). Samples were diluted in 1x PBS and mixed well to get 10-100 particles per image. Diluted samples were then injected into the laser chamber. The

following settings were used for data acquisition: camera level 12, acquisition time 20ms, and detection threshold 7.

2.6. Multiplex immunoassay

Isolated EVs and plasma samples were stored at -20°C and - 80 °C, respectively, until analysis. Sample aliquots had not been previously thawed before use in the multiplex cytokines and chemokine assay. The levels of IFN gamma, IL-6, MIP-1 alpha (CCL3) and TNF alpha were measured using the ProcartaPlex 4 Plex, 1plate 4-Plex (CAT # PPX-04-MXT2AYK) according to the manufacturer's instructions.

In a 96-well plate, HGF (Hepatocyte Growth Factor) and HGFR-captured (Hepatocyte Growth Factor Receptor) antibody-coupled magnetic beads were added to each well and washed twice. 25 µL of Standards, plasma samples, PBS (blanks) and EV suspension were then added into respective wells (in duplicates) and left to incubate for 16–18 h at 2–8 °C before washing three times with wash buffer on an automated magnetic plate washer (Bio-Plex Pro™ /Bio-Plex Pro II Wash Station). The concentration of EVs suspension used in this study ranged from 1.53 x 10^7 to 2.6 x 10^8 EVs/mL and therefore normalization has been done to eliminate the effect of concentration variations and cytokine concentrations were expressed as pg/ 10^8 EVs. After that, the biotinylated-detection antibody was pipetted into each well and incubated for 1 h at room temperature. After washing, the plate was incubated for 30 minutes at room temperature with streptavidin–phycoerythrin (SAPE). Prior to resuspending each well with Drive fluid, the plate was lastly cleaned. A Biorad Bio-Plex Magpix system was used for detection. Raw data was extracted using Bio-Plex Manager software version 4.1 and the Bio-Plex®MAGPIXTM Multiplex Reader (Bio-Rad Laboratories Inc., USA).

2.7. Statistical analyses

All data analyses and graphical representations were generated using GraphPad Prism 8.0 (C.A., La Jolla). Statistical analyses were done using the unpaired t-test and significance was considered when p-value < 0.05.

3. Results

3.1 Clinical characteristics

Relevant clinical information about each group, i.e. Control and COVID-19 +ve are summarized in Table 1 as mean ± standard deviation due to their parametric distribution. Maternal age, maternal weight, systolic blood pressure, diastolic blood pressure, heart rate, body temperature, gestational age, gravidity and parity were non-significantly (ns) different between the Control and COVID-19 +ve study groups, as shown in Table 1.

Table 1. Patient demographics of Control and COVID-19 +ve pregnant women.

Results are represented as the mean (±SD), ns = Not significant (P > 0.05)

	Maternal age (years)	**Maternal weight (kg)**	**Systolic BP (mmHg)**	**Diastolic BP (mmHg)**	**Heart Rate (Beats per minute)**	**Body Temperature (°C)**	**Gestational age (weeks)**	**Gravidity**	**Parity**
Control (n = 10)	29.40 (±5.50)	127.3 (±38.05)	130.0 (±26.73)	68.20 (±17.33)	89.30 (±15.17)	36.31 (±0.18)	36.20 (±1.99)	2 (±1.5)	1.4 (±1.43)
COVID19+ ve (n = 10)	28.60 (±4.97)	100.30 (±42.83)	113.5 (±15.59)	64.20 (±7.54)	90.60 (±10.27)	36.30 (±0.22)	36.80 (±2.25)	2.5 (±2.1)	1.8 (±2.1)
Significance	ns	ns	ns	ns	ns	ns	ns	ns	ns

3.2 Morphology and concentration of circulating EVs

3.2.1 Transmission Electron Microscopy

Transmission Electron microscopy analysis of EVs isolated from the plasma of both group are shown in **Figure 7**. EVs isolated from COVID19-+ve group revealed oval-shaped vesicles, while COVID19-ve group showing round-shaped vesicles with almost similar size. TEM image also showed lipoprotein aggregates (white circular particles), which were isolated with EVs due to using of precipitation-based EV isolation method.

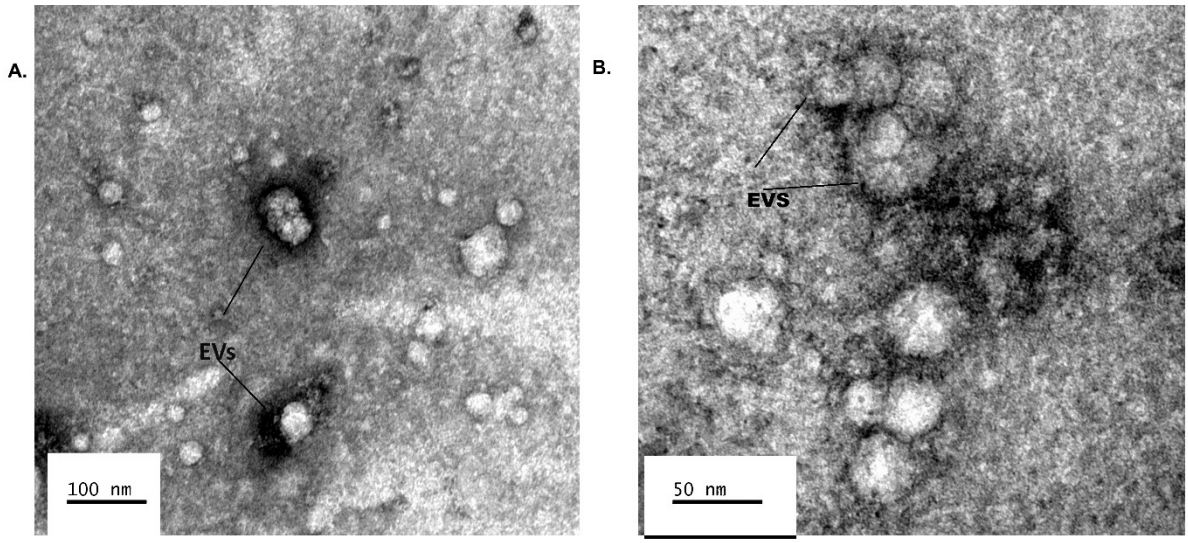

Figure 1. Transmission Electron microscopy images of EVs.

A). Covid +ve pregnancy showing oval-shaped vesicles, (scale bar 100 nm). **B).** Covid -ve pregnancy showing round-shaped vesicles (scale bar 50 nm). Lines indicate individual EVs and circular lipoprotein aggregates are also present with the isolated EVs

3.2.2 Nanoparticle Tracking Analysis

EVs isolated from both groups have shown a monodispersion size distribution as presented in **Figure 8**. The hydrodynamic average particle size of EVs from COVID-19 -ve pregnancies was 172.48 ± 47.21 nm with an average EV concentration of $1.3 \times 10^8 \pm 0.77 \times 10^8$ EVs/ mL, particle size span 1.92 ± 0.58 and D90/D10 ratio of 4.72 ± 1.88. The average size of EVs from COVID-19-+ pregnancies was 167.6 ± 51.18 nm with an average EV concentration of 6.73 ×

$10^7 \pm 5.86 \times 10^7$ EVs/ mL, particle size span 1.63 ± 0.41 and D90/D10 ratio of 3.91 ± 0.41. Statistical analysis of Nanoparticle tracking analysis (NTA) results revealed that there were no significant differences between EVs isolated from the plasma of COVID-19 +ve and COVID-19 -ve pregnancies in terms of concentration and average particle size. In contrast, COVID19 infection have significantly reduced EVs polydispersity as inferred from the reduction of both particle size span and D90/D10 ratio values.

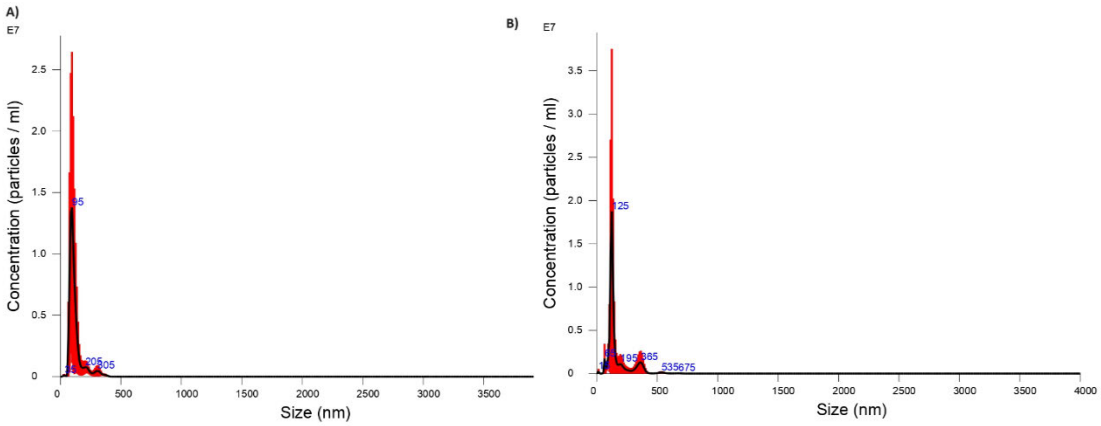

Figure 2. Graphical representation of the particle size distribution of EVs isolated from plasma.

A) COVID-19 + ve pregnancies, **B)** COVID-19 - ve pregnancies. This monodispersion size distribution obtained from Nanoparticle Tracking Analysis highlights the particle size distribution and concentration of the plasma-derived EVs.

3.3 Plasma and EV Pro-inflammatory cytokines levels in COVID-19-negative and positive pregnancies

Table 2 illustrates that the plasma from COVID-19-+ ve pregnancies has shown significantly higher levels of all investigated pro-inflammatory cytokines (IFN gamma, IL-6, MIP-1 alpha and TNF alpha) when compared to COVID-19 - ve pregnancies. There were no significant differences observed when comparing the levels of IL-6, MIP-1 alpha and TNF alpha) in the EVs fraction isolated from the plasma of COVID-19-+ ve pregnancies to their levels in COVID-19 -ve pregnancies. The only exception is IFN gamma where its level is significantly

higher in the EVs isolated from the plasma of COVID-19-+ ve pregnancies, compared to its level in the EVs isolated from plasma of COVID-19 -ve pregnancies.

Table 2. Pro-inflammatory cytokine level (pg/Ml) in the plasma and EVs fraction of COVID-19 -/+ pregnant women.

Data represented as mean ± STD, (n = 10).

MARKER	PLASMA			EVs Fraction		
	COVID –	**COVID +**	*p-value*	**COVID –**	**COVID +**	*p*-value
IL-6	10.46 ± 0.99	70.26 ± 29.21	0.04*	7.89 ± 1.01	17.16 ± 7.11	0.190
IFN-γ	7.07 ± 0.34	8.29 ± 0.29	0.02*	6.52 ± 0.17	7.91 ± 0.41	0.01**
TNF-α	6.58 ± 0.08	7.26 ± 0.26	0.03*	6.64 ± 0.16	7.10 ± 0.33	0.19
MIP-1α	3.23 ± 0.32	4.95 ± 0.66	0.04*	1.12 ± 0.09	1.23 ±0.23	0.64

Results are represented as the mean ± *SEM*, *$p < 0.05$., **$p < 0.01$.

Interestingly, unlike EVs fraction cytokine levels, normalized EVs cytokine level per fixed number of EVs (10^8 EVs) have shown significant increase due to COVID-19 infection (**Figure 9**). In addition, this study also notes that 3 neonates from COVID-19 positive mothers experienced distress and presented with low birthweights. 1 of these neonates were born premature while the other 2 presented with abnormalities and died. 1 of neonates from COVID-19 negative pregnancies experienced distress and was born prematurely with a low birthweight.

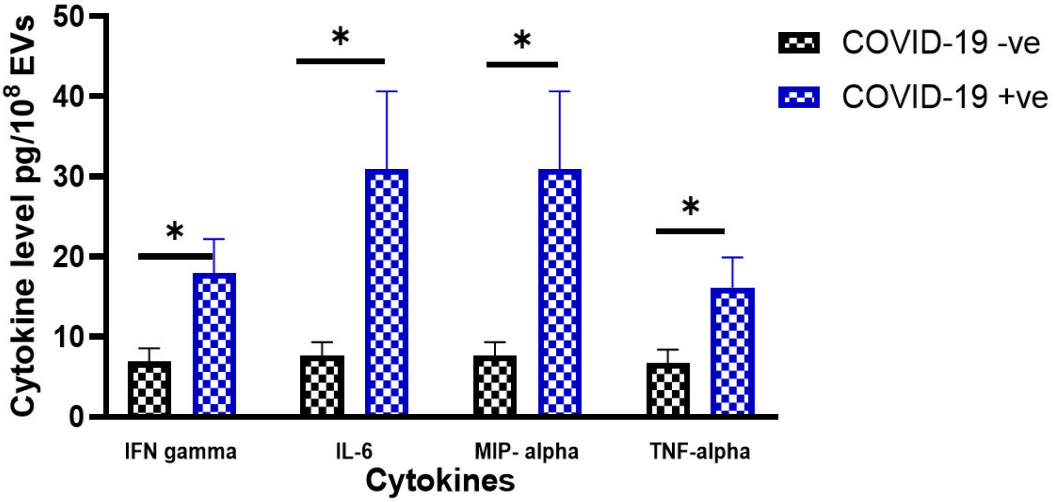

Figure 3. Pro-inflammatory cytokine level in EVs isolated from COVID-19 -/+ pregnant women.

Data represented as mean ± STD, (n = 10). * p-value < 0.05.

4. Discussion

In the present study, COVID-19 infection has significantly increased the levels of key pro-inflammatory cytokines (IL-6, TNF-α and MIP-1α) in the plasma from COVID-19-positive pregnant women compared to COVID-19-negative pregnancies. These results are consistent with previous reports of alterations in plasma cytokine profile due to COVID-19 infection during pregnancy (Tanacan *et al.*, 2021, Liu *et al.*, 2020b, Fatih *et al.*, 2022, Irwinda *et al.*, 2021, Chen *et al.*, 2021, Fenizia *et al.*, 2020, Sabharwal *et al.*, 2023, Phoswa and Khaliq, 2020, Vásquez-Procopio *et al.*, 2022, Aminsobahni *et al.*, 2023, Bernier *et al.*, 2024). Our study also reported on the EVs isolated from the plasma of COVID-19 -/+ ve South African pregnant women and subsequent characterization of these isolated EVs using TEM and NTA analyses. COVID-19 infection in pregnancy have significantly reduced EVs polydispersity, while did not significantly affect the release, concentration and average particle size of isolated EVs. Furthermore, the key pro-inflammatory cytokines content in EVs fractions (IL-6, TNF-α and

MIP-1α) was not affected by COVID-19 infection in pregnancy. While the EV fraction level of multifunctional cytokine IFN-γ was significantly elevated in response to COVID-19 infection in pregnancy. In contrast, normalized cytokine level per number of isolated EVs has shown a significant increase in EVs contents of all investigated cytokines due to COVID-19 infection during pregnancy, this highlights the importance of EVs content normalization to EVs count and/or protein content in evaluation of EVs characteristics.

Concerns of excessive inflammation during pregnancy in response to viral infections, like COVID-19, are therefore raised due to the resultant predisposition of gestational complications like pre-eclampsia, preterm birth, intrauterine growth restriction, and stillbirth which could affect the development of the foetus consequently resulting in severe postnatal repercussions (Seymen, 2021, Heeralall *et al.*, 2023, Jamieson and Rasmussen, 2021). Alterations in the pro-inflammatory cytokine profile are suggestive of a pro-inflammatory state being favoured, which raises a multitude of concerns, as the balance amongst anti/pro-inflammatory cytokines and chemokines is critical in ensuring that pregnancy is safe (Liu *et al.*, 2020a, Tanacan *et al.*, 2021). On the other hand, EVs have shown a dual role in inhibiting or enhancing viral infection pathogenesis (Caobi *et al.*, 2020). Therefore, in this study, we have investigated the pro-inflammatory cytokines profile in both plasma and EVs from COVID-19 -/+ ve pregnant women to explore their role in COVID-19 pathogenesis during pregnancy.

Elevated plasma levels of pro-inflammatory cytokines associated with COVID-19 in pregnancy, in particular IL-6, have been linked to systemic inflammation in pregnancy, which is thought to have an impact on foetal development (Martins-Filho *et al.*, 2020, Hoffmann *et al.*, 2020). IL-6 in particular is considered to have a direct impact on the development of the foetal brain, as maternal IL-6 can pass through the placental barrier with the placenta being considered the site of action for this cytokine (Dahlgren *et al.*, 2006, Sandovici *et al.*, 2012, Tsukada *et al.*, 2019). Therefore, IL-6 can impact long term brain development and can

increase in the foetal and postnatal brain as a result of maternal immune activation (Garay *et al.*, 2013, Gallagher *et al.*, 2013).In addition, increased expressions in TNF-α and IFN-γ have been further linked to organ damage, miscarriage, preterm delivery with recent reports raising concerns that this pro-inflammatory state can result in neurodevelopmental disorders in the offspring (Vásquez-Procopio *et al.*, 2022, Fenizia *et al.*, 2020, Rosen *et al.*, 2022, Taglauer *et al.*, 2022, Sabharwal *et al.*, 2023). Therefore, we believe that the alterations observed in this study suggest that COVID-19 infection during pregnancy can increase the risk of gestational complications and possibly impact foetal development. This could be further linked to the increase in adverse neonatal outcomes in COVID-19 positive pregnancies observed in this study. Moreover, increases in chemokines such as CCL2, CCL5 and CCL3 (MIP-1α) could been further linked to premature delivery and possible placental malfunction (Fenizia *et al.*, 2020). As a result, we propose that this pro-inflammatory state observed in COVID-19 positive pregnancies could be linked to altered placental functioning and structure reported in these pregnancies (Heeralall *et al.*, 2023).

Herein, we investigated for the first time, to the best of our knowledge, the effect of COVID-19 on the pro-inflammatory cytokine profile in plasma-derived EVs from South African pregnant women to explore their role in COVID-19-associated pro-inflammation in pregnancy. This study demonstrates that COVID-19 infection during pregnancy was significantly reduced EVs size poly dispersion and significantly increased their key pro-inflammatory cytokines content, with no significant impact on EV release, concentration and average particle size. Interestingly, COVID-19 during pregnancy triggers production of pro-inflammatory cytokines and IFN-γ in EVs as EVs isolated from COVID-19 +ve pregnancies have shown significantly higher levels compared to their COVID-19 -ve counterparts. This elevation in the level of multifunctional cytokine IFN-γ and other pro-inflammatory cytokines investigated in this study suggests that EVs may contribute to maternal immune activation and could have possible

proliferative effects during COVID-19 infection in pregnancy. Furthermore, the lack of significant difference in the EV release, concentration and average particle size between COVID-19 positive and negative pregnancies could also suggest a possible protective role of EVs / major immune-modulatory effects in COVID-19. Studies have suggested that alterations in EV concentrations and sizes are linked to gestational complications (Sokolov *et al.*, 2016, Lok *et al.*, 2009, Rice *et al.*, 2015). Similar studies have also reported that EVs from COVID-19 patients could exert an inflammatory response through stimulation of endothelial cells' release of pro-inflammatory cytokines like IL-6 due to an increase in expression of fibrinogen-β (FGB) and tenascin-C (TNC), which are known to stimulate pro-inflammatory cytokines like IL-6 (Sur *et al.*, 2021). Another report further documented that COVID-19 exosomes activate IL-1β secretion by triggering the NLRP3 inflammasome, thereby resulting in an inflammatory response (Sur *et al.*, 2022). In addition, similar findings have also been noted in studies on HIV, Hepatitis B and pre-eclampsia, with these alterations in EVs being linked to disease pathogenesis (Konadu *et al.*, 2015, Holder *et al.*, 2012, Tannetta *et al.*, 2014, Kouwaki *et al.*, 2016). Moreover, EVs have been linked to a host of pregnancy complications, including pre-eclampsia and preterm birth (Bai *et al.*, 2021). EV's isolated from the placenta and umbilical cord could also shed insight on its function in these pregnancies with COVID-19, as EV's have been suggested to play thrombo-inflammatory role in the placentae from preeclampsia pregnancies (Kohli *et al.*, 2016). Whilst human umbilical mesenchymal stem-cell-derived EVs have been reported to reduce damage as a result of inflammation in preeclampsia (Yu *et al.*, 2023). However, this possible protective role of EVs in COVID-19 are yet to be fully understood and could suggest that EVs could suppress inflammation as a protective mechanism in COVID-19 as noted in another study (Cao *et al.*, 2022). Therefore, we believe our study indicates that EVs are in fact involved and implicated in COVID-19 pregnancies but suggest

further investigations into EV's from the placenta and umbilical cord to elucidate their exact role.

5. Conclusion

This study demonstrates that the plasma pro-inflammatory cytokine profile is impacted in South African pregnancies with COVID-19 infection, suggesting the elevated risk of systemic inflammation and its associated harmful complications. Furthermore, this study demonstrated that plasma-derived EVs have no direct pro-inflammatory activities during COVID-19 infection in pregnancy and suggested their possible protective and proliferative effects due to their higher content of IFN-γ compared to COVID-19 – ve pregnancies. These findings however, raise much concern for the functioning of the placenta in these pregnancies and the subsequent altered environment neonates were exposed to. We believe this proinflammatory state could be possibly linked to the placental dysfunction observed in COVID-19 pregnancies and therefore suggest further investigation. Consequently, we believe COVID-19 poses a greater threat for long-term complications, especially in the neonates from these pregnancies. Therefore, monitoring of neonates from COVID-19 pregnancies is recommended and their neurodevelopment should be under strict observation.

6. Limitations

During the final wave of the COVID-19 pandemic, the collecting site still followed strict regulations and access to patients was restricted thereby contributing to a limited sample size and lack of information about confounding factors such as foetal sex, marital status, socioeconomic status. Furthermore, using the precipitation-based method to isolate EVs could affect the accuracy of the determination of EV cytokines levels due to the presence of co-isolated lipoproteins aggregate and residual serum cytokines. As well as lack of assessing EV-specific markers. Nonetheless, we believe our findings contribute to the fundamental

understanding that COVID-19 infection during pregnancy has an impact on the cytokine profile in the South African cohort.

7. Ethics Approval and Consent to participate

Regulatory ethical and Institutional approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004591/2022), South Africa. Patients were recruited based on the inclusion and exclusion criteria. Once they were identified, the purpose and requirements of this study were explained to them. Upon agreeing to participate, all participants signed a consent form.

8. Availability of data and material

Data will be made available on request.

9. Declaration of competing interest

The authors declare that there is no conflict of interest.

10. Funding

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11. Consent for publication

Not applicable

12. Acknowledgements

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13. Authors contributions

C Heeralall: Conceptualization, methodology, writing of the original draft, data curation and revision of the manuscript. U H Ibrahim: Conceptualization, Writing the original draft and data curation, reviewing and editing of the manuscript. M Jenneker: Methodology. S Singh: Methodology and data curation. L Lazarus: Conceptualization and supervision. I Mackraj: Conceptualization, supervision, reviewing and editing of the manuscript.

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BRIDGING TEXT

FROM CHAPTER THREE TO FOUR

The previous chapters highlighted the alterations reported in placental morphology due to COVID-19 globally and examined the cytokine profile in South African pregnancies. These results demonstrated a hyperinflammatory state which is not considered to be conducive for optimal placental development and functioning. The placental histopathology in COVID-19 pregnancies in this paper are investigated by evaluating the kisspeptin expression, to establish whether proper placental development and functioning occurred in these pregnancies from the South African cohort. The placental morphology was then further examined to understand if placental insufficiency occurred as a result of COVID-19 alterations. The findings of this paper provide evidence for impaired placental functioning possibly due to altered kisspeptin levels in COVID-19 pregnancies.

4.0 CHAPTER FOUR

MANUSCRIPT THREE

The effect of COVID-19 on placental functioning in South African pregnancies: Investigation of kisspeptin expression, vascular and inflammatory alterations

C Heeralall [1], U H Ibrahim[2#], M Jenneker[3], S Singh[4], M Matjila[5], L Lazarus[1], I Mackraj[2#]

[1]Discipline of Clinical Anatomy, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

[2] Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

[3]Discipline of Obstetrics and Gynaecology, School of Clinical Medicine, University of KwaZulu-Natal, Durban, South Africa.

[4]Optics & Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban 4041, South Africa.

[5]Department of Obstetrics and Gynaecology, Faculty of Health Sciences, University of Cape Town, Groote Schuur Hospital, Cape Town 7925, South Africa.

[#] Corresponding authors: Professor Irene Mackraj and Dr Usri Ibrahim

Address: Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville Campus, Private Bag X54001, Durban, South Africa.

Email address: Mackraji@ukzn.ac.za, IbrahimU@ukzn.ac.za

Tel: +27 729085646, +27 651489693

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Introduction. The COVID-19 pandemic has passed; however, its long-term effects are yet to be determined. Pregnant women and their neonates faced an increased risk for complications during this pandemic as COVID-19 was reported to result in oxidative and inflammatory stress, and the cytokine storm, which would impact upon pregnancy viz. trophoblast invasion, placental development and functioning. Therefore, this study aims to determine the effect of COVID-19 on the placental functioning in South African pregnancies through the analysis of kisspeptin and placental morphology.

Methods. Immunohistochemical analysis of placental samples was performed to detect the expression of kisspeptin. Histopathological analysis was conducted to identify vascular and inflammatory alterations.

Results. This study demonstrated that COVID-19 results in a significantly increased expression of placental kisspeptin in both the central ($p = 0.001$) and peripheral ($p < 0.0001$) regions as compared to the placentae from control pregnancies. Upon further analysis, the placentae from COVID-19 pregnancies also presented with severe inflammation and maternal and foetal vascular malperfusion compared to the control placentae.

Discussion. A significantly increased expression of placental kisspeptin was observed in COVID-19 positive pregnancies inferring impaired placental functioning. This was further supported by vascular and inflammatory alterations observed in COVID-19 positive placentae which may suggest that trophoblast invasion was compromised. To date, there still exists small clusters of COVID-19 outbreaks and our findings highlights the importance for future surveillance of these mothers and neonates from COVID-19 pregnancies in South Africa, as neonates from other countries have presented with abnormalities.

Keywords: COVID-19, placenta, kisspeptin, implantation, inflammation

Introduction

The coronavirus pandemic (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to millions of confirmed cases globally, with South African recording the highest prevalence on the African continent (WHO, 2024). The outbreak of SARS-CoV-2 emerged in late 2019 resulted in a widespread pandemic, which continues to have significant health, social and economic consequences (Chuang *et al.*, 2024, Gheorghita *et al.*, 2024). In the United States, more than 400,000 women have been impacted by this pandemic, including 23,434 pregnant women (Zambrano *et al.*, 2020). Given the ongoing challenges posed by COVID 19, it remains crucial to investigate the effects of this virus on pregnant women and foetal health, as long-term consequences on maternal and foetal health are not yet fully understood.

The severity of COVID-19 ranged from fevers and fatigue to pneumonia with decreased oxygen saturation (Velavan and Meyer, 2020). These symptoms were more severe in pregnant women than in non-pregnant women, who were more likely to require intensive care unit admission (Wenling *et al.*, 2020, Zambrano *et al.*, 2020). These women were reported to be more vulnerable to pneumonia and other respiratory diseases due to differences in oxygen consumption and T lymphocyte immunity compared to that of a normal individual (Wenling *et al.*, 2020, Zambrano *et al.*, 2020). In addition, COVID-19 could further induce acute inflammation in pregnant mothers, causing respiratory distress and organ failure which could impact foetal development (Granja *et al.*, 2021, Seymen, 2021). Moreover, adverse consequences in the placenta of these pregnant women as a result of COVID-19 were observed, which presented as signs of inflammation, vascular alterations, hypoxia along with an increased risk of pre-eclampsia (PE), preterm birth, and stillbirth (Wenling *et al.*, 2020, Abedzadeh-Kalahroudi *et al.*, 2021, Wastnedge *et al.*, 2021, Jamieson and Rasmussen, 2021, Khoiwal *et al.*, 2022).

Proper implantation, placental development and functioning is fundamental for the success of all pregnancies, thus, understanding if these processes were impacted in COVID-19 pregnancies would be beneficial, in order to comprehend the outcomes and long-term impact associated with these pregnancies (Boss *et al.*, 2018). Any alteration from as early as implantation has the potential to result in abnormal placentation (Burton and Jauniaux, 2023). There is an increase in evidence that suggests complications, as a result, can extend beyond gestation as it has been further linked to predisposing infants to neuropsychiatric, metabolic, and cardiovascular disorders (Burton and Jauniaux, 2023). Trophoblast migration and invasion

are fundamental to implantation required for the development of the placenta and foetus; and are dependent on factors such as oxygen tension, angiogenic and growth factors (Matjila, 2015, Kapustin *et al.*, 2020, Silva and Serakides, 2016). Kisspeptins (group of peptide fragments encoded by the metastasis suppressor gene viz. KISS 1) are key components in trophoblast migration and invasion that possess a major influence in placental formation and development (Hu *et al.*, 2019a, Akhtar *et al.*, 2020), through an array of mechanisms including inhibition of certain matrix metalloproteinases (MMPs) enzymes (Cao *et al.*, 2019, Gorbunova and Shirshev, 2020). The invasion of extravillous trophoblasts into the maternal tissue is a pivotal factor in the oxygenation of the placenta and foetus to prevent hypoxia (Huppertz *et al.*, 2014). Alterations in kisspeptin expression have been linked to PE, preterm birth, intra-uterine growth restriction (IUGR) and an increased risk for miscarriage (Cao *et al.*, 2019, Kapustin *et al.*, 2020). Kisspeptin has been shown to significantly influence preeclampsia where insufficient invasion can result in hypoperfusion, the release of proinflammatory markers and poor vascular remodelling (Gomes and Sones, 2021). Therefore, investigations involving kisspeptin in COVID-19 pregnancies would provide an understanding of the effect that COVID-19 infection has on placental formation and functioning. This could possibly be further linked to the multitude of adverse pathological alterations observed in the placentae from COVID-19 pregnancies.

According to studies, histopathological alterations have been documented in the placentae from COVID-19 pregnancies (Heeralall *et al.*, 2023), including signs of maternal and foetal malperfusion (Algarroba *et al.*, 2020, Baergen and Heller, 2020, Hecht *et al.*, 2020, Hosier *et al.*, 2020, Mulvey *et al.*, 2020, Shanes *et al.*, 2020, Sisman *et al.*, 2020, Smithgall *et al.*, 2020, Hsu *et al.*, 2021, Patberg *et al.*, 2021, Schwartz *et al.*, 2021, Watkins *et al.*, 2021, Huynh *et al.*, 2022, Mao *et al.*, 2022, Schwartz *et al.*, 2022). Similarly, some studies also documented inflammatory changes in the placenta (Baud *et al.*, 2020, Facchetti *et al.*, 2020, Mongula *et al.*, 2020, Pulinx *et al.*, 2020, Richtmann *et al.*, 2020, Vivanti *et al.*, 2020, Gao *et al.*, 2021, Giordano *et al.*, 2021, Ikhtiyarova *et al.*, 2021, Jaiswal *et al.*, 2021, Jang *et al.*, 2021, Liu *et al.*, 2021, Menter *et al.*, 2021, Shchegolev *et al.*, 2021, Dubucs *et al.*, 2022, Kato *et al.*, 2022) with no placental expression studies linking hypoperfusion to kisspeptin. Furthermore, these studies were conducted globally, with limited information available on the placental alterations caused by COVID-19 on the African population, particularly in South Africa (Govender *et al.*, 2021).

Therefore, this study aims to assess the expression of kisspeptin, coupled with morphological changes, in the placentae of COVID-19 positive pregnant South African women. To the best

of our knowledge, this is the first study to investigate the impact COVID-19 on kisspeptin levels in pregnant women. These findings have then been further linked to the results from extensive analysis of the morphological structure and alterations in these placentae. The results obtained from the immunohistochemical analysis of kisspeptin together with the histological analysis of the morphology of placentae from COVID-19 positive and negative pregnant women are herein reported.

Material and Methods

Ethics Approval and consent to participate

Regulatory ethical and Institutional approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004591/2022), South Africa. Patients were recruited based on the inclusion and exclusion criteria. Once suitable patients were identified for this study, its purpose and requirements were explained to them. Upon agreeing to participate, all participants signed a consent form.

Study Population (inclusion and exclusion criteria)

The Inkosi Albert Luthuli Central Hospital was under the triage system for COVID-19 diagnoses, and therefore upon arrival, all patients were tested prior to admission. Black South African women over 34 weeks of gestation who were confirmed either COVID-19 positive (n=6 central and n=6 peripheral) and negative (n=6 central and n=6 peripheral) for SARS-CoV-2 RNA via real-time polymerase chain reaction (RT-PCR) on nasopharyngeal swabs were considered for this study. Only those who could provide full consent and did not present with other major co-morbidities such as hypertension, preeclampsia, IUGR, or other major health issues were then recruited due to overlapping pathology. Placental samples were taken from women who had elective caesarean sections. Within 10 minutes of delivery placental tissue were dissected from central and peripheral regions of the placenta. Within ten minutes of delivery, the placental tissue was removed, washed with PBS (1X, pH 7.5), and preserved in 10% phosphate-buffered formalin. These samples were then transported to UKZN for further processing.

Placental tissue processing

A central and peripheral piece (including chorionic and basal plate) of placental tissue (n=24) was collected from COVID-19 positive and negative patients. According to standard laboratory

procedure, all samples were immediately fixed in 10% buffered formalin and embedded into paraffin wax blocks (Burton *et al.*, 2014).

Using a rotary microtome, 3 µm thin sections of placental tissue were floated onto frosted glass slides, de-paraffinized and rehydrated for Haematoxylin and Eosin (H&E), and Masson's Trichrome (MT) staining according to the methodology described in Bancroft's Theory and Practice of Histological Techniques (Fischer *et al.*, 2008, Suvik and Effendy, 2012, Suvarna *et al.*, 2018).

Immunohistochemistry

The slides were incubated overnight at 37 °C, then de-waxed, rehydrated, and blocked with 3% hydrogen peroxide (H2O2) for 15 minutes, washed with distilled water thereafter. Antigen retrieval was then performed using Tris-EDTA buffer for 30 minutes in a pressure cooker. The slides were then washed in TBS and subsequently blocked with normal donkey serum. Incubation with a primary antibody against Kisspeptin ([EPR23770-189] manufactured by ABCAM raised in rabbit) at a dilution of 1: 500 was conducted at 4 °C overnight. The slides were cleaned in TBS the following morning. The Goat Anti-Rabbit Peroxidase (1: 100) secondary antibody (Dako Cat no. P0448) was then applied. After another TBS wash, the slides were incubated for 10 minutes at room temperature with 2 drops/1 ml of the 3,3'-Diaminobenzidine (DAB) substrate chromogen system (DAKO K3468, Agilent, United States). Thereafter the slides were washed again to counterstain with Haematoxylin for 5 minutes, rinsed, dehydrated, and cleared with xylene. Mounting medium was then to the slides along with a coverslip. These slides were then visualised under the microscope and scanned using a Nikon scanner.

Morphometric analysis

The Axioscope A1 microscope (Carl Zeiss, Germany) was used to view placental sections. Using AxioVision software (Carl Zeiss, Germany; version 4.8.3), pictures were taken at 20× objective magnification in four fields of vision each slide. Fiji ImageJ software's colour deconvolution was used to calculate the proportion of immunostaining specific to Kisspeptin antibody expression (Jensen, 2013, Crowe and Yue, 2019). Colour deconvolution is the process of dividing an image's colours into three channels: blue, green, and red. Haematoxylin staining is represented by the blue channel, whereas DAB staining is represented by the red channel. To calculate the Kisspeptin expression percentage, the proportion of DAB staining was divided by the total tissue area.

Statistical analysis

Patient demographics were examined for normality using the D'Agostino and Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests. In order to compare the differences between the control and COVID-19+ve groups, the Unpaired T-Test was used. Statistical significance was defined as a probability threshold of $p = 0.05$. GraphPad Prism 8.4.3 (San Diego, CA) was used for all statistical analyses.

Results

Clinical characteristics

Relevant clinical information about each group, i.e. Control and COVID19+ve are summarized in Table 3 as mean ± standard deviation due to their parametric distribution. Maternal age, maternal weight, systolic blood pressure, diastolic blood pressure, heart rate, and gestational age were non-significantly (ns) different between the Control and COVID19+ve study groups, except for body temperature which was elevated in the COVID19+ve group ($p = 0.460$), as shown in Table 3.

Table 1. Patient demographics of Control and COVID19+ve pregnant women.

	Maternal Age (years)	**Maternal Weight (kg)**	**Systolic BP (mmHg)**	**Diastolic BP (mmHg)**	**Heart Rate (Beats per minute)**	**Body Temperature (°C)**	**Gestational Age (weeks)**	**Birthweight (kg)**	**Neonatal Distress**
Control (n = 6)	30.67 (±6.82)	119.7 (±39.52)	114.2 (±18.33)	73.17 (±19.14)	96.50 (±21.68)	36.17 (±0.21)	37.17 (±1.72)	2.93 (±0.83)	1/6 (16.67 %)
COVID19 +ve (n = 6)	25.50 (±4.64)	99.78 (±36.95)	121.5 (±14.25)	70.17 (±9.22)	81.33 (±14.69)	36.42 (±0.17)	37.00 (±1.90)	2.65 (±0.91)	3/6 (50%)
Significance	ns	ns	Ns	ns	ns	$p = 0.460$ *	ns	ns	

Key: Results are represented as the mean (±SD), * $p < 0.05$

Immuno-localization of placental kisspeptin

The expression of kisspeptin was localized in the central (Fig 10) and peripheral (Fig 11) regions of the placentae obtained from COVID-19 positive and negative pregnant women. The immuno-expression of kisspeptin was significantly increased in both the central ($p = 0.001$) and peripheral ($p < 0.0001$) placental regions from pregnancies with COVID-19 infection as compared to healthy pregnancies.

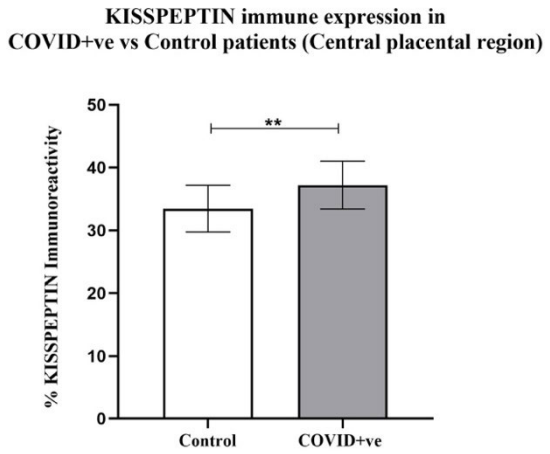

Figure 1 shows the expression of kisspeptin in the central placental region of COVID-19 positive and negative pregnant women.

(n=6 per group)

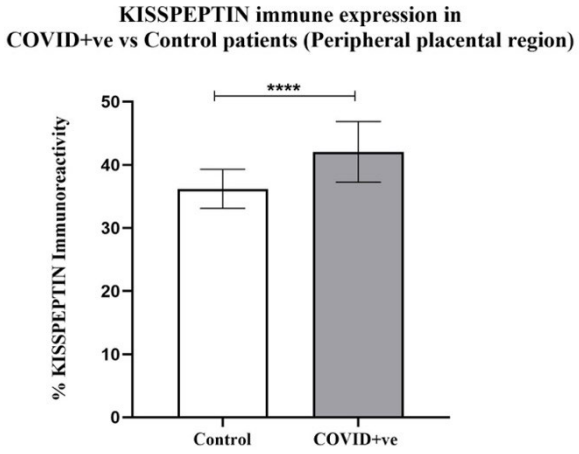

Figure 2 shows the expression of kisspeptin in the peripheral placental region of COVID-19 positive and negative pregnant women.

(n=6 per group)

This is further depicted in Figure 12, where kisspeptin immunostaining is localized to the villous syncytiotrophoblast and cytotrophoblast cell layers of the placental villi which is indicated by DAB (brown) staining, with the nuclei counterstained blue with haematoxylin. More intense and complete kisspeptin immunostaining was observed in the syncytiotrophoblast and cytotrophoblast layers from central and peripheral regions of placentae from pregnancies with COVID-19 infection in comparison to healthy pregnancies.

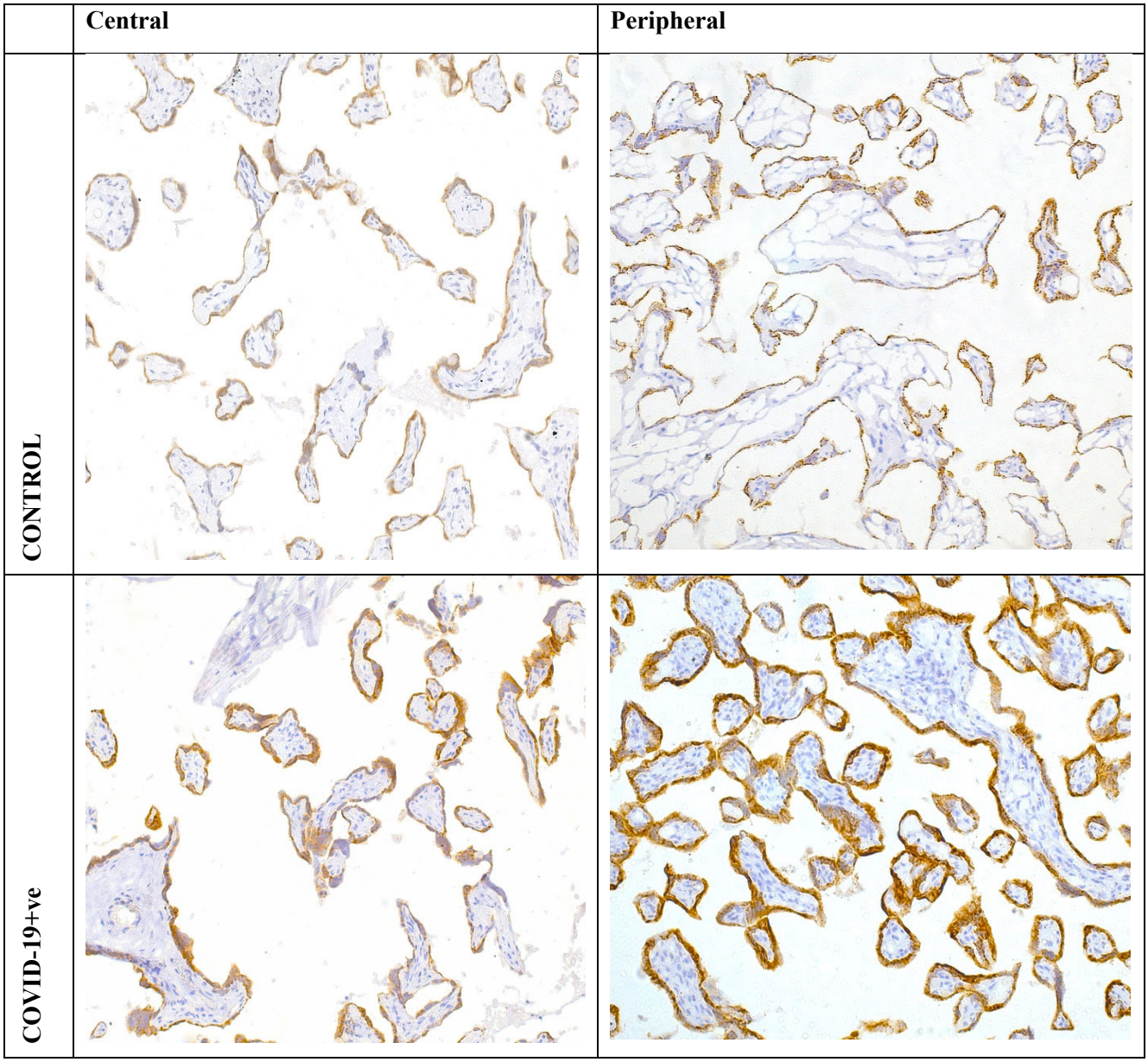

Figure 3 Represents immunohistochemical expression of kisspeptin in the central and peripheral regions of the placentae from both control and COVID-19 positive pregnancies.

The tile scans show kisspeptin staining (brown) of the villous syncytiotrophoblast and cytotrophoblast cell layers, with nuclei (blue) that were counterstained with haematoxylin.

Histopathological features

The histopathological findings in the Control and COVID19+ve groups are shown in Figure 13 and Figure 14 with a comparative evaluation in Table 4. Vascular alteration characteristics indicate maternal vascular malperfusion (MVM) or foetal vascular malperfusion (FVM), also

considering inflammatory lesions. This semi-qualitative evaluation (Table 2) was performed using a grading scale of 0 to 3, where: 0 = no effect → 0%, 1 = mild effect → 1-25%, 2 = moderate effect → 26-50%, 3 = severe effect → >50%.

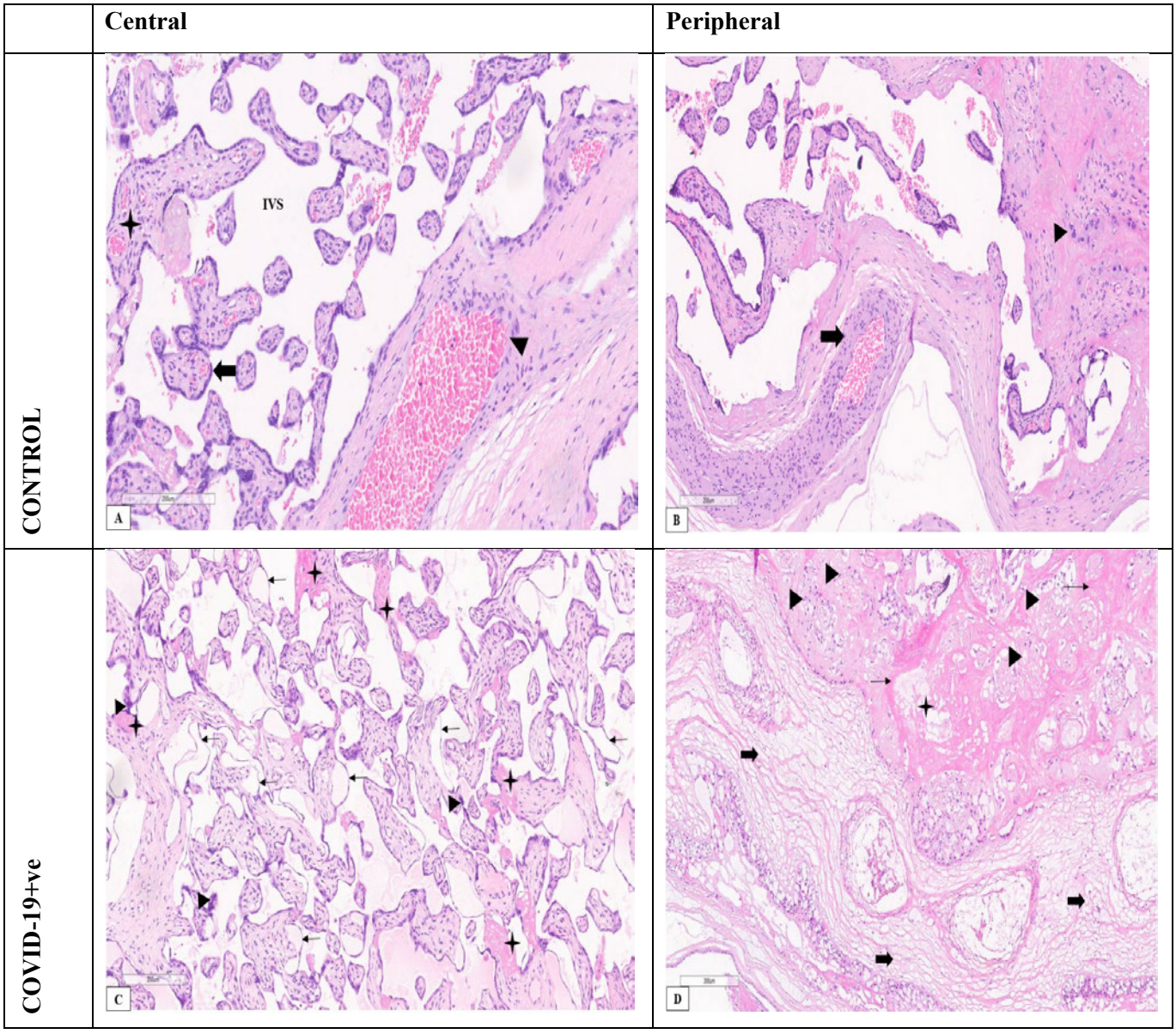

Figure 4 Central and peripheral placenta of control and COVID-19+ve pregnancies, stained with H&E.

Figure 4 A: The image shows cross-sections of the villous tree of the placenta – each chorionic villus is lined with syncytiotrophoblast. Inside the villi, foetal capillaries are observed. The intervillous space (IVS) is shown in white and contains maternal red blood cells. Normal well defined intermediate villi – arrow head. Syncytiotrophoblast layer surrounding villi – block arrow. Normal chorionic villi showing villous core with foetal vessels and stroma – star. Figure 4 B: Normal spiral artery – block

arrow. Decidua with decidual cells characterized as epithelioid and polygonal – arrow head. Figure 4 C: Suggestive increased fibrin deposit with disappearance of nuclear material suggestive of hyalinization – star Hypovascular villi with villous edema and stromal fibrosis. Villi show widespread trophoblast abnormalities with thinning of the villous trophoblast layer – line arrow. Presence of syncytial knots – arrowhead. Figure 4 D: Villous infarction – arrowhead suggestive by Villous necrosis – star with Vascular wall edema – block arrow.

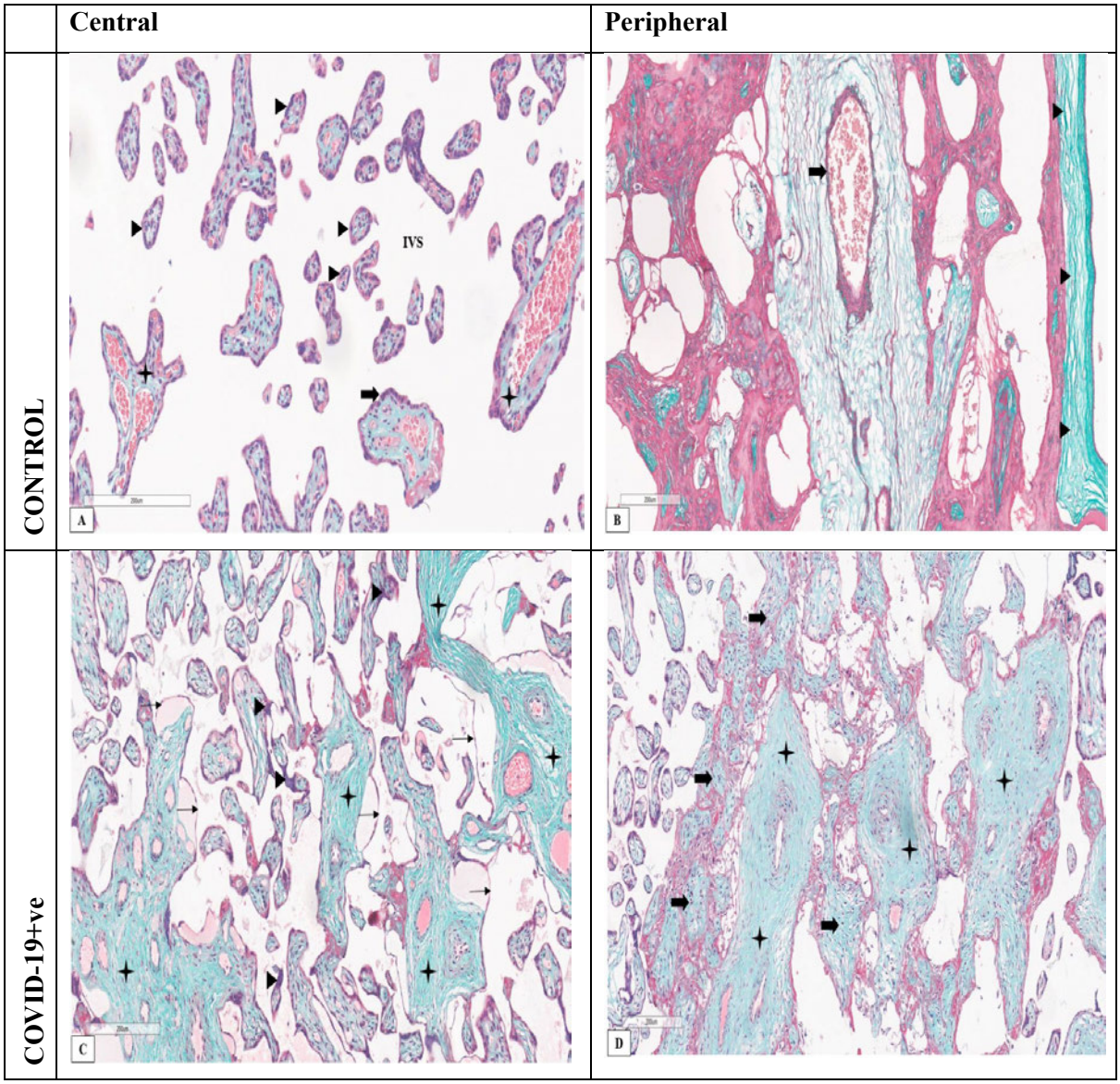

Figure 5 Central and peripheral placenta of control and COVID-19+ve pregnancies, stained with MT.

Figure 5 A: The image depicts cross-sections of the placenta's villous tree, with each chorionic villus lined with syncytiotrophoblast cells. Inside the villi, foetal capillaries are seen. The intervillous space (IVS) is depicted in white and contains maternal red blood cells. Normal well defined terminal villi –

arrowhead. Syncytiotrophoblast layer surrounding villi – block arrow. Normal chorionic villi showing villous core with foetal vessels and stroma – star. Figure 5 B: Normal stem artery – block arrow. Normal chorioamniotic membranes with no evidence of chronic inflammatory cell infiltration in the amnion and choriodecidua – arrowhead. Figure 5 C: Increased fibrin deposit and possible hyalinization of villi – star. Hypovascular villi with villous edema and stromal fibrosis. Villi show widespread trophoblast abnormalities with thinning of the villous trophoblast layer – line arrow. Increased presence of syncytial knots – arrowhead. Figure 5 D: Massive perivillous fibrin deposition is noted with excessive deposition of fibrous tissue around the stem villi of the placenta – star. Hypovascular villi – block arrow.

Table 2. Histopathological evaluation of placenta.

	HISTOPATHOLOGICAL FEATURES		CONTROL						COVID-19 POSITIVE					
			C1	C2	C3	C4	C5	C6	P7	P8	P9	P10	P11	P12
Vascular Alterations	Maternal Vascular Malperfusion (MVM)	Accelerated villous maturation (Increased syncytial knots)	1	1	2	2	0	1	3	3	3	2	3	3
		Increased fibrin deposition	1	1	0	2	2	2	3	2	2	3	3	3
		Decidual arteriopathy	1	1	2	1	3	1	3	3	3	3	3	2
		Intervillous thrombosis	1	1	1	1	0	2	2	2	2	3	2	2
		Retroplacental haemorrhage	0	0	0	0	1	1	0	2	2	0	0	0
		Villous infarction/necrosis	0	0	0	0	0	1	0	3	3	1	2	1
	Foetal Vascular Malperfusion (FVM)	Avascular fibrotic villi	0	1	0	1	2	2	2	2	1	0	2	2
		Distal villous hypoplasia	0	2	1	1	2	0	3	2	2	3	3	3
		Chorangiosis	0	2	1	0	0	1	3	1	1	3	2	3
		Delayed villous maturation (DVM)	0	0	0	0	1	1	1	2	2	1	2	2
		Foetal thrombotic vasculopathy (FTV)	1	2	1	1	2	1	3	2	2	3	3	3
		Intramural fibrin deposition	1	2	0	1	1	2	1	2	3	3	3	3
		Vascular ectasia	1	1	0	0	1	1	3	1	2	3	3	1
		Villous stromal vascular karyorrhexis	0	0	0	1	0	0	2	2	2	0	2	1
Inflammatory Alterations		Chronic Villitis	0	0	0	0	0	0	2	1	1	2	2	2
		Villous Edema	0	1	1	1	0	0	0	3	3	3	2	2

Grading Scale:
0 = no effect → 0%
1 = mild effect → 1-25%
2 = moderate effect → 26-50%
3 = severe effect → >50%

Histopathology of the COVID19+ve placentae demonstrated a higher grade of MVM and FVM with increased villous edema and chronic villitis compared to the control placentae.

Maternal Vascular Malperfusion in COVID19+ve vs. Control

A severe effect of accelerated villous maturation was observed in 5/6 COVID19+ve placentae with moderate effect in 1/6, is indicated by increased syncytial knots and villous agglutination. The control group showed 1/6 with no effect, 3/6 with mild effect and 2/6 with moderate effect. Massive perivillous fibrin deposition is the term used to describe the excessive deposition of fibrous tissue around the chorionic villi of the placenta. A severe effect of increased fibrin deposition was noted in 4/6 COVID19+ve placentae with moderate effect seen in 2/6 in the group. In the control placentae group, moderate (3/6), mild (2/6) and no effect (1/6) of increased fibrin deposition was displayed.

Decidual arteriopathy presented with thickening or fibrinoid necrosis of the vessel wall, endothelial swelling and detachment was noted. Severe effects of decidual arteriopathy was demonstrated in 5/6 COVID19+ve placentae with moderate effects in 1/6 in the group. However, a mild effect of decidual arteriopathy was shown in 4/6, moderate effect in 1/6 and severe effect in 1/6 of the control placentae.

Blood coagulation in the intervillous space can cause several pathologic lesions, the majority of which seem laminated and are known as thrombi. A severe effect of intervillous thrombosis was observed in 1/6 COVID19+ve placenta, whilst a moderate effect was noted in 5/6 placentae. In the control placentae group, moderate (1/6), mild (4/6) and no effect (1/6) of intervillous thrombosis was displayed.

When the placenta detaches over an extensive area and forms a hematoma between the uterine wall and the placenta, it produces retroplacental haemorrhage. A moderate effect of retroplacental haemorrhage was noted in 2/6 COVID19+ve placentae with no effect observed in 4/6 of the group. Similarly, no effect (4/6) of retroplacental haemorrhage with a mild effect in 2/6 control placentae was exhibited. Villi that have undergone ischemic necrosis as a result of a focused reduction in placental (maternal) blood flow create placental parenchymal lesions. A severe effect of villous infarct/necrosis lesions were demonstrated in 2/6 COVID19+ve placentae with a further 1/6 showing moderate effect. Moreover, a mild effect in 2/6 and no effect (1/6) was also seen in the COVID19+ve group. In contrast, a greater number of control placentae (5/6) displayed no effects and 1/6 with mild effects of villous infarct/necrosis lesions.

Foetal Vascular Malperfusion in COVID19+ve vs. Control

Loss of villous vascularity with replacement of the villus core by dense fibroblastic material is known as avascular fibrotic villi. A moderate effect in 4/6 COVID19+ve placentae of avascular fibrotic villi was observed, whilst mild (1/6) and no effect (1/6) was also noted in the group. In the control placentae group, moderate (2/6), mild (2/6) and no effect (2/6) of avascular fibrotic villi was displayed.

A sparse, poorly developed distal villous tree with abnormally shaped, elongated, slender villi and widened intervillous space were the hallmarks of distal villous hypoplasia. The villi displayed widespread trophoblast abnormalities, including an increase in wave-like syncytial knots, a reduction in cytotrophoblast numbers, and thinning of the villous trophoblast layer. There was also evidence of an increase in syncytiotrophoblast nuclear senescence. A severe effect of distal villous hypoplasia was observed in 4/6 COVID19+ve placentae with 2/6 showing moderate effect. No effect (2/6) of distal villous hypoplasia was demonstrated in the control placentae with 2/6 and 2/6 showing mild and moderate effects, respectively. Capillary hyperplasia in the terminal villi is known as chorangiosis, and it results from low grade tissue hypoxia or persistent placental hypoperfusion. A severe effect of chorangiosis was demonstrated in 3/6 COVID19+ve placentae with a further 1/6 showing moderate effect. Moreover, a mild effect of chorangiosis was reported in 2/6 of the COVID19+ve group. However, no effect of chorangiosis was displayed in 3/6 control placentae with 2/6 placentae showing mild effect, and 1/6 moderate effect in the group.

Delayed villous maturation (DVM) is distinguished by decreased tertiary villus production, decreased vasculo-syncytial membrane formation, and, in more severe cases, enlarged bullous villi. Moderate effect of DVM was demonstrated in 4/6 COVID19+ve placentae with mild effect noted in 2/6. In contrast, no effect of DVM was displayed in 4/6 control placentae with only 2/6 placentae showing mild effect.

A vascular thrombotic disorder called foetal thrombotic vasculopathy (FTV) causes blockage of arteries and veins in the placenta's foetal circulation, which leads to ischemic alterations in the villi surrounding the blockage. A severe effect of FTV was demonstrated in 4/6 COVID19+ve placentae with moderate effect noted in 2/6. However, a moderate effect of FTV was demonstrated in 2/6 control placentae with 4/6 placentae showing mild effect.

Intramural fibrin deposition occurs when abnormal amounts of fibrin is deposited in the intima of the vessel thus escalating FVM. A severe effect of intramural fibrin deposition was displayed in 4/6 COVID19+ve placentae with moderate (1/6) and mild (1/6) noted in the group. In the

control placentae group, moderate (2/6), mild (3/6) and no effect (1/6) of intramural fibrin deposition was observed.

Luminal dilatation (vascular ectasia) resulting from elevated venous pressure, is characterized by a pair of large foetal arteries in the chorionic plate or stem villi, one of which has a luminal diameter at least four times greater than the adjacent vessel. A severe effect of vascular ectasia was observed in 3/6 COVID19+ve placentae with moderate (1/6) and mild (2/6) effect also noted in the group. No effect (2/6) of vascular ectasia was demonstrated in the control placentae with 4/6 showing mild effects.

Loss of vascular wall integrity, fragmentation and extravasation of red blood cells in the stroma, and early septation are signs of villous stromal-vascular karyorrhexis. Moderate effect of villous stromal-vascular karyorrhexis was reported in 4/6 COVID19+ve placentae with mild effect displayed in 1/6 and no effect 1/6 of the group. In contrast, no effect of villous stromal-vascular karyorrhexis was exhibited in 5/6 control placentae with only 1/6 placentae showing mild effect.

Inflammatory Lesions of COVID19+ve vs. Control

The classic chronic inflammatory placental lesion known as chronic villitis is defined by the presence of chronic inflammatory cells infiltrating the chorionic villi, which ultimately results in villous agglutination and loss of placental function. A moderate effect of chronic villitis was noted in 4/6 COVID19+ve placentae with a mild effect seen in 2/6. In contrast, no effect of chronic villitis in 6/6 control placentae was reported.

Placental villous edema was identified by locating open spaces within the cytoplasm of intervillous cells and in the interstitium of the villi. A severe effect of villous edema was reported in 3/6 COVID19+ve placentae with moderate effect seen in 2/6, and no effect (1/6) within the group. However, control placentae group showed no effect (3/6) and mild effect (3/6) of villous edema.

Discussion

The findings from this study corroborate that COVID-19 had a severe pathogenic impact on the morphology of the placenta, which may be indicative of impaired placental function. A significant elevation in the expression of kisspeptin was observed in the central and peripheral regions of the placentae from COVID-19 positives pregnancies in this study when compared to COVID-19 negative pregnancies. The placentae from these pregnancies with COVID-19 infection further presented with vascular and inflammatory alterations.

Kisspeptin has an essential role in trophoblast invasion, angiogenesis and placentation, hence any alterations in its expression could result in adverse pregnancy outcomes or placental dysfunction (Kapustin *et al.*, 2020). This is due to kisspeptin inhibiting the matrix metalloproteinase-9 (MMP9) expression in the placenta, which negatively impacts the maturation and invasion; thus, the elevated expression of KISS can consequently result in gestational complications (Hu *et al.*, 2019a, Kleimenova *et al.*, 2019, Zhang *et al.*, 2011). Elevations in placental kisspeptin have therefore been linked to inadequate trophoblast invasion, which is inhibited by kisspeptin (Cartwright and Williams, 2012, Matjila *et al.*, 2016). Hence, it is evident from the results reported herein that COVID-19 has a significant impact on placental functioning via altering the placental kisspeptin expression, which could possibly impact sufficient trophoblast invasion in these pregnancies. To the best of our knowledge, these results are yet to be supported by other studies. However, studies on preeclampsia, gestational diabetes mellitus, and preterm birth have also noted similar elevations in the placental expression of kisspeptin (Matjila *et al.*, 2016, Kapustin *et al.*, 2020, Zhang *et al.*, 2011, Vodneva *et al.*, 2014, Qiao *et al.*, 2012, Torricelli *et al.*, 2008). We believe this alteration in kisspeptin could be linked to the inflammation observed in COVID-19 pregnancies. Furthermore, the cytokine profile was noted to be altered in the plasma and exosomes of the current cohort where we observed a pro-inflammatory state (unpublished data). KISS-1 systems' signalling is susceptible to inflammatory conditions hence this environment could disrupt its signalling accounting for the altered kisspeptin expression observed in our study (Iwasa *et al.*, 2008). We believe that the placental dysfunction observed in COVID-19 pregnancies could be attributed to the kisspeptin alterations observed in this study and could have further manifested as morphological alterations.

The alteration in this key protein was further supported by the placental histopathological changes observed in the placentae from COVID-19 positive pregnancies in the South African cohort. Signs of morphological alterations indicative of maternal and foetal vascular

malperfusion were evident in all the COVID-19 positive placentae when compared to placentae from COVID-19 negative pregnancies. This was identified in the South African cohort, through the increased presence of pathological changes, including increased fibrin deposition, intervillous thrombosis, necrosis, chorangiosis, and foetal thrombotic vasculopathy when compared to the control group. Other South African studies also noted alterations in most of the COVID-19 positive placentae analyzed in their studies (Vannevel *et al.*, 2021, Ramphal *et al.*, 2022, Nunes *et al.*, 2022). Similar observations have been noted in other countries, including Switzerland, Italy, USA, India, Netherlands, Belgium, Brazil, France, China, Korea and Uzbekistan, whereby an increased prevalence of MVM and FVM in COVID-19 placentae was also identified when compared to controls (Baud *et al.*, 2020, Facchetti *et al.*, 2020, Hecht *et al.*, 2020, Mongula *et al.*, 2020, Pulinx *et al.*, 2020, Richtmann *et al.*, 2020, Shanes *et al.*, 2020, Sisman *et al.*, 2020, Vivanti *et al.*, 2020, Gao *et al.*, 2021, Ikhtiyarova *et al.*, 2021, Baergen and Heller, 2020, Londhe *et al.*, 2024, Tripathy *et al.*, 2024, Mulvey *et al.*, 2020, Smithgall *et al.*, 2020, Jaiswal *et al.*, 2021, Jang *et al.*, 2021, Menter *et al.*, 2021, Patberg *et al.*, 2021, Watkins *et al.*, 2021, Dubucs *et al.*, 2022).

In addition to the increased prevalence of malperfusion, a significant increase in inflammation was observed in all the COVID-19 placentae which presented with severe chronic villitis, and villous edema compared to the placentae from the control group. This could possibly be a result of the altered pro-inflammatory cytokine profile noted in the South African cohort from this study (unpublished data). Similar findings were also recorded worldwide in the Italian, American, Brazilian, Chinese, Korean, Swiss, French, Indian, Indonesian, and Turkish population confirming that COVID-19 results in inflammatory and vascular alterations (Facchetti *et al.*, 2020, Hecht *et al.*, 2020, Richtmann *et al.*, 2020, Gao *et al.*, 2021, Jang *et al.*, 2021, Menter *et al.*, 2021, Dubucs *et al.*, 2022, Huynh *et al.*, 2022, Garg *et al.*, 2023, Milot *et al.*, 2023, Altuntaş *et al.*, 2024, Ryan *et al.*, 2024, Umamaheswari *et al.*, 2024, Wardhana *et al.*, 2024).

The altered placental kisspeptin expression, increased vascular and inflammatory alterations observed in this study can be further linked to the increase in distress and death in neonates from mothers infected with COVID-19. This study notes that 3 of 6 (50%) neonates from COVID-19 positive mothers experienced distress compared to only 1 of 6 (16.67%) neonates from COVID-19 negative mothers. Furthermore, 3 neonates from COVID-19 positive pregnancies were born premature or presented with abnormalities and died, whilst no abnormalities or deaths were noted in neonates from COVID-19 negative pregnancies.

Similarly, an increased prevalence of alterations was reported in the COVID-19 positive placentae from HIV+ patients when compared to HIV- patients in the South African cohort, which was further reported to be associated with preterm delivery (Barbera *et al.*, 2021, Ramphal *et al.*, 2022, Nunes *et al.*, 2022). Furthermore, this population could have been more susceptible to COVID-19 complications due to the increased prevalence of social and healthcare issues which could have exacerbated the effects of COVID-19 (Lone and Ahmad, 2020b).

Therefore, from the results presented herein we believe that the altered cytokine profile observed in this population impacted kisspeptin signaling which was demonstrated through increased placental kisspeptin expression in COVID-19 positive pregnancies. This alteration could have impacted trophoblast invasion suggesting that spiral artery conversion is impacted, along with blood flow which could lead to poor placental perfusion and hypoxia. These conditions along with hyperinflammation could be the reason for the placental dysfunction observed through the altered histopathology in the COVID-19 placentae. However, the neonates from these pregnancies remain a concern as it is clear that they were exposed to environments that were not conducive for optimal development and growth. Studies have already reported complications impacting on foetal cardiac function and morphology in infants from these pregnancies affected by COVID-19 (Zhu *et al.*, 2024).

Conclusion

This study demonstrates that placental development and function are impacted by COVID-19 infection during pregnancy due to the potential influence of altered placental kisspeptin expression. This alteration could be underlying cause for the vascular and inflammatory morphological alterations observed in the COVID-19+ve placentae in this study. This study suggests a plausible link between elevated placental kisspeptin expression with histopathological alterations and adverse neonatal outcomes.

Limitations

During the COVID-19 pandemic's last wave, the collecting site still followed strict regulations, and access to patients was restricted, thereby contributing to a limited sample size. Nevertheless, our findings provide valuable insights into the critical role that kisspeptin plays in COVID-19 pregnancies and highlights the importance of conducting more research within the South African population to deepen our understanding of its implications.

Future Recommendations

The long-term effects of COVID-19 in pregnancies remain poorly elucidated, necessitating further investigation into potential impacts. Investigating placentation in COVID-19 pregnancies is essential in understanding pathology, identifying adverse outcomes, and exploring potential therapeutic interventions in managing these complications. In particular, more focussed research should be conducted on kisspeptin in COVID-19 positive pregnancies. Additionally, it is vital for the mothers and neonates from these pregnancies, to be rigorously monitored so that any detrimental outcomes as a result of COVID-19 can be identified and treated swiftly minimizing long-term consequences in these patients.

Ethics Approval and Consent to participate

Regulatory ethical and Institutional approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00004591/2022), South Africa. Patients were recruited based on the inclusion and exclusion criteria. Once they were identified, the purpose and requirements of this study were explained to them. Upon agreeing to participate, all participants signed a consent form.

Availability of data and material

Data will be made available on request.

Declaration of competing interest

The authors declare that there is no conflict of interest

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Consent for publication

Not applicable

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Authors contributions

C Heeralall: Conceptualization, methodology, writing of the original draft, data curation and revision of the manuscript. U H Ibrahim: Writing the original draft and data curation. M Jenneker: Methodology. S Singh: Methodology and data curation. Mushi Matjila: Methodology. Lelika Lazarus: Supervision and reviewing of the manuscript. Irene Mackraj: Conceptualization, supervision, reviewing and editing of the manuscript.

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5.0 CHAPTER FIVE

SYNTHESIS AND CONCLUSION

5.1 Synthesis

The year 2019 proved to be significant due to the identification of SARS-CoV-2, which impacted the world through its severe mortality rate (Yuki *et al.*, 2020). This pandemic presented a substantial threat to older, immunocompromised, and pregnant individuals (Simbar *et al.*, 2023). Inflammation is considered one of the greatest concerns, as it remains the leading cause for premature delivery and pregnancy loss (Ragab *et al.*, 2020, Vesce *et al.*, 2022). This raised further concerns during the pandemic due to COVID-19's strong association with the cytokine storm (Ragab *et al.*, 2020, Vesce *et al.*, 2022). This storm, along with the hypoxic conditions related to the virus, is of importance as it could affect proper placental development and functioning (Cavezzi *et al.*, 2020). Kisspeptin has been recognized as an essential regulator in this process, with alterations being linked to placental dysfunction in preeclampsia, preterm birth, but its relationship to COVID-19 had not been explored (Vodneva *et al.*, 2014, Tsoutsouki *et al.*, 2022). Hence, the assessment of the cytokine profile, placental development and functioning was critical. This study is the first to report altered kisspeptin levels in COVID-19 pregnancies, thus possibly providing a link to the cytokine storm, and to vascular and inflammatory placental alterations observed globally. Therefore, the data presented in this study forms the basis of evidence for possible altered placental development and functioning in COVID-19 pregnancies.

5.1.1 Assessing the impact of COVID-19 on the placental morphology

The review conducted in this study has focused on highlighting the impact COVID-19 had on placental morphology in detail. This review has critically evaluated the pathophysiological impact of COVID-19 on the placenta by reviewing the histopathological changes observed. These vascular and inflammatory alterations reported worldwide were then further linked to the gestational outcomes and complications that have occurred, including the increased prevalence of preeclampsia and preterm births. The implications of the COVID-19 vaccine and therapeutic interventions on pregnancy have elucidated in this study, with thoughts and recommendations for future studies to consider. From this review, it is evident that COVID-19

has severely impacted pregnancies by predisposing women to complications and altering the placental pathology. This is what formed the basis for our subsequent experiments.

Thereafter, the impact of contracting COVID-19 during pregnancy on the cytokine profile was examined due to the inflammatory nature of this virus, possibly life-threatening to pregnant women (Rosen *et al.*, 2022). In addition, the review has hypothesized that any alterations in this immune response could possibly be linked to the placental dysfunction observed in COVID-19 pregnancies. As a result, the second aim of this study was undertaken, which was to identify how pregnant South African women were impacted by severe immune dysregulation in the form of a cytokine storm and to identify if EVs played a role in this storm. It has been of particular interest to investigate this impact within the context of the Black South African cohort due to the immunocompromised nature within this cohort, as a result of the increased prevalence of HIV and preeclampsia.

5.1.2 Evaluation of the cytokine profile in the plasma and EVs

The cytokine profile in the plasma and EVs of the pregnant Black South African women were investigated in Manuscript Two of this study **(Chapter Three- Aim Two)**. Notably, pregnancy is considered an inflammatory state, with cytokines aiding in numerous processes for optimal functioning (Phoswa and Khaliq, 2020, Zanza *et al.*, 2022). However, dysfunction arises when they are produced in excess, thus resulting in the cytokine storm (Phoswa and Khaliq, 2020, Zanza *et al.*, 2022). EVs have also been linked to this storm due to their ability to release cytokines, which possibly contribute to dysfunction in the immune response (Jung *et al.*, 2020). Furthermore, EVs and their role in pregnancy have become of great relevance due to their association with viral transmission, with alterations in their cargo being linked to gestational diseases, thereby making them pertinent in COVID-19 pregnancies (Konadu *et al.*, 2015, Babaei *et al.*, 2022). A dysregulated immune response has been reported in pregnancies worldwide so in the South African cohort, it was critical to assess the cytokine profile in the plasma and EVs from these COVID-19 pregnancies.

The quantification of plasma and EV proinflammatory cytokines and chemokines (IL-6, IFN-γ, TNF-α, and MIP-1α) were selected for investigation due to their apparent link and role in critical gestational processes and the cytokine storm (Fenizia *et al.*, 2020). Analysed data from this study indicated increased IL-6, IFN-γ, TNF-α, and MIP-1α levels in the plasma and EVs content, together with EVs fraction level of IFN-γ levels in COVID-19-positive pregnant South

African women. This suggests that the cytokine profile is impacted in both the plasma and EVs of pregnant women, importantly linking EVs to COVID-19 pathogenesis in pregnancy. Such an imbalance in the cytokine profile is indicative of a pro-inflammatory state in COVID-19-positive pregnancies. This is concerning due to hyperinflammation being linked to the predisposition of gestational complications, including IUGR, preterm birth, preeclampsia, stillbirth, and further dangers to foetal development (Fenizia *et al.*, 2020, Rosen *et al.*, 2022). Therefore, the findings from this study could be further linked to the increased prevalence of low birthweight, neonatal distress, foetal abnormalities and death observed in COVID-19 positive pregnancies. Furthermore, this is suggestive that COVID-19 infection during pregnancy results in an imbalance in Th1 and Th2 responses. The proinflammatory Th1–type immune response is considered to be incompatible with pregnancy, leading to concerns for optimal placental development and functioning in these pregnancies (Raghupathy, 1997, Vesce *et al.*, 2022). This proinflammatory state also increases the risk of cytokine passing through the placenta, suggesting that this altered cytokine profile observed in COVID-19 pregnancies is linked to the placental alterations observed worldwide. This study suggests that this hyperinflammatory state has the ability to impact the placenta, as well as its development and functioning, and ultimately to foetal health.

5.1.3 Elucidating the impact of COVID-19 on placental functioning and development in the South African cohort

The effect of COVID-19 on pregnant women has been significant, as this virus made mothers more susceptible to complications due to exposure to hypoxic and inflammatory conditions (Cavezzi *et al.*, 2020, Granja *et al.*, 2021). Subsequently, studies globally have reported that COVID-19 infection during pregnancy results in vascular and inflammatory placental alterations. In some of these studies, signs of inflammation and malperfusion were documented in the placentae from these pregnancies (Jamieson and Rasmussen, 2021, Wastnedge *et al.*, 2021). However, the mechanism through which COVID-19 could have potentially caused these alterations had not yet been investigated in South Africa (Dubucs *et al.*, 2022, Londhe *et al.*, 2024). Therefore, Manuscript Three **(Chapter Four- Aim 3)** of this study has focused on how placental development and functioning were impacted by COVID-19 infection. It also assessed any placental morphological alterations that could have emerged in the South African population.

The present study hypothesizes that placental development could have been impacted by COVID-19 infection during pregnancy, thereby resulting in impaired placental functioning. This subsequently resulted in assessment of kisspeptin in the placentae from COVID-19 pregnancies as kisspeptin plays a critical role in implantation, thereby influencing placental development and functioning (Hu *et al.*, 2019a). This study found a significantly altered expression in kisspeptin levels in placentae from COVID-19 pregnancies using immunohistochemistry. This observation suggests COVID-19's direct involvement in dysregulating key gestational processes during placental development, which is instrumental for proper vascular remodelling, oxygenation of the foetus, and essentially a safe, healthy pregnancy. These alterations, therefore, illustrate that COVID-19 may have predisposed mothers to placental dysfunction via altering kisspeptin levels. Such alterations have also been linked to preterm birth, preeclampsia, IUGR, and miscarriage, thereby further indicating that COVID-19 could increase the risk of developing these gestational complications. Our findings included observations that 50% of the neonates from COVID-19 positive pregnancies were born prematurely or presented with abnormalities and died, whilst no abnormalities or deaths were noted in neonates from COVID-19 negative pregnancies. These findings also suggest that altered kisspeptin levels may be the underlying mechanism for the placental dysfunction observed in other cases, which also manifested as vascular and inflammatory alterations in the South African population. Importantly, these alterations in kisspeptin levels could therefore, be the underlying cause for the foetal anomalies that are currently arising as a result of placental dysfunction observed in these pregnancies. These observations can also be useful for therapeutic interventions for these infants in later years.

This study shows that COVID-19 altered the cytokine profile in South African pregnancies, possibly impacting placental development by altered kisspeptin levels, consequently affecting optimal placental functioning. This could be why extensive alterations in the placental morphology were observed indicating that the placenta was not functioning optimally in the COVID-19 pregnancies.

5.2 Conclusion

The findings presented have identified an altered cytokine profile in pregnancies impacted by the COVID-19 infection in a South African cohort. This presented as an increase in IFN gamma, IL-6, MIP-1 alpha, and TNF alpha plasma and EV content levels. Further investigation revealed an elevated EV fraction level of IFN-γ in response to the COVID-19 infection during

pregnancy. In addition, this study is the first to report elevated kisspeptin expression in the placentae from COVID-19-positive pregnancies in South Africa. Such an imbalance in kisspeptin levels is associated with placental development and functioning. Therefore, this is linked to the vascular (intervillous thrombosis, increased fibrin deposition, chorangiosis) and inflammatory (chronic villitis, villous edema) alterations observed in these placentae, verifying impaired placental functioning. This could be further linked to the elevated pro-inflammatory cytokine profile identified in the plasma and EVs of these mothers. Importantly, the impaired placental functioning observed in COVID-19 pregnancies in this study sheds light on why such severe morphological alterations were present. This altered placental functioning observed in COVID-19 pregnancies in this study suggests that foetal development could be impacted in these pregnancies. These findings could therefore be linked to the foetal abnormalities and neurodevelopmental issues that are arising. Therefore, the observations made in this study warrant investigation into the health of these neonates from COVID-19 pregnancies. Children from such pregnancies must be monitored for any neurodevelopmental disorders that can arise according to our observations. Furthermore, our findings could be utilised to establish prognostic, diagnostic, and therapeutic interventions for future impending viral pandemics.

5.3 Recommendations

Future research should focus on elucidating the mechanisms through which these alterations observed in COVID-19 pregnancies could impact foetal neurodevelopment. Understanding these mechanisms will be crucial for developing future targeted therapies to prevent and treat emerging foetal abnormalities that are now arising. Additionally, ongoing monitoring of infants born from these pregnancies is recommended to enable early detection and intervention for any developmental concerns. Furthermore, studies investigating the angiogenesis in these pregnancies are already underway, providing valuable insight into how impaired placental development influences vascular formation.

5.4 Limitations of the study

Even though the present study concurs with other studies that COVID-19 impacts the cytokine profile, placental development and functioning in South African pregnancies, further validation in a larger patient cohort is required, as this study experienced challenges in obtaining samples during the last wave of COVID-19. Nevertheless, this study has formed a foundation for establishing the severity of the impact of COVID-19 on South African pregnancies, and has shed light on the challenges faced by these women during a pandemic in a developing country.

5.5 Summary of Findings

- COVID-19 infection during pregnancy significantly impacts the pro-inflammatory cytokine profile in the South African cohort by altering plasma and extracellular vesicle cytokine levels.
- COVID-19 increases the IL-6, IFN-γ, TNF-α, and MIP-1α levels in the plasma and EVs content of pregnant women.
- COVID-19 infection during pregnancy significantly increases the IFN-γ EV fraction levels.
- COVID-19 infection does not impact the average particle size and concentration of extracellular vesicles in pregnant women.
- COVID-19 increases the placental kisspeptin expression with implications on placental development and function. As a result, vascular and inflammatory alterations are present in the placentae from COVID-19 pregnancies.
- These findings are possibly linked to the increased prevalence of premature birth, neonatal distress, foetal abnormalities and death in pregnant women with COVID-19
- Infants from these pregnancies need to be monitored in the future.

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6.0 CHAPTER SIX

APPENDICES

6.1 Human Ethics Approval

21 December 2022

Miss Chanel Heeralall (214528607)
School Of Laboratory Medicine & Medical Science
Westville

Dear Miss Heeralall,

Protocol reference number: BREC/00004591/2022
Project title: Detection of histopathological changes, vascular changes and novel biomarkers in COVID-19 positive pregnancies in black South African women
Degree: PhD

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 21 December 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 21 December 2022. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 14 February 2023.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

6.1.1 Human Ethics Approval Recertification

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

29 December 2023

Miss Chanel Heeralall (214528607)
School of Laboratory Medicine & Medical Science
Westville

Dear Miss Heeralall,

Protocol reference number: BREC/00004591/2022
Project title: Detection of histopathological changes, vascular changes and novel biomarkers in COVID-19 positive pregnancies in black South African women
Degree: PhD

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**21 December 2023**
Expiration of Ethical Approval:	**20 December 2024**

I wish to advise you that your application for recertification received on for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC). The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 13 February 2024.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

6.2 Hospital Approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

Amended letter
17 June 2021

Prof I Mackraj
Department of Human Physiology
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear I Mackraj

PROTOCOL: Characterizing Trophoblastic Debris in Pregnant Women.
REF: BE036/12

We wish to advise you that your response to queries to BREC letter dated 10 June 2021 has been noted by a subcommittee of the Biomedical Research Ethics Committee. Your application for amendments listed below received on 11 May 2021 for the above study has now been **approved** by a subcommittee of the Biomedical Research Ethics Committee.

Amendments noted and approved:

1. Addition of a sepsis cohort.
2. Additional Investigators: Ms R Bhagwan (PhD Student) and Ms C Heeralall (PhD)
3. Addition of Pregnant women who are COVID Positive
4. To include the testing of placenta for changes as outlined.

The committee will be notified of the above approval at its next meeting to be held on 13 July 2021.

Yours sincerely

[redacted]

.....
Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

KWAZULU-NATAL PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

DIRECTORATE:

INKOSI ALBERT LUTHULI CENTRAL HOSPITAL
OFFICE OF THE MEDICAL MANAGER
Private Bag X03, Mayville, 4058
800 Vusi Mzimela (Bellair) Road, Mayville,4091
Tel: 031 240 1059 Fax: 031 240 1005 Email: Ursula.john@ialch.co.za

Reference: BE 036/12
Enquiries: Medical Management

1 July 2021

Prof I Mackraj
Department of Human Physiology
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Prof Mackraj

RE: PERMISSION TO CONDUCT RESEARCH AT IALCH

I have pleasure in informing you that permission has been granted to you by the Medical Manager to conduct research on: **Characterizing Trophoblastic Debris in Pregnant Women.**

Kindly take note of the following information before you continue:

1. Please ensure that you adhere to all the policies, procedures, protocols and guidelines of the Department of Health with regards to this research.
2. This research will only commence once this office has received confirmation from the Provincial Health Research Committee in the KZN Department of Health.
3. Kindly ensure that this office is informed before you commence your research.
4. The hospital will not provide any resources for this research.
5. You will be expected to provide feedback once your research is complete to the Medical Manager.

Y[redacted]
...
Dr L P Mtshali
Medical Manager

KWAZULU-NATAL PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

DIRECTORATE:

INKOSI ALBERT LUTHULI CENTRAL HOSPITAL
OFFICE OF THE MEDICAL MANAGER
Private Bag X03, Mayville, 4058
800 Vusi Mzimela (Bellair) Road, Mayville,4091
Tel: 031 240 1059 Fax: 031 240 1005 Email: Ursula.john@ialch.co.za

1 July 2021

Prof I Mackraj
Department of Human Physiology
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Dear Prof Mackraj

Re: Approved Research: Ref No: BE 036/12: Characterizing Trophoblastic Debris in Pregnant Women.

As per the policy of the Provincial Health Research Committee (PHRC), you are hereby granted permission to conduct the above mentioned research once all relevant documentation has been submitted to PHRC inclusive of Full Ethical Approval.

Kindly note the following.

1. The research should adhere to all policies, procedures, protocols and guidelines of the KwaZulu-Natal Department of Health.
2. Research will only commence once the PHRC has granted approval to the researcher.
3. The researcher must ensure that the Medical Manager is informed before the commencement of the research by means of the approval letter by the chairperson of the PHRC.
4. The Medical Manager expects to be provided feedback on the findings of the research.
5. Kindly submit your research to:

   The Secretariat
   Health Research & Knowledge Management
   ɔalle Street, Pietermaritzburg, 3200
   X9501, Pietermaritzburg, 3201
   -3123, Fax 033394-3782
   Email: hrkm@kznhealth.gov.za

Yours faithfully
[redacted].....
Dr L P Mtshali
Medical Manager

6.3 South African Department of Health Approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

Physical Address: 330 Langalibalele Street, Pietermaritzburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3189/ 3123 Fax: 033 394 3782
Email:
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management

NHRD Ref: KZ_202107_008

Dear Prof I. Mackraj
(UKZN)

Approval of research

1. The research proposal titled **'Characterising Trophoblastic Debris and biomarkers in a Pregnancy and a normal cohort'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at Inkosi Albert Luthuli Central Hospital.

2. You are requested to take note of the following:
   a. *All research conducted in KwaZulu-Natal must comply with government regulations relating to Covid-19. These include but are not limited to: regulations concerning social distancing, the wearing of personal protective equipment, and limitations on meetings and social gatherings.*
   b. *Kindly liaise with the facility manager BEFORE your research begins in order to ensure that conditions in the facility are conducive to the conduct of your research. These include, but are not limited to, an assurance that the numbers of patients attending the facility are sufficient to support your sample size requirements, and that the space and physical infrastructure of the facility can accommodate the research team and any additional equipment required for the research.*
   c. *Please ensure that you provide your letter of ethics re-certification to this unit, when the current approval expires.*
   d. *Provide an interim progress report and final report (electronic and hard copies) when your research is complete to* ***HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200*** *and e-mail an electronic copy to* hrkm@kznhealth.gov.za
   e. *Please note that the Department of Health shall not be held liable for any injury that occurs as a result of this study.*

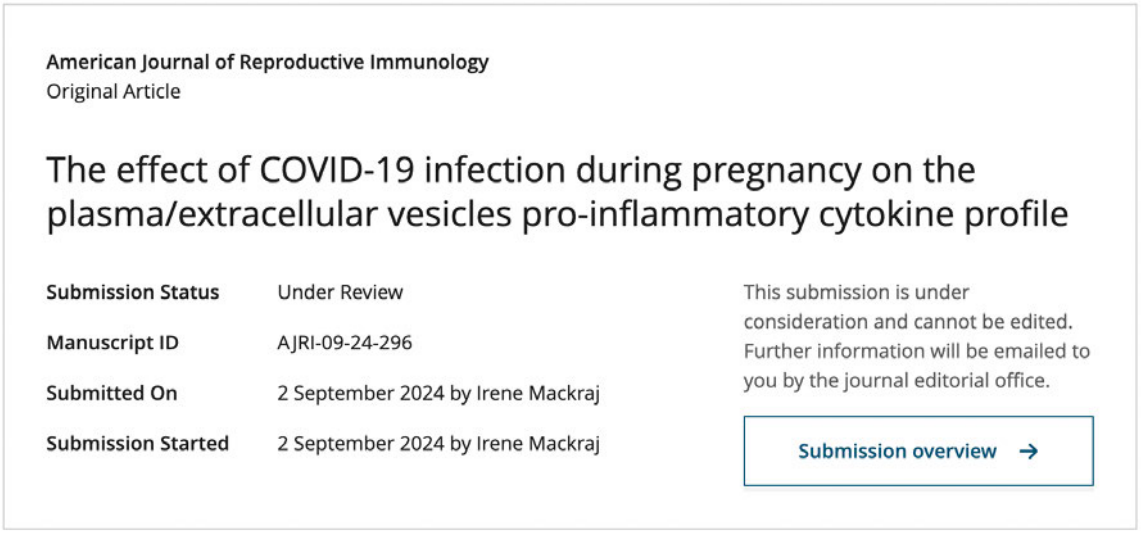
For any additional information please contact Mr X. Xaba on 033-395 2805.

Y[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 29/07/2021

Fighting Disease, Fighting Poverty, Giving Hope

6.4 Manuscript 2 (chapter three) submission confirmation



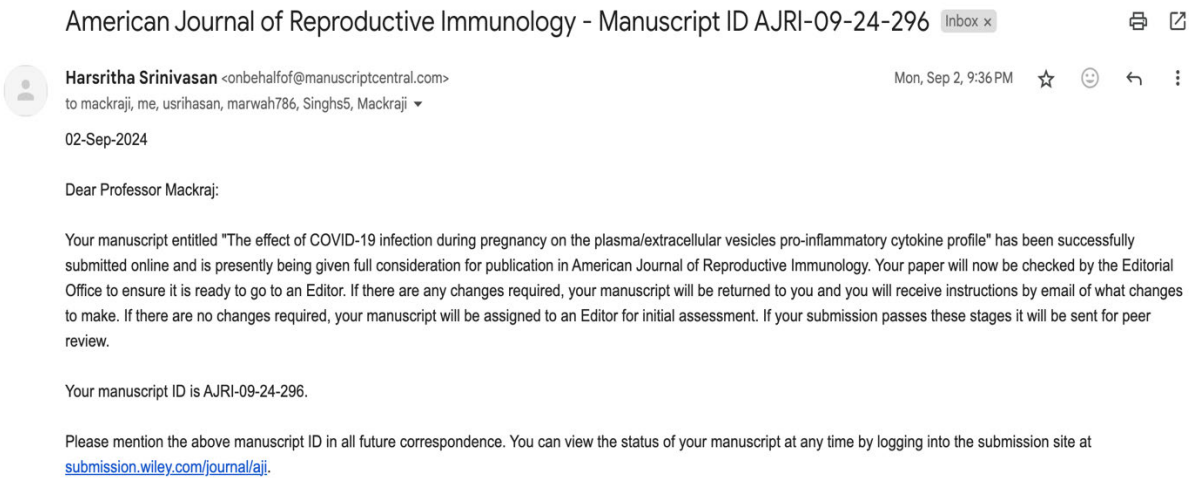
American Journal of Reproductive Immunology
Original Article

The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile

Submission Status	Under Review
Manuscript ID	AJRI-09-24-296
Submitted On	2 September 2024 by Irene Mackraj
Submission Started	2 September 2024 by Irene Mackraj

This submission is under consideration and cannot be edited. Further information will be emailed to you by the journal editorial office.

Submission overview →



American Journal of Reproductive Immunology - Manuscript ID AJRI-09-24-296 Inbox ×

Harsritha Srinivasan <onbehalfof@manuscriptcentral.com> Mon, Sep 2, 9:36 PM
to mackraji, me, usrihasan, marwah786, Singhs5, Mackraji

02-Sep-2024

Dear Professor Mackraj:

Your manuscript entitled "The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile" has been successfully submitted online and is presently being given full consideration for publication in American Journal of Reproductive Immunology. Your paper will now be checked by the Editorial Office to ensure it is ready to go to an Editor. If there are any changes required, your manuscript will be returned to you and you will receive instructions by email of what changes to make. If there are no changes required, your manuscript will be assigned to an Editor for initial assessment. If your submission passes these stages it will be sent for peer review.

Your manuscript ID is AJRI-09-24-296.

Please mention the above manuscript ID in all future correspondence. You can view the status of your manuscript at any time by logging into the submission site at submission.wiley.com/journal/aji.

American Journal of Reproductive Immunology
Original Article

The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile

Submission Status	Under Review (Revision 1)
Manuscript ID	AJRI-09-24-296.R1
Revised On	19 February 2025 by Irene Mackraj
Submitted On	2 September 2024 by Irene Mackraj

This submission is under consideration and cannot be edited. Further information will be emailed to you by the journal editorial office.

Submission overview →

American Journal of Reproductive Immunology - Manuscript ID AJRI-09-24-296.R1 Inbox ×

Harsritha Srinivasan <onbehalfof@manuscriptcentral.com> 5:52 AM (13 hours ago)
to mackraji, me, usrihasan, marwah786, Singhs5, ramsaroopl, Mackraji

19-Feb-2025

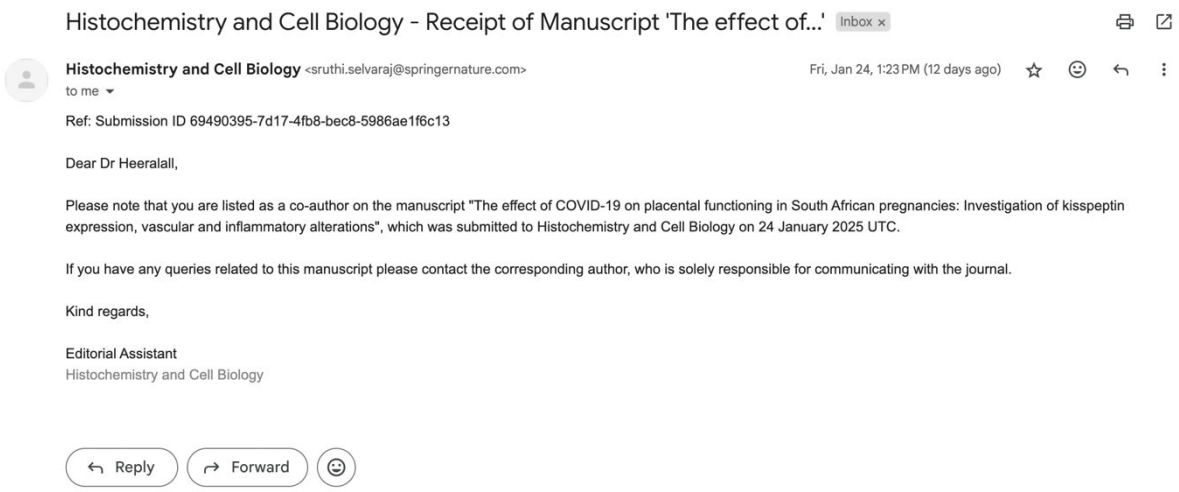
Dear Professor Mackraj:

Your manuscript entitled "The effect of COVID-19 infection during pregnancy on the plasma/extracellular vesicles pro-inflammatory cytokine profile" has been successfully submitted online and is presently being given full consideration for publication in American Journal of Reproductive Immunology. Your paper will now be checked by the Editorial Office to ensure it is ready to go to an Editor. If there are any changes required, your manuscript will be returned to you and you will receive instructions by email of what changes to make. If there are no changes required, your manuscript will be assigned to an Editor for initial assessment. If your submission passes these stages it will be sent for peer review.

Your manuscript ID is AJRI-09-24-296.R1.

Please mention the above manuscript ID in all future correspondence. You can view the status of your manuscript at any time by logging into the submission site at submission.wiley.com/journal/aji.

6.5 Manuscript 3 (chapter four) submission confirmation



Histochemistry and Cell Biology - Receipt of Manuscript 'The effect of...' Inbox ×

Histochemistry and Cell Biology <sruthi.selvaraj@springernature.com> Fri, Jan 24, 1:23 PM (12 days ago)
to me

Ref: Submission ID 69490395-7d17-4fb8-bec8-5986ae1f6c13

Dear Dr Heeralall,

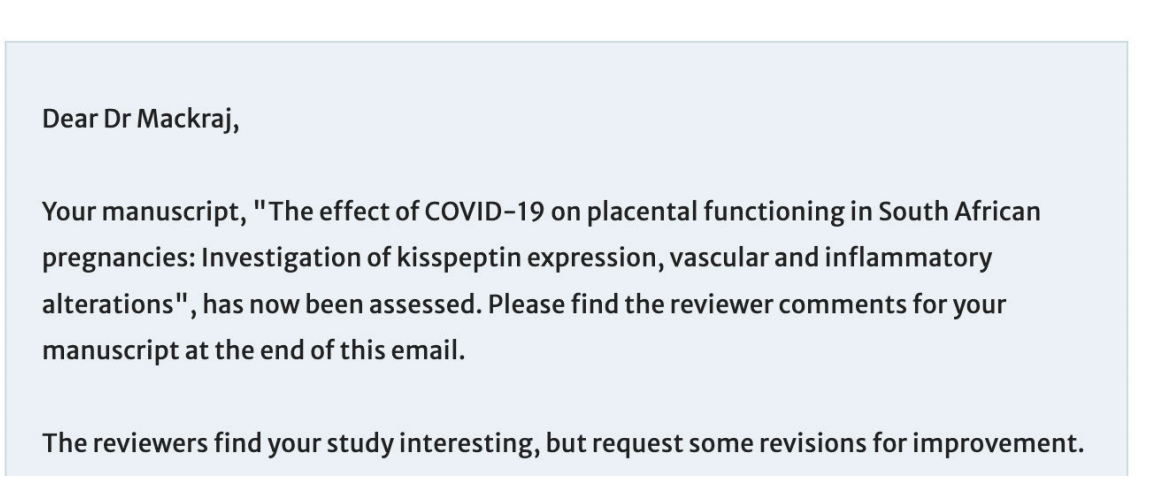
Please note that you are listed as a co-author on the manuscript "The effect of COVID-19 on placental functioning in South African pregnancies: Investigation of kisspeptin expression, vascular and inflammatory alterations", which was submitted to Histochemistry and Cell Biology on 24 January 2025 UTC.

If you have any queries related to this manuscript please contact the corresponding author, who is solely responsible for communicating with the journal.

Kind regards,

Editorial Assistant
Histochemistry and Cell Biology

Reply Forward



Dear Dr Mackraj,

Your manuscript, "The effect of COVID-19 on placental functioning in South African pregnancies: Investigation of kisspeptin expression, vascular and inflammatory alterations", has now been assessed. Please find the reviewer comments for your manuscript at the end of this email.

The reviewers find your study interesting, but request some revisions for improvement.

6.6 Abstract in UKZN CHS Symposuim Abstract Book

ASSESSING THE IMPACT OF COVID-19 INFECTION DURING PREGNANCY ON THE CYTOKINE PROFILE IN THE SOUTH AFRICAN COHORT

C Heeralall[1], U H Ibrahim[2#], M Jenneker[3], S Singh[4], I Mackraj[2#]

1. Discipline of Clinical Anatomy.
2. Discipline of Human Physiology.
3. Discipline of Obstetrics and Gynaecology
4. Optics & Imaging Centre.

Introduction/Aims

The Coronavirus disease (COVID-19) has impacted pregnant women significantly, with increased mortality and morbidity. The implications of this virus is linked to maternal inflammation due to the cytokine storm. Hence, this study aims to investigate the impact of COVID-19 on the cytokine profile in both plasma and exosomes of South African pregnant women.

Methods

Plasma samples were obtained from pregnant women in the third trimester, from which exosomes were extracted using the Invitrogen™ Total Exosome Isolation Kit. These plasma-derived exosomes were characterized using nanoparticle tracking analysis and transmission electron microscopy. The levels of IFN gamma, IL-6, MIP-1 alpha and TNF alpha were analysed in the plasma and circulating exosomes through a multiplex assay.

Results

A significant increase in IL-6 ($p = 0,0451$), IFN γ ($p = 0,0207$), TNF-α ($p = 0,0326$) and MIP-1α ($p = 0,0368$) were observed in the plasma, along with elevated exosomal IFN γ ($p = 0,0094$) from COVID-19 pregnancies. A reduction in the average size and concentration of plasma-derived exosomes were observed in these pregnancies when compared to COVID-19 negative pregnancies.

Conclusion

These findings suggest that COVID-19 infection seems to impact the cytokine profile in the plasma and exosomes of South African pregnant women.

6.7 English Editor Certificate

St Charles College,
Harwin Road,
Scottsville
Pietermaritzburg 3201
Tel: [redacted]
admin@kznlanguageinstitute.com
www.kznlanguageinstitute.com

Registration number: 131 804 NPO

Certificate of editing 27 February 2025

Name: Chanel Heeralall

Title: The effect of COVID-19 on the cytokine profile, placental function and morphology during pregnancy

This serves to confirm that the first and last chapters of the above document were edited substantively by a member of the KZN Language Institute's professional English language editing team. The document was returned to the author with tracked changes and comments intended to correct errors and to clarify meaning. It was the author's responsibility to attend to these changes.

[redacted]

J. Kerchhoff

Director of the KwaZulu-Natal Language Institute

KZN Language Institute - Transforming Words

4 December 2024

TO WHOM IT MAY CONCERN

RE: The effect of COVID-19 on the cytokine profile, placental development and functioning during pregnancy

I hereby confirm that I, Prem Michelle Chetty (Editor and Proofreader), have edited the thesis titled '**The effect of COVID-19 on the cytokine profile, placental development and functioning during pregnancy**' by student **Chanel Heeralall (214528607)**.

The manuscript was edited for all English related typographical, grammatical and formatting errors as well as editorial layout. A plagiarism check was not conducted.

Please contact me on [redacted] for any queries related to the editing of the document.

Sincerely,

[redacted]

Prem Michelle Chetty

Senior Editor

PROOFIT

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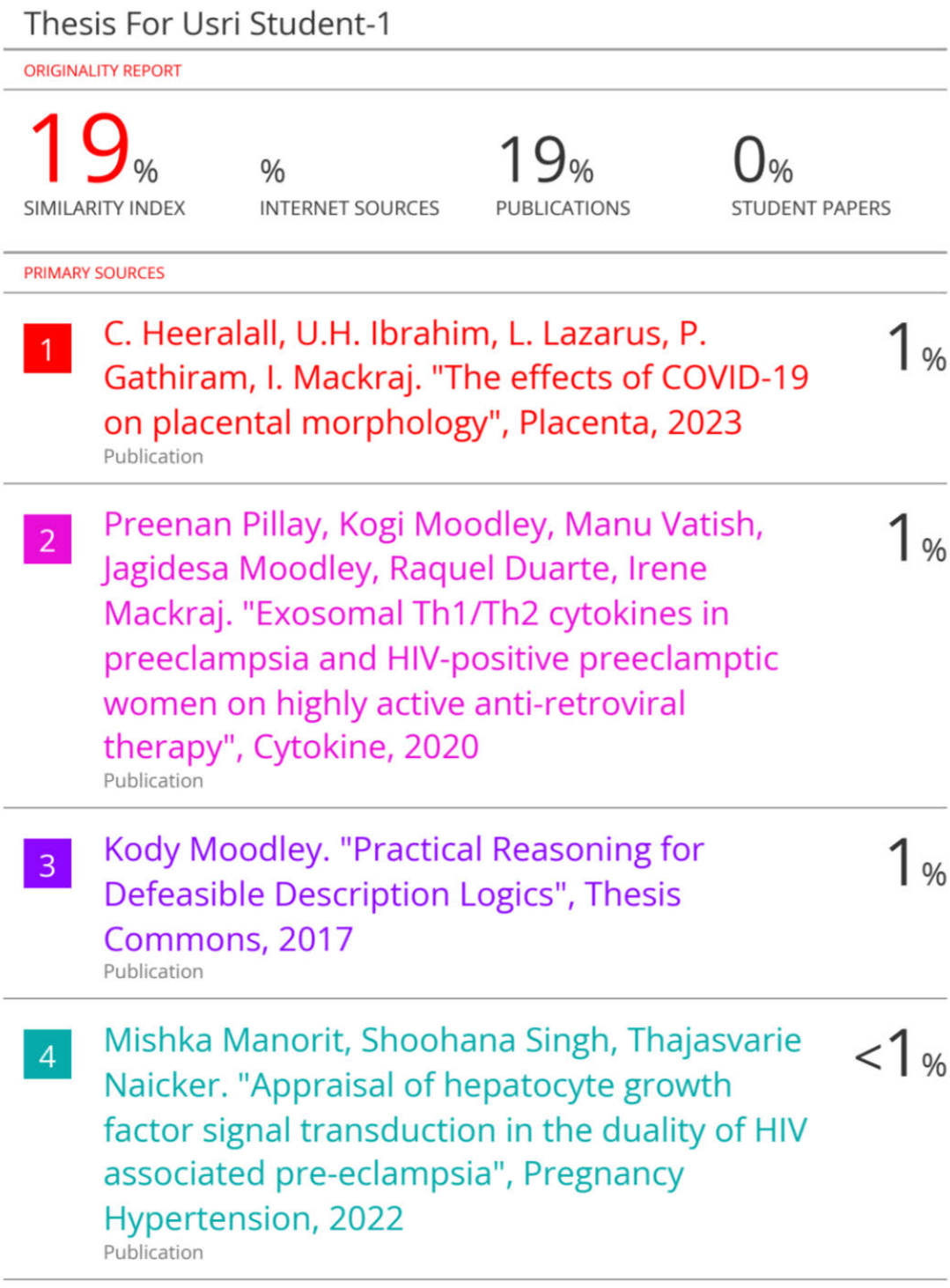

Thesis For Usri Student-1

ORIGINALITY REPORT

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PRIMARY SOURCES

1	C. Heeralall, U.H. Ibrahim, L. Lazarus, P. Gathiram, I. Mackraj. "The effects of COVID-19 on placental morphology", Placenta, 2023 Publication	1%
2	Preenan Pillay, Kogi Moodley, Manu Vatish, Jagidesa Moodley, Raquel Duarte, Irene Mackraj. "Exosomal Th1/Th2 cytokines in preeclampsia and HIV-positive preeclamptic women on highly active anti-retroviral therapy", Cytokine, 2020 Publication	1%
3	Kody Moodley. "Practical Reasoning for Defeasible Description Logics", Thesis Commons, 2017 Publication	1%
4	Mishka Manorit, Shoohana Singh, Thajasvarie Naicker. "Appraisal of hepatocyte growth factor signal transduction in the duality of HIV associated pre-eclampsia", Pregnancy Hypertension, 2022 Publication	<1%