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Elucidating the Placental Histopathological and Molecular Biological Markers of Preterm Birth

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Abstract

Preterm birth (PTB) remains a major global burden; its heterogeneous nature results in challenges in prediction and prevention. Inflammation contributes to increased PTB risk, yet limited data exist on the placenta's role in PTB and the contribution of placental inflammation or dysfunction on birth timing. Thus, there remains a need for histopathological characterisation of preterm placentas and comparison with term placentas. Additionally, there are limited molecular biomarkers available that provide information on placental health during pregnancy or predict the presence of PTB-associated inflammatory or malperfusion lesions.

Placentas were histopathologically examined following spontaneous preterm (*n*=47) or term (*n*=51) birth and gross morphology recorded before dissection and sampling according to the Amsterdam Criteria to determine macroscopic and histologic variations between cohorts. To investigate the contribution of viral placentitis to PTB, immunohistochemical staining was performed to quantify and localise cytomegalovirus (CMV), herpes simplex virus-1/2 (HSV-1/2) and severe acute respiratory virus coronavirus-2 (SARS-CoV-2). Hofbauer cells, and maternal macrophages were assessed using immunofluorescence to determine changes in distribution, quantity and phenotype between preterm and term cohorts and placentas with histologically-diagnosed inflammatory responses. Finally, ELISAs were performed to ascertain differences in preterm and term placental expression levels of a panel of antimicrobial and inflammation-associated proteins implicated in PTB.

Distinct morphological and histopathological characteristics were defined in preterm placentas, the severity of reported inflammatory and non-inflammatory lesions negatively correlated with gestational age and distinct histopathological placental phenotypes were identified for PTB subgroups. CMV and HSV-1/2 were undetectable in the cohort, but one positive SARS-CoV-2 case was observed. Three potential biomarkers of inflammation-associated PTB, Interleukin-6, secretory leukocyte protease inhibitor and soluble CD163, were identified through analysis of macrophages and target proteins. These preliminary data hold promise for providing additional information on placental pathology reports which may benefit neonatal health, and aid in developing novel biomarkers of PIR-associated PTB.

Dedication

For Michael

"You know, I'm really very, very clever"[1]

[1]Zippy, *Rainbow*, ©Thames Television

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Awards, Presentations and Publications Related to this Thesis

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- Parris, Kerry M., Amabebe, Emmanuel, Cohen, Marta C. & Anumba, Dilly. Preterm Birth-Associated Placental Microbial-Metabolite and Inflammatory Mechanisms – What's the Global Difference? Sheffield Global Health Symposium, Sheffield. January 2020 (Oral)
- Parris, Kerry M., Cohen, Marta C. & Anumba, Dilly. Histopathological and Immunohistochemical Characterisation of Placental Features Associated with Infection at Term and Preterm Delivery. Sheffield Medical School Annual Research Conference, Sheffield. June 2020 (Poster: Virtual)
- Parris, Kerry M., Cohen, Marta C. & Anumba, Dilly. Differential phenotype, density and distribution of maternal and fetal macrophages in infected and non-infected preterm versus term placentas. Paediatric Pathology Society 66th Annual European Meeting, Sheffield. December 2020 (Oral: Virtual)
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Declaration of Authorship Contribution

Except where due acknowledgment is made by reference, the studies undertaken in this thesis are the work of the author.

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Table of Contents

Table of Figures

Table of Tables

Abbreviations and Acronyms

ACE2	Angiotensin converting enzyme 2
AKI	Acute kidney injury
AMA	Advanced maternal age
AMP	Antimicrobial protein
ANOVA	Analysis of variance
APH	Antepartum haemorrhage
AROM	Assisted rupture of membranes
BMI	Body mass index
CCA	Clinical chorioamnionitis
CD	Cluster of differentiation
CHIV	Chronic histiocytic intervillositis
ChLA	Chorion laeve accreta
CI	Confidence interval
CL	Cervical length
CMV	Cytomegalovirus
Covid-19	Coronavirus disease-19
CRP	C-reactive protein
CS	Caesarean section
CTb	Cytotrophoblast
CV	Coefficient of variability
CVF	Cervicovaginal fluid
CXCL-	C-X-C motif chemokine ligand-
DAB	3,3'-diaminobenidine
DAMP	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
DNA	Deoxyribonucleic acid
dNK	Decidual natural killer cells
DOHaD	Developmental origins of health and disease
DSC	Decidual stromal cell
E.coli	Escherichia coli
ECM	Extra-cellular matrix
ELISA	Enzyme-linked immunosorbent assay
EPT	Extremely preterm
EVT	Extravillous trophoblasts
fFN	Fetal fibronectin
FFPE	Formalin fixed paraffin embedded
FIR	Fetal inflammatory response
FMU	Feto maternal unit
FPWC	Fetoplacental weight centile
FVM	Fetal vascular malperfusion
GBS	Group B Streptococci
GDM	Gestational diabetes mellitus
GEC	Glandular epithelial cell
GP	General practitioner
GPCR	G-protein-coupled receptors
H&E	Haematoxylin and Eosin
HBC	Hofbauer cells
HCA	Histologically-diagnosed chorioamnionitis
hCAP18	Human cathelicidin antimicrobial protein 18
hCG	Human chorionic gonadotrophin
HDP	Host defence peptide
HIC	High-income country
HIF-1α	Hypoxia inducible factor-1 alpha
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Hsp	Heat shock protein
HSV	Herpes simplex virus
IF	Immunofluorescence
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
ILR1	Interleukin receptor-1
IMS	Industrial methylated spirit
iNOS	Inducible nitric oxide synthase
IQR	Interquartile range

IUGR	Intrauterine growth restriction
IVH	Intraventricular haemorrhage
IVS	Intervillous space
JMIS	Jessop Wing Maternity Information System
JW	Jessop Wing
LFT	Lateral flow test
LGA	Large for gestational age
LMIC	Low-middle-income country
LMP	Last menstrual period
LPS	Lipopolysaccharide
LW	Labour ward
MAPK	Mitogen-activated protein kinase
MERS-CoV	Middle East respiratory syndrome-coronavirus
MIAC	Microbial invasion of the amniotic cavity
MIR	Maternal inflammatory response
MMP	Matrix metalloproteinases
MP	Maternal pyrexia
MPT	Moderate to late preterm
mRNA	Messenger ribonucleic acid
MVM	Maternal vascular malperfusion
NBF	Neutral buffered formalin
NE	Neutrophil elastase
NEC	Necrotising enterocolitis
NET	Neutrophil extracellular traps
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NK	Natural killer cells
NO	Nitric oxide
NPV	Negative predictive value
NTD	N-terminal subdomain
OD	Optical density
ONS	Office for National Statistics
OR	Odds ratio
P Value	Probability value
PAMG-1	Placental alphamicroglobulin-1
PAMP	Pathogen-associated molecular patterns
PAPP-A	Pregnancy-associated plasma protein-A
PAS	Placenta accreta spectrum
PBC	Preterm birth clinic
PBS	Phosphate buffered saline
PEX	Hemopexin domain
PE	Preeclampsia
PG	Prostaglandin
phIGFBP-1	Phosphorylated insulin-like growth-factor binding protein-1
PIR	Placental inflammatory response
PlGF	Placental growth factor
PMN	Polymorphonuclear leukocytes
PoC	Point of care
PPH	Post-partum haemorrhage
PPROM	Preterm prelabour rupture of membranes
PPV	Positive predictive value
PRIME	Preterm Birth Prevention and Management Research Group
PRR	Pattern recognition receptor
PTB	Preterm birth
PTL	Preterm labour
PVFD	Perivillous fibrin deposition
RBD	Receptor-binding domain
RCPath	Royal College of Pathologists
RDS	Respiratory distress syndrome
RFM	Reduced fetal movements
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPoC	Retained product of conception
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome Coronavirus-2

SCA	Standard clinical assessment
sCD163	Soluble CD163
sFlt-1	Soluble FMS-like tyrosine-kinase-1
SGA	Small for gestational age
SLPI	Secretory leukocyte protease inhibitor
sFlt-1	Soluble FMS-like tyrosine-kinase-1
SLPI	Secretory leukocyte protease inhibitor
SNP	Single nucleotide polymorphism
sPTL	Spontaneous preterm labour
SROM	Spontaneous rupture of membranes
STb	Syncytiotrophoblast
STH	Sheffield Teaching Hospitals
SUDI	Sudden unexplained death in infancy
Tb	Trophoblast
Th-	T-helper cell
thPTL	Threatened preterm labour
TIMP	Tissue inhibitor of metalloproteinases
TMB	Tetramethylbenzidine
TMPRSS2	Transmembrane protease serine 2
TNF	Tumour necrosis factor
TReg	T regulatory cell
TVU	Transvaginal ultrasound
UC	Umbilical cord
UCI	Umbilical coiling index
UCT	University of Cape Town
UoS	University of Sheffield
VE	Vaginal examination
VEGF	Vascular endothelial growth factor
VOC	Variants of concern
VPT	Very preterm
VSC	Vascular stromal cell
VUE	Villitis of unknown aetiology
WAP	Whey acidic protein
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Preterm birth

Preterm birth (PTB), defined as a delivery after gestation lasting fewer than 259 days since the last menstrual period (LMP) or 37 weeks, occurs in approximately 10% of all livebirths annually (1, 2). As the leading cause of death in children under 5 years of age, and a major contributor to long-term morbidity, PTB places significant economic and societal burden upon healthcare systems and families. The mechanisms by which the placenta may contribute to PTB through aberrant inflammatory responses or vascular impedance are poorly understood (3). Determining the correlations between placental histopathological findings and PTB may provide new insights into placental regulation and the effects of inflammation and malperfusion on placental function, potentially leading to improved predictive and preventative care of PTB.

1.1.1 Definition and epidemiology

Classification of PTBs is generally into three subgroups according to gestational age, as defined by the World Health Organisation (WHO) (4). Extremely preterm (EPT), very preterm (VPT) and moderate to late preterm (MPT) define infants born at gestations < 28 weeks, 28 - <32 weeks and 32 - <37 weeks, respectively (Figure 1.1) (5, 6), however, differences and difficulties in the measurement and recording of gestation between and within countries complicate PTB categorisation (5). Further, limited access to ultrasound technology and late presentation to antenatal care in many low-middle-income country (LMIC) settings results in a reliance on LMP dates being used as a measure of gestation, a scenario in itself which is only effective when cycle characteristics and the exact date of LMP is definitively ascertained (7).

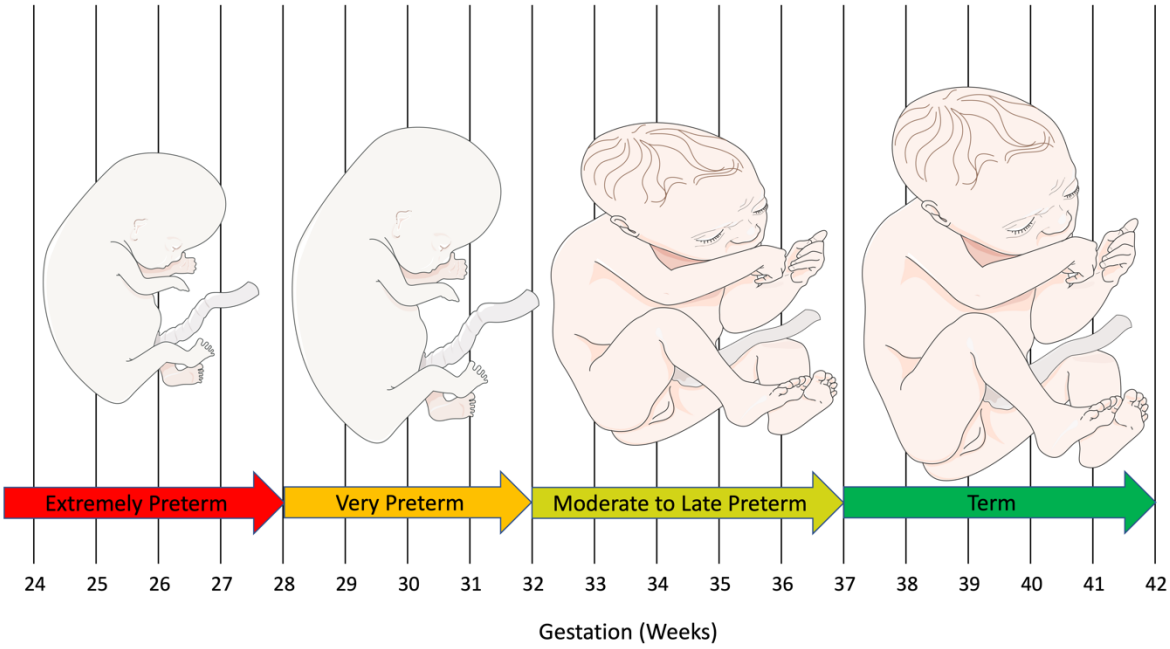

Figure 1. 1 Subcategories of preterm birth.

Deliveries occurring prior to 37 weeks' completed gestation are defined as preterm, however, this description spans a wide range of gestations. PTB is therefore subcategorised to extremely preterm for births occurring <28 weeks' gestation, very preterm if delivery occurs between 28 and <32 weeks and moderate to late preterm if >32 weeks' gestation but less than the defined gestation of term at 37 weeks. Figure partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

Another complication when accurately defining PTB is the consideration of viability, the gestational age at which there is a 50% chance of survival. In high-income countries (HIC), viability is commonly considered to be 24 weeks gestation, however, for infants born in LMICs, the likelihood of a 50% survival rate is sometimes not achieved until 34 weeks' gestation (8-10).

1.1.2 Global preterm birth prevalence

PTB is a worldwide problem; based on data from 107 countries, the 2014 global PTB rate was estimated at 10.6%, equating to 14.84 million babies being born too soon each year (2). Intercontinental and individual country PTB rates vary considerably but, over 80% of all PTBs occur in Asia (52.9%) and sub-Saharan Africa (28.2%), predominantly because of high numbers of total births in Asia but high PTB rates in Sub-Saharan Africa (2, 11). Unsurprisingly, of the top ten countries with the highest total number of PTBs, eight are located within these two regions; among these countries, Bangladesh, Tanzania, India, Ethiopia, Nigeria and Brazil all reported a PTB rate greater than the global rate as detailed in Table 1.1 (2). It is accepted

that there are limitations when analysing estimates of PTB, including country-specific robustness of reporting, variable data quality and frequent adjustments in defining PTB. Despite this, global and national trends show PTB to be increasing in many regions (1, 2, 6, 8, 12).

Table 1. 1 Top ten countries ranked by estimated total number of preterm births

Rank	Country	Total Number of Preterm Births	Preterm Birth Rate (% of Livebirths)
1	India*	3,519,947	13.6
2	China*	1,168,126	6.9§
3	Nigeria*	803,178	11.4
4	Bangladesh*	603,698	19.1
5	Indonesia†	527,672	10.4
6	Pakistan*	454,104	8.4
7	USA§	383,257	9.6
8	Ethiopia†	376,730	12.0
9	Brazil§	339,239	11.2
10	Tanzania*	336,025	11.6

Preterm births data adapted from Chawanpaiboon et al., (2018) (2). *Modelled national estimates, †modelled regional estimates, § directly reported data. §National data on PTB are not reported in China, therefore total numbers and PTB rates were collected and calculated based on literature published in the six most highly-cited medical journals accessed through the Chinese database, Sinomed.

1.1.2.1 Preterm birth prevalence in England

Current data provided by the Office for National Statistics (ONS) revealed PTBs accounted for 7.4% of all live births in England in 2020, a 0.4% decrease on the previous year (13). Additionally, a recent study reported a PTB incidence of 6.1% of singletons delivered between 1 April 2015 and 31 March 2017 in England, but also found that over 50% of these PTBs were iatrogenic (14). As is observed on a global scale, disparities in PTB rates exist between ethnic groups in England. Neonates born to mothers of Black Caribbean and Any Other Black background not only had the highest incidence of PTB, 10.6% and 10.2%, respectively, but were the only ethnicity in England where PTB rates increased in the year 2019-2020 (13).

1.1.3 Consequences of preterm birth

PTB is recognised as a complex syndrome resulting in severe complications for neonates (2), plus, preterm babies are at significant risk of mortality. Globally, PTB accounts for more than 1 million neonatal deaths per annum and is currently the leading cause of death in children under five (1, 2). Although there has been recent increases in neonatal survival rates due to advances in antenatal and neonatal care (15), studies report a greater risk of both short- and long-term morbidities in preterm infants and that the risk of adverse outcome is inversely proportional to gestational age (16). In the short-term, respiratory distress syndrome (RDS), intraventricular haemorrhage (IVH), necrotising enterocolitis (NEC) and sepsis occur more frequently in preterm neonates than their term counterparts (16-19). Complications which may not manifest until weeks, months or even years following PTB are also more likely. Preterm babies are at increased risk of bronchopulmonary dysplasia, retinopathy of prematurity (16), neurodevelopmental disorders including cerebral palsy, visual impairment, and hearing loss (19-21). In parallel to these reported sequelae, children born preterm often experience reduced cognitive ability and impaired educational performance, further adding to the psychosocial and financial burden experienced by their families (2). Alongside this vast personal cost to preterm babies and their parents and carers, the high rates of short- and long-term mortality and morbidity are estimated to cost health services in England and Wales £3.4bn per annum. Data showing an annual NHS saving of £994 million if PTB is delayed by an additional week of gestation (22) supports the need for improved PTB prevention and prediction strategies and research into the intrauterine contributors of poor neurological and physical outcomes for preterm babies.

1.1.4 Preterm birth risk factors

Prior obstetric history and maternal factors contribute vital detail in assessing PTB risk (23). A wide range of maternal factors have been implicated in increasing a woman's risk of PTB, including adolescent or advanced maternal age (24), low or high pre-pregnancy BMI (25, 26), smoking status, alcohol consumption and psychoactive substance use (27). PTB has been associated with maternal nutritional status, previous PTB, and short inter-pregnancy interval, for example, estimates of the PTB recurrence rate vary from 15-50% and periods of <6 months between pregnancies have been associated with a 2-fold increase in PTB risk (28-32). In addition, surgical interventions such as large loop excision of the transformation zone (LLETZ) for treatment of cervical intraepithelial neoplasia, multiple pregnancy, potentially arising from assisted reproductive technologies, and infections are documented as being associated with PTB (31, 33-35). Multiple studies have detailed the link between obesity, defined as a body mass index (BMI) ≥30 kg/m^2, and PTB (26, 36-38), an issue of concern when considering the increased prevalence of women of reproductive age being obese or overweight (BMI 25.0–29.9 kg/m^2) (26). Whilst the mechanisms explaining this relationship are poorly defined, modification of the amniotic and placental environments to a proinflammatory state has been shown in the presence of maternal obesity (36-38). Not only may high BMI be a contributory factor to PTB, alterations to the intrauterine environment are associated with maternal and fetal metabolic complications such as gestational diabetes mellitus (GDM) and fetal macrosomia, plus a greater risk of type 2 diabetes (TIID) in later life for both the mother and neonate (37, 38). Spontaneous abortion, or miscarriage, is a frequent pregnancy outcome, predominantly occurring in the first trimester of up to 15.3% of recognised pregnancies (39, 40). Whilst late miscarriage rates are estimated to be less than 1% of all pregnancies, a miscarriage at any gestation has been shown to increase the risk of a subsequent preterm birth (41), more so where the loss occurred between 16 and 20 weeks' gestation (23). Nulliparous women are more likely to experience PTB than those in their second pregnancy, but the risk of early delivery increases again by the fifth pregnancy (42).

Ethnicity is significantly associated with PTB, and multiple studies have shown Black women to have an increased risk of delivering before 37 weeks than White women. Rates of PTB have been suggested as being 1.5-, 2- and 4-times higher in Black women, although most studies consider this variance in ethnically-diverse countries such as the United States of America

(USA) and the United Kingdom (UK) rather than in countries where women are predominantly of Black ethnicity (43-45). Conversely, women of Asian and Hispanic ethnicity have been shown to be less likely than Black and White women to deliver preterm (44). Whilst no exclusive biological, clinical or epidemiological cause has been determined to explain this disparity, over thirty factors have been identified which may provide mutual contributions to PTB in Black women. Recent consensus highlights how the biological mechanisms leading to PTB may be influenced by upstream, midstream and downstream factors, or interactions of each of these (46). The greatest effect sizes among Black American women compared to their White counterparts were identified as the upstream factor of racism (in multiple forms), midstream factors of local socioeconomic disadvantage and stress, and downstream genetic factors and local environmental hazards. A March of Dimes-convened multi-disciplinary group showed pre-pregnancy chronic hypertension, obesity and short inter-pregnancy intervals to contribute to increased PTB rates in Black women, but they could not explain why Black women without hypertensive disorders, with normal BMIs or those with longer inter-pregnancy intervals were still at increased risk of PTB (46). No research considering the effects of ethnicity on PTB risk in White and Black pregnant female populations in sub-Saharan countries has been published; however, a 2015 study authored by Olorunfemi *et al*. (47) emphasised a persistent significant ethnic disparity in the burden of cervical cancer in South Africa. Despite establishment of prevention schemes following multi-ethnic democracy 20 years prior, the results of which included better access to reproductive health services, Black women were 5.8-times more likely to die from cervical cancer than White women. Furthermore, although 80.4% of South Africa's 2015 population were Black, and only 8.3% and 2.4% identified as White or Indian/Asian, respectively, cervical cancer 5-year survival rates in these non-Black groups were higher (60-80%) than women identifying as Black (40-50%) (47). Factors identified as being causative in ethnic disparities in cervical cancer mortality and survival rates were analogous to those suggested by the March of Dimes consensus group in respect of PTB. Notably, socioeconomic disadvantage was linked with lower rates of access to healthcare, higher parity, greater incidence of stress and late presentation to hospital with pathologic conditions such as hypertension (46, 47). It is, therefore, not unreasonable to suggest the ethnic disparities contributing to increased cervical cancer rates in Black women in the South African population translate to the ethnic disparities which lead to a higher frequency of PTB in the same population. How and why

these same influences appear to be interchangeable irrespective of where a woman of Black ethnicity is born, raised and educated is undefined. As noted in the first of a series of papers published in 2008 in which Goldenberg *et al.* (34) discussed the epidemiology of PTB, the mechanisms by which PTB relates to these maternal characteristics remain elusive (45). Despite continued research, and improvements in predictive technologies and treatments, our understanding of the complex interactions between the physiological, clinical and epidemiological factors influencing birth timing is lacking (34, 46).

1.2 Aetiology and pathogenesis of preterm birth

PTB can be further classified as iatrogenic or spontaneous (sPTB). Accounting for 30-40% of PTBs, iatrogenic PTBs are those which are initiated by an obstetric-care provider in order to expedite delivery due to maternal or fetal complications, most commonly preeclampsia (PE) or severe intrauterine growth restriction (IUGR) (48). sPTB may be preceded by preterm prelabour rupture of membranes (PPROM) or preterm labour (PTL), yet in many cases there is no identifiable cause (45, 49, 50). The syndromic nature of sPTB arises from the complexity and heterogeneity of its aetiology and pathophysiology (51) and, although discrete activators of these pathophysiological pathways may remain elusive, the significance of bacterial infection, colonisation of fetal tissue and the subsequent maternal inflammatory response has been recognised in the pathogenesis of PTB (52, 53).

1.2.1 Inflammation and Preterm Birth

1.2.1.1 Microbial-Associated Inflammation

As described, PTB is a heterogenous condition with multiple causative social, environmental and clinical factors, however, intrauterine infection is a known significant contributor to sPTB. Nonetheless, determining the principal factor in PTB, particularly in cases without obvious infection, remains a challenge (45, 49, 54, 55). While the 'sterile womb' paradigm is frequently debated, the increased likelihood of PTB and PPROM are definitively associated with positive amniotic fluid cultures resulting from vertical transmission of pathogens and/or microbial invasion of the amniotic cavity (MIAC) (56-59). Diagnosing MIAC presents a challenge in that invasive amniocentesis is the 'gold standard' methodology but is a procedure which by its nature may also contribute to an increased risk of PTB (60, 61). MIAC following ascension of

microorganisms through the vagina and cervix, and subsequent colonisation of the choriodecidua, elicits the release of lipopolysaccharide (LPS) and downstream synthesis of a raft of pro-inflammatory cytokines, such as interleukin-1β, (IL-1β), interleukin-6 (IL-6), prostaglandins PGE_2 and PGE_{2a}, and tumour necrosis factor-alpha (TNF-α), in tandem with chemokines including CXC motif ligand 1 and 8 (CXCL1 and CXCL8) (55, 62). The resultant feed-forward effect initiates an inflammatory cascade, instigates and sustains myometrial contractility and upregulates matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expression whilst simultaneously diminishing expression of the tissue inhibitor of metalloproteinases-1 (TIMP-1) (63-65). Consequently, cervical and amniotic membranal extracellular matrix (ECM) proteins are degraded, permitting further ascension of microorganisms with the potential to result in rupture of the chorioamniotic membranes (PPROM) (66, 67). In parallel, PGE_2 and PGE_{2a} trigger further release of MMPs and stimulation of myometrial contractions, thus amplifying the risk of preterm labour and/or PPROM (67). MIAC is estimated to be present in up to 22% of pregnant women presenting with PTL and intact membranes and in over 32% of women experiencing PPROM. However, the inability of conventional culture techniques to detect all microbial agents may mean the true prevalence of MIAC is vastly underestimated (68, 69).

1.2.1.2 Sterile Inflammation

PTB has been shown to correlate with elevated IL-1β and IL-6 in the absence of identifiable bacteria and LPS stimulation (65), suggesting it is the downstream inflammatory effects rather than the infection itself which results in initiation of preterm labour. Cases of 'sterile' inflammation and resultant PTB outweigh infection-associated PTB (70, 71) as demonstrated by the lack of inhibition of inflammatory pathways or prevention of PTB through prophylactic antibiotic administration (58). Whether the inflammatory cascade is initiated by a microbial or sterile stimulus, such as damage-associated molecular patterns (DAMPs), pattern recognition receptors (PRR) in immune and non-immune cells of the placenta activate nuclear factor kappa B (NF-κB) and p38 mitogen-activated protein (MAP) kinase signalling which stimulates inflammatory gene expression (70, 72). Placental dysfunction-induced hypoxia, ischemia or inflammation are implicated in PTB through trophoblastic cell death, alarmin release and subsequent increased placental inflammation and further trophoblast necrosis (71). A model example of sterile stimulation of damaging inflammation is that of Interleukin

1-alpha (IL-1α) transcription and expression which results in the same downstream proinflammatory effects as IL-1β transcription and expression due to their common receptor (IL1R). Whilst IL-1β expression is stimulated by LPS as described previously, expression of the alarmin IL-1α occurs in response to signals from necrotic cells, however, neither of these members of the IL-1 family have the ability to effect the ILR1 receptor structural change necessary to elicit a strong proinflammatory signal and initiate downstream cascades which may lead to labour activation (73) (Figure 1.2). Therefore, understanding the normal inflammatory processes of parturition is crucial in advancing knowledge of infectious and sterile inflammatory-induced preterm parturition.

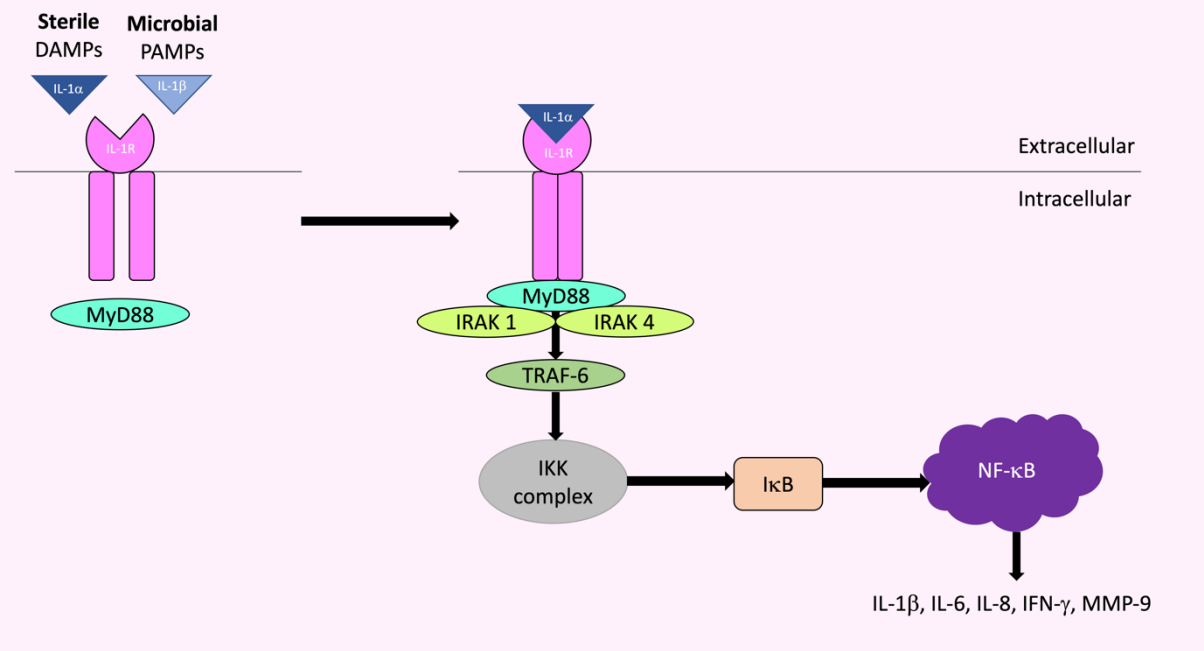

Figure 1. 2 Simplified schematic of IL-1 receptor sterile and microbial inflammatory signalling pathways linked to preterm birth.

In response to endogenous damage-associated molecular patterns (DAMPs) released from necrotic cells IL-1α binds to the pattern recognition receptor (PRR), IL-1 receptor (IL-1R) on placental immune and non-immune cells; where these signals are pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), IL-1β binds to IL-1R. Binding of either IL-1α or IL-1β leads to a structural change in IL-R1 and associated binding of myeloid differentiation primary response 88 (MyD88) which becomes phosphorylated. Through interactions with Interleukin receptor activated kinases 1 and 4 (IRAK1-4), tumour necrosis factor receptor-associated factor-6 (TRAF-6) activates the inhibitor of nuclear factor-κB kinase (IKK) complex which, in turn, phosphorylates the inhibitor of nuclear factor-κB (IκB). IκB is ubiquitinated and degraded by the proteasome permitting NFκB to translocate to the nucleus where it activates a range of proinflammatory genes associated with labour (72, 73). *Il-6;* interleukin-6, *IL-8;* interleukin-8, I*FN-γ*; interferon-gamma; *MMP-9*; matrix metalloproteinase-9.

1.2.2 Genetic and racial disparities in inflammatory responses leading to preterm birth

Virulence of microorganisms, environmental factors and genetic variation in the inflammatory response are likely to be important aspects of the establishment and severity of gestational tissue infections. Moreover, these divergent circumstances may in part explain the racial and socioeconomic differences seen in PTB prevalence (45). The risk of preterm born women delivering preterm themselves is elevated compared to women born at term, and a history of PTB magnifies this risk, further adding credence to a genetic predisposition to inflammation contributing to preterm delivery (74, 75). Polymorphisms in the promoter region of the *TNF-*α gene have been identified. Carriers of the *TNF2* allele, a polymorphism at position -308 of the nucleotide sequence, have an increased likelihood of developing sepsis than those with the *TNF1* allele, most likely due to an exaggerated inflammatory response to infectious stimuli (74, 76). In a 1999 study of 55 women experiencing PPROM, as described in a review conducted by Holst and Garnier (2008) (74), a three-fold increase in the risk of delivering preterm was reported in women with the *TNF2* allele (OR 3.18; 95% CI 1.33-7.83) compared to patients where it was not present. These findings were corroborated in a larger cohort where *TNF2* allele carriers were also found to have an elevated risk of PTB (OR 2.7; 95% CI 1.9-21.0) (74). Also increasing the risk of PPROM, a single nucleotide polymorphism (SNP) at position -656 of the nucleotide sequence in the promoter of the *SERPINH1* gene was identified in a study by Wang *et al.*, (2006) (75). The result of this SNP in a gene which reduces promoter function in amnion fibroblast cells was decreased heat shock protein 47 (Hsp47) expression, a molecular chaperone essential for interstitial collagen synthesis, procollagen deposition and stabilisation of the collagen triple helix (75, 77). Subsequently, it is suggested that the reduction in fibrillar collagen laid down in the amnion, the fetal membrane layer contributing the greatest proportion to the overall tensile strength, results in membrane rigidity, weakness and increased PPROM risk (75). Interestingly, the *SERPINH1* SNP led to enrichment of the minor -656T allele over the major -656C allele in people of African descent, potentially providing some explanation as to why PTB is more prevalent in women of African origin, irrespective of their place of birth or country of residence at the time of delivery (75, 76). Other studies have also demonstrated racial disparity in immune responses leading to PTB, including increased IL-1β and TNF-α in Black women and, in contrast, increased interleukin-8 (IL-8; also known as CXCL8) in White women (78). Although these data indicate a potential to predict PTB in specific racial cohorts, to date, no robust associations with SNPs

have been identified. Work to address this is ongoing with genome-wide studies and multilocus allele analyses promising to reveal stronger associations between genetic variation and PTB (79-81).

1.2.3 Placenta and preterm birth

Progress in PTB research has revealed intraamniotic infection and MIAC may contribute to PTB less frequently than previously thought. Alternative theories suggest placental inflammation or insufficiency to be underlying reasons for PTL and subsequent sPTB. Histopathological evaluation of preterm placentas has revealed a thorough record of the pregnancy, including adverse events and evidence of aberrant activation of parturition-associated inflammatory pathways, and demonstrated the placenta's ability to provide sufficient detail to predict future risk of chronic disease development or PTB recurrence (82, 83). It is therefore imperative to better understand these placental factors which may lead to PTB.

1.3 The human placenta

1.3.1 Placentation and placental development

Although fertilisation of an oocyte by a single sperm cell may be the first step in the formation of a new human life, an expertly choreographed process of implantation and placental development must then follow to ensure a correctly timed, healthy delivery (84). By day three post-fertilisation, the zygote has proliferated to a 12-30 cell morula, a name derived from the Latin *morus* given its resemblance to a mulberry (85). Stage 3 of embryogenesis begins around day 4 post-fertilisation, and it is at this point cell-cell junctions are reinforced in the blastomeres of the morula leading to alignment and adhesion in a process known as compaction which results in the blastocyst. The newly divided blastocyst is comprised of an inner cell mass of blastomeres and an outer single epithelial layer of trophoblasts surrounding the blastocoel or blastocytic cavity. From this point on, these blastomeres are destined to become the embryo (85). The trophoblast is the first cell lineage to differentiate, giving rise to larger parts of the placenta and the extraembryonic (fetal) membranes and propelling the steps to implantation (86). Polarised outer cells of the blastocyst attach to the uterine epithelium before syncytial fusion creates oligonucleated syncytiotrophoblasts, formed from cytotrophoblast stem cells capable of invading and anchoring to the uterine wall (Figure 1.3)

(86, 87). In addition, trophoblastic differentiation enables the formation of the maternal-fetal interface, or placental barrier (88).

Early placental development sees extensive remodelling, including regression of the villi from the superficial pole to form the chorion laeve, a loss of smooth muscle and elastin from the maternal spiral artery walls, and terminal segment dilation to support histiotrophic nutrition (89). To achieve this remodelling, the differentiated interstitial trophoblasts invade through the stroma into the underlying decidua. In unison, endovascular trophoblasts travel down the spiral artery lumen, effectively plugging the arterial lumen for the majority of the first trimester (86, 89, 90). Early stages of placental development occur in a state of physiological hypoxia (2-5% O_2), an essential requirement to protect the developing embryo and placenta from teratogenic reactive oxygen species (ROS) during crucial organogenesis (87). At the end of the first trimester, there is a switch from uterine histiotrophic nutrition of the developing fetus to a placental haemotrophic supply from the maternal circulation (91). This early regulated process of remodelling ensures high volumes of low-velocity/low pressure maternal blood continually flow through the placenta to facilitate optimum maternal-fetal exchange for the remainder of gestation (89).

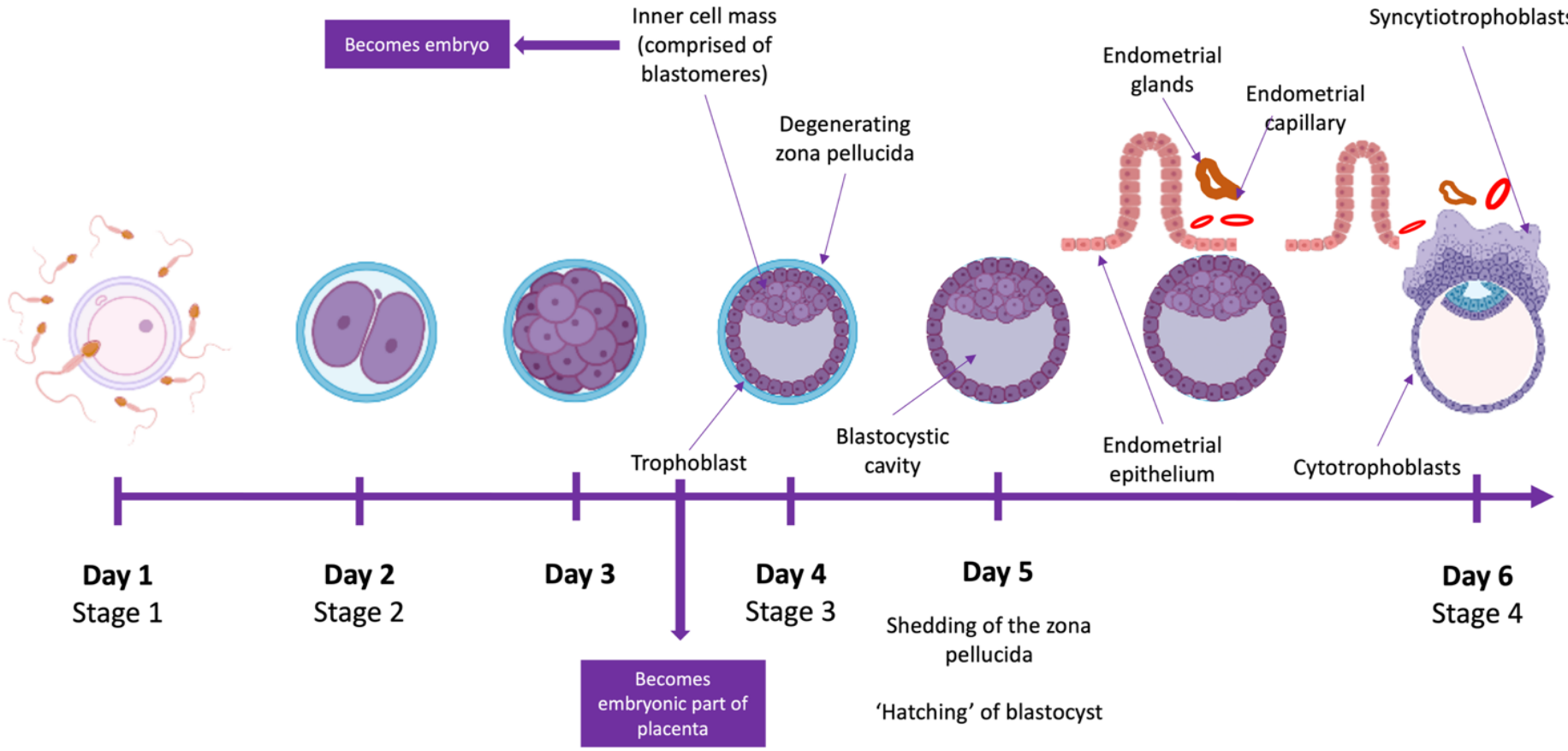

Figure 1. 3 Early development of the placenta.

Within five days of fertilisation the two-cell zygote has divided rapidly to become the blastocyst, a trophoblast layer enclosing blastocoelmic or blastocytic cavity, this will form the extraembryonic structures including the amniotic membranes, and the inner cell mass which will form the embryo. The outer zona pellucida degenerates around day four and the blastocyst 'hatches'. The syncytiotrophoblast layer, which will become the placenta, is exposed and invades the endometrial epithelium and underlying connective tissue. By day seven, the blastocyst is superficially implanted in the endometrium. Figure created with Biorender.com.

1.3.2 Anatomy of the human placenta

The ephemeral placenta is the largest fetal organ (89) with a pivotal role in maternal and fetal health, both for the short- and long-term (92-94). At term, the placenta is discoid with an average diameter of 22cm, a central thickness of 2.5cm and an approximate mass of 500g (93-95). Whilst the exact mechanisms determining placental morphology are unclear, events which occur in the first trimester are believed to influence the final size and elliptical shape (89, 96-99). The placental disc comprises two surfaces, the chorionic plate or fetal surface and the basal plate or maternal surface which is only examinable following separation of the placenta from the maternal endometrium at delivery (Figure 1.4) (94). By term, between thirty and forty highly branched fetal villous trees protrude into the intervillous space which lies between the chorionic and basal plates, subsequently one to four of these villous trees occupy each placental lobe or cotyledon, each separated by placental septa (93, 94, 100). Elaborate branching of the terminal villi located at the ends of the fetal villous trees results in a surface area of 12-14m^2 in the term placenta and, due to high vascularisation and thin interhaemal membranous separation of the maternal and fetal circulations, it is here where the majority of maternal-fetal exchange takes place (93). The maternal and fetal surfaces converge at the chorion laeve, a triple layer comprised of the amniotic epithelium and mesenchyme, the chorionic mesenchyme and a sheet of extravillous trophoblast cells (94). Covering each villous tree is a layer of syncytiotrophoblast epithelium, a thin, multinucleated and polar syncytium involved in protection from vertical transmission of pathogens and, in early pregnancy, invasion of the uterine epithelium (93, 94). Underlying the syncytiotrophoblast is a basal membrane adjacent to the cytotrophoblast layer, cells of which fuse together and with the syncytiotrophoblast to become an inherent component of the syncytium. Pyknotic and apoptotic processes of trophoblastic nuclei condense nuclear chromatin before they are sloughed off the membrane and form syncytial knots in a normal ageing process of the placenta (94, 101).

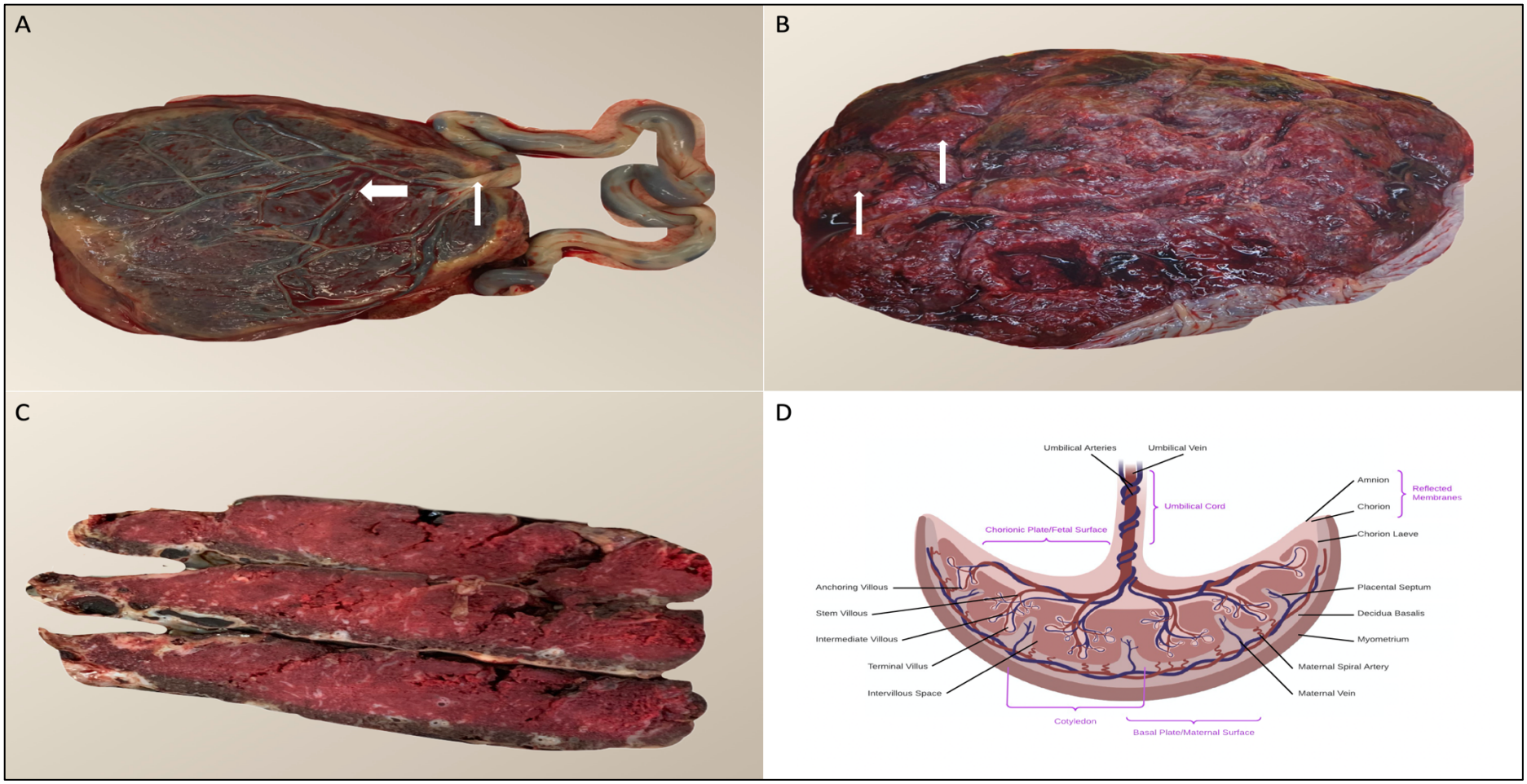

Figure 1. 4 Representative images of placental surfaces and schematic diagram of a cut surface of the placenta.

A Image of fresh fetal surface displaying chorionic vessels (wide arrow); note marginal cord insertion (narrow arrow). **B** Image of fresh maternal surface with demarcated cotyledons visible (arrows). **C** Three cut surfaces of the placental disc. **D Schematic diagram** Cross section representative of cut surface (C) of the placenta showing the chorionic plate, (fetal surface) and the basal plate (maternal surface). The maternal-placental circulation supplies the placenta with oxygen and nutrients for exchange to the fetus and removes waste products and carbon dioxide through the spiral arteries and endometrial veins. Intervillous spaces are filled with maternal blood, bathing the numerous villi of the villous tissue and allowing exchange of nutrients to take place at this maternal-fetal interface. (Schematic created with Biorender.com).

1.3.3 Anatomy of amniotic (fetal) membranes

Comprised of two distinct layers, namely the amnion and chorion, and extending out from the placental margin, extraembryonic tissue surrounds the developing fetus and contains the amniotic fluid throughout pregnancy (102, 103). The thin internal surface of this bilayer is composed of amnion epithelial cells and is devoid of any smooth muscle cells, nerves, lymphatics and vasculature (102). In contrast, the cellular chorion is up to four times thicker than the amnion and is vascularised but contributes considerably less tensile strength or structural protection. It does, however, provide an immunological barrier for the fetus from the maternal immune system (102, 104). A single layer of cuboidal epithelial cells connected to a collagen IV-rich basal membrane characterises the amnion layer of fetal membranes (105). Within the chorion, tightly-packed cytotrophoblasts border the chorionic basement membrane with superficial fibroblasts and maternal decidual cells contributing to the total cellular component of the membranes (106) (Figure 1.5).

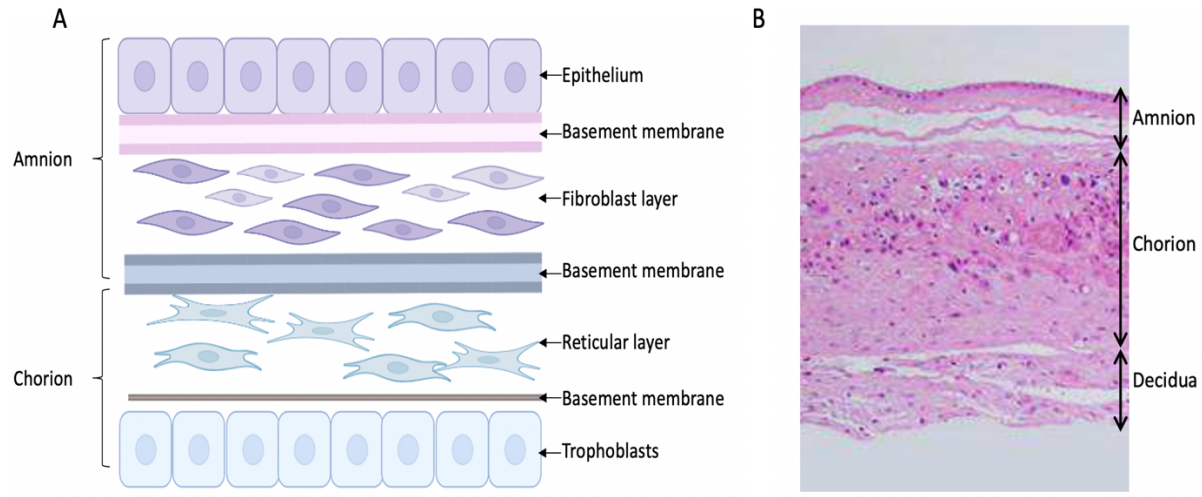

Figure 1. 5 Cross-sectional schematic of the fetal membranes (A) and corresponding haematoxylin and eosin-stained cross-section (10X original magnification) (B).

The single amnion layer is made up of cuboidal epithelial cells connected to a collagen-rich membrane layer which, on histology, may appear as a large gap between layers. In contrast, the chorion comprises a chorionic basement and tightly-packed, sometimes multinucleated, cytotrophoblast cells with superficial fibroblasts throughout. Maternal decidual cells make up the remaining component of the fetal membranes and are found fused to the thick chorion layer (Schematic created with Biorender.com).

1.3.4 Anatomy of the umbilical cord

Aristotle (384-322 BCE) originally identified the umbilical cord as the connection between mother and fetus (107). At only 4-8 weeks' gestation, the rudimentary umbilical cord is formed from the envelopment of tissue arising from the omphalomesenteric duct, the connecting stalk and the umbilical coelom; blood flow is established as early as the 5th week of embryogenesis (107). Once fully developed, the helical, tubular umbilical cord contains the remnants of the allantois, two umbilical arteries and a single umbilical vein, all embedded in Wharton's jelly, a mucous connective tissue encircling the umbilical vessels, and surrounded by a single amnion layer (107) (Figure 1.6).

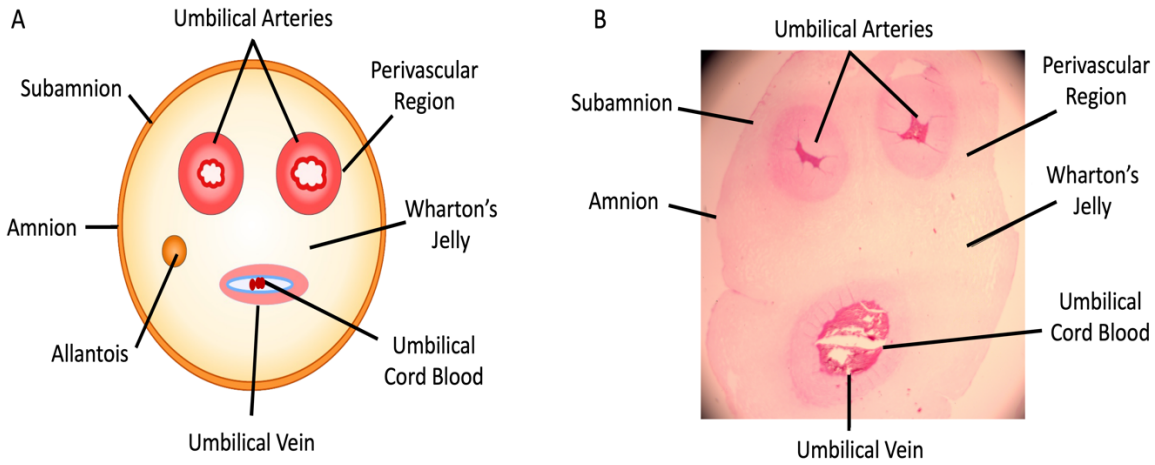

Figure 1. 6 Cross-sectional schematic of the umbilical cord (A) and corresponding haematoxylin and eosin-stained cross-section (2X original magnification) (B).

Two umbilical arteries with thick perivascular muscle return deoxygenated blood from the fetus back through the placenta, conversely, one umbilical vein transports oxygenated blood to the fetal circulation. The umbilical vein is more elliptical in shape than the umbilical arteries and has noticeably less perivascular muscle. Wharton's jelly, a mucous connective tissue, surrounds the three vessels in the umbilical cord and the vestigial allantois, all of which are enclosed in a single amnion layer.

The umbilical cord is fully developed by week 12 and, by term, has a length of approximately 50-60 cm, an important factor since long (>100cm) and short (<30cm) cords pose issues in that long cords may become entangled around the fetus leading to distress and possibly fetal demise, or they may prolapse. Short cords are associated with premature placental separation, intrauterine growth restriction (IUGR), congenital abnormalities, delayed fetal descent, placental abruption and, as with long cords, fetal distress and demise (107, 108).

Umbilical cord diameter is also important for healthy fetal development and this too increases during gestation from an average of 3.19 mm at 10 weeks to approximately 16.72 mm between 33-35 weeks (107). Reduced water content of the Wharton's jelly at term sees a decline in overall average diameter to 12-14.5 mm (107, 108) but, prior to term, reduced umbilical cord diameters are linked to IUGR and increased diameters are associated with GDM and fetal hydrops (109). Characteristic of the human umbilical cord is the cylindrical helical pattern which is formed by the umbilical arteries spiralling around the umbilical vein, thus creating a coil at every 5cm cord length and defining the Umbilical Coiling Index (UCI) (107, 110). At term, the umbilical cord may comprise up to forty coils across its length (107, 108) and, in some cases, pseudo knots or true knots (108). Some studies describe almost 90% of umbilical cord coils as being dextral (right twists) with only the remaining 10% twisting to the left, or sinistrally (107), but a consensus on sampling the placenta and defining lesions for clinical assessment states the converse in that left twists are more commonly observed (103). Whilst it is unclear if the direction of coils is relevant to healthy or pathological pregnancy, hypo- and hypercoiled cords are associated with IUGR and preterm birth (PTB) (107, 110).

1.3.5 Physiology of the human placenta

The human placenta provides the interface between mother and fetus and plays a crucial role in sustaining the developing pregnancy (92, 96). Whilst the essential functions of the placenta are undisputed, the early simplification by Harland Mossman that the placenta primarily exists as a conduit for fetal nourishment and waste removal (111) perpetuates today and completely overshadows its true physiological complexity (96). Until safe delivery of the neonate, the placenta performs multiple functions in unison: physiological exchange, endocrine activity, metabolism and immune protection and immunotolerance of the developing fetus (92, 112, 113). A combination of the diffusing capacity of the placenta, blood oxygen levels and flow rate in the umbilical and uterine arteries regulates the fetus' availability of oxygen (66). In recent years, more effective approaches to identifying how villous tree geometry affects this placental exchange have been developed (114), yet a true understanding of how dysregulated diffusion relates to placental pathophysiology remains elusive. Placental hormones are necessary from early developmental stages up to labour and delivery with the major contributor to the synthesis of these hormones being the syncytiotrophoblast layer (113). Many critical placental hormones have been identified,

including human chorionic gonadotropin (hCG), commonly used as a biochemical pregnancy test, placental growth hormone, progesterone, oestrogens and numerous adipokines. Whilst beyond the scope of this thesis, placental hormones have been extensively reviewed by Costa (113). Their significance as potential biomarkers of adverse pregnancy outcomes, however, cannot be underestimated.

Placental metabolism contributes greatly to fetal development through maternal health with key substrates of metabolism being identified as glucose, amino acids and lipids. As the primary placental and fetal energy source, studies have demonstrated fetal glucose demand to be the driving factor in transplacental glucose transport (38, 93). Lipid metabolism also contributes to fetal growth and the placenta contains multiple lipoprotein receptors, lipases and fatty-acid binding proteins which facilitate transplacental transport. Another important substrate required for fetal growth and development is amino acids, and their transport is facilitated by placental expression of fifteen amino acid transport systems (38). One of the most important physiologic functions of the placenta is immunotolerance of the fetus and immune protection through provision of an anatomical barrier to pathogens (112). Paradoxically, the gestational shift towards a T-helper (Th) 2, anti-inflammatory dominance necessary to facilitate maternal-fetal tolerance and prevent rejection of the semi-allogenic fetus exposes the mother to a greater susceptibility to some infectious organisms, evidenced most recently by the increased risk of acquisition and acute disease in relation to severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (72, 115, 116). Breakdowns in immunotolerance, either as a result of placental dysfunction, preeclampsia or infections, have been implicated in PPROM and PTB (117).

1.3.6 Physiology of the fetal membranes

The fetal membranes and amniotic fluid provide both mechanical and antimicrobial protection to the fetus throughout gestation (102, 105, 106, 118). The amniochorion bilayer supports the growing fetus and has adequate strength and stretch to accommodate the increasing uterine volume (104) whilst absorbing the forces of fetal movement (118). Furthermore, fetal membranes produce bioactive compounds required by the fetus for normal physiological function and development and play a key role in the maternal-fetal communication which activates MMPs and subsequently degrades ECM (104). Timing of

these events is critical as dysregulation can lead to PPROM and preterm delivery (106). Interestingly, membranes sampled from women experiencing PPROM are less hydrophobic, therefore, friction is increased between the amnion and chorion layers (102). Increased membrane friction has been postulated as a cause of iatrogenic PPROM following fetoscopy (119) which could go some way to explain similar spontaneous PPROM effects. Moreover, bacterial LPS consists of a hydrophobic membrane anchor portion known as lipid A; recognised by the Toll-like receptor 4 (TLR4)/myeloid differentiation factor2 (MD-2) complex, lipid A is responsible for endotoxic activity of bacteria and induction of inflammatory responses (120, 121). Hydrophilic microorganisms, such as some strains of *Escherichia coli* (*E. coli*), are more transient and move through water-rich environments to colonise tissues (122), additionally, they demonstrate less adherence to hydrophilic surfaces (122), and, as such, it is feasible that a reduced hydrophobicity and concomitant increase in hydrophilicity in the fetal membranes could lead to colonisation of the amniotic fluid and placenta by these microorganisms. Considered together, it is reasonable to postulate the breakdown in mechanical protection through increased frictional forces and the subsequent modification of antimicrobial protection and immune response leads to the interlinked cases of PPROM and MIAC commonly seen in clinical practice.

1.3.7 Physiology of the umbilical cord

Within the umbilical cord, the vein transports oxygenated blood to the fetal heart whilst the arteries return oxygen-depleted blood to the placenta (107, 110). Found within approximately 3-5 cm of the cord insertion into the placental surface, the Hyrtl's anastomosis is the site of umbilical artery convergence critical for equalising blood flow and pressure before the umbilical arteries enter the placenta and bifurcate into the chorionic vessels (107, 123). Oxygenated blood travels from the placenta to the fetus by way of a pressure gradient created from the umbilical vein pressure being higher than the fetal inferior cava pressure. As the quantity of blood flowing in both directions, i.e., from the placenta to the fetus and back from the fetus to the placenta, is near-equal, the fetus is considered to be a closed system (107). Fetal pulmonary and systemic circulations differ to those seen in neonates in that the majority of venous return to the fetal left atrium is from the placenta and passes via the umbilical vein through the ductus venosus, inferior vena cava and foramen ovale. Conversely, in neonates and adults, this supply to the left heart is directly from the lungs. Blood exiting the right fetal

ventricle bypasses the lungs and enters the thoracic aorta via the ductus arteriosus before it travels to the head and body and returns to the placenta via the aorta (124).

1.3.8 Histological overview of villous tissue

On histology, normal term placentas reveal long intermediate villi with branched mature terminal villi resulting from attenuation of cytotrophoblasts leaving a single layer of syncytiotrophoblasts to which the villous capillary epithelium become closely positioned (3, 125, 126). As chorionic villi mature, the intervillous space (IVS) decreases (3, 125). Syncytial knot formation is increased, whereas the presence of vasculosyncytial membranes decreases and, where they remain, they are less well-developed (3) (Figure 1.7).

Microscopic assessment of the preterm placenta shows reduced numbers of syncytial knots correlating directly to decreased gestational age. Syncytial knots, formed from debris of degrading syncytiotrophoblast cells, are characteristic of the ageing placenta, thus their observation in early preterm placentas indicates accelerated chorionic villous maturation and potential malperfusion disorders (101, 127) (Figure 1.7). There is a lack of connective tissue in the stem villi of preterm placentas, instead, perivascular capillary sheaths are visible in these immature villi and, in gestations closer to term, Langhan's fibrinoid replaces the syncytiotrophoblast layer of proximal stem villi (128). Preterm distal villi consist of greater numbers of mature intermediate villi compared to later gestations and at term when terminal villi are the greatest components of distal villi (129). Of observable difference, the intervillous space is generally greater in term than preterm placentas, largely due to the decreased presence of cell islands in the latter, however, no distinct limit of acceptability has been established for any gestational age (128).

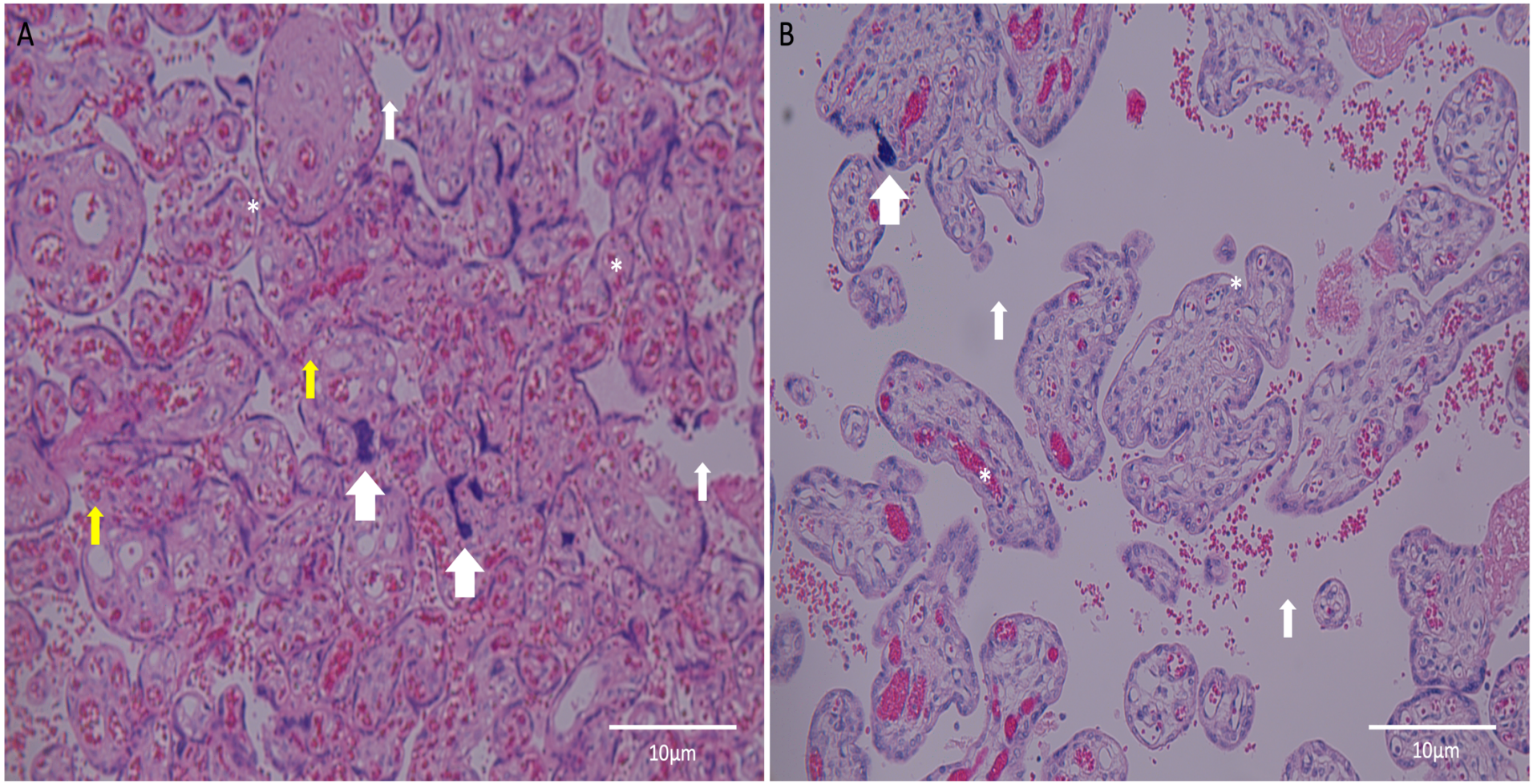

Figure 1. 7 Placental histology of term and preterm villous tissue stained with H&E. 20X original magnification.
A appropriate for gestational age term placenta (40.5 weeks). Normal maturation of villi (white asterisk) and decreased intervillous space (narrow white arrow). Mature terminal villi and attenuated syncytiotrophoblast layers are highlighted by yellow arrows. Multiple syncytial knots (wide black arrows) are present at term. **B** appropriate for gestational age preterm placenta (26.3 weeks) demonstrating immature villi (white asterisk) and increased intervillous space. Only one distinct syncytial knot is present in the field of view (wide white arrow).

1.4 Current process of placental histopathological examination

Determining early pathological pathways of obstetric complications related to the placenta is inherently difficult due to 1) the complexity and heterogeneity of these syndromes, 2) the organ's inaccessibility, 3) the invasive nature of placental biopsies and, 4) ethical implications of increasing risks to the ongoing pregnancy. Consequently, current understanding of this complex organ, which singularly performs the functions of numerous fetal organs, is insufficient (130, 131). Existing knowledge most often derives from studies in animal models, which do not accurately reflect the human placenta (132), or studies of term placentas which are known to differ from early placentas (133). An absence of functional experimental models further adds to the difficulties (134). Histopathological placental examination elucidates valuable clinical data following adverse pregnancy outcome and, in many cases, poor neonatal outcomes following an apparently normal pregnancy such as hypoxic brain injury or neonatal death (135). Findings of placental examinations are useful in their provision of potential explanations of pregnancy loss or neonatal death and/or understanding of risk for future pregnancies. Additionally, they may indicate a need for immediate care and management of the mother or neonate, for example, administering antifungal treatment to a preterm infant following diagnosis of *Candida albicans* presence in the placenta (135, 136). However, the period taken to assess the placenta macroscopically and microscopically may impede timely communication of this information (135). Conversely, routine placental examination is unnecessary and economically unviable in most settings (135). In the UK, The Royal College of Pathologists (RCPath) outlines a guide to reasonable practice in its 'Tissue Pathway for Histopathological Examination of the Placenta' document (last updated September 2022) (136) whilst noting the wide variation of placental pathology services across the UK.

Amplifying this variation globally indicates the challenges in determining 'routine' placental examination and the discrepancies in reporting placental pathology. The RCPath does not define the indications for referral of a placenta for histopathological analysis but does make a list of suggestions which include essential referrals for stillbirth, late miscarriage, fetal growth restriction, maternal pyrexia (>38°C) and preterm delivery <32 weeks' gestation. Desirable indications for referral, such as placental abruption, uncomplicated multiple pregnancy, gestational diabetes, maternal group B streptococcus and prematurity 32+0 to 36+6 weeks' gestation, will be dependent upon the pathology services of each trust. Placental

histopathological examination is not indicated for a range of conditions, notably normal pregnancy, as well as maternal HIV or hepatitis infections, maternal disease with normal pregnancy outcome and post-partum haemorrhage (136).

Whilst undeniably valuable for clinicians, histopathological examination of the placenta and the resulting report may bring comfort and support to parents and families experiencing adverse pregnancy outcomes or fetal loss. Mothers experiencing stillbirth or PTB are at a greater risk of post-partum depression and, with no explanation of the reason for the adverse outcome in many cases, women report feeling guilty or shameful and experience high rates of self-blame (137, 138). These feelings can persist and lead to relapses to depressive or anxious states, particularly if a preterm infant has long-term health conditions (138), or manifest as unrealistically high expectations of self in future pregnancies and with any other children (137). There is a paucity of evidence on the effect receiving a placental examination report has on mothers and families, however, a 2022 study conducted by Redline and colleagues (139) revealed an increased awareness of placental pathology amongst patients and patient advocacy groups. Although this appears a step in the right direction in improving knowledge of the placenta's role in PTB and long-term neonatal conditions resulting from PTB, the authors refer to personal communications in which patients and their supporters have questioned why placental pathology is not integrated into clinical care for mothers or their babies (139). Nonetheless, placental histopathological examination and the placental pathology report are not without limitation, and it is naïve to believe the placenta has the ability to explain and justify every adverse obstetric outcome. Many placental histopathological examinations find no clear reason for an adverse pregnancy event and in some cases placental histopathological lesions do not correlate to clinical outcome and are considered to be incidental findings (140). Despite an increase in placental research over recent years, there is much more to learn about the underlying physiologic and immune processes leading to placental dysfunction or injury and subsequent adverse outcome (139). Furthermore, infrequent examination of the placenta, a shortage of pre-defined standards, examiner variability and inconsistent placental pathology reports have contributed to this shortfall in truly understanding the placenta (135).

1.4.1 Placental inflammatory lesions observed on histology

As discussed, inflammation in the placenta may arise from microbial or sterile insults, either of which may be associated with premature activation of labour and PTB. On histopathological examination of the placenta, lesions resulting from this inflammatory activation may be observed and are classified according to the Amsterdam Placental Workshop Group Consensus Statement (hereafter referred to as the Amsterdam Criteria) (103). Each lesion is then staged based on progression of neutrophils through placental compartments and graded for severity based on neutrophilic confluence. Depending on where neutrophils have infiltrated, inflammatory responses are termed maternal (MIR) or fetal (FIR) (Table 1.2). The characteristic features of the principal placental inflammatory responses (PIR) are described below.

Table 1. 2 Staging and grading of maternal and fetal inflammatory responses

Placental Inflammatory Response	Stage			Grade		
	1 Early	**2 Intermediate**	**3 Advanced**	**1 Mild**	**2 Severe**	**Other**
Maternal (MIR)	Acute subchorionitis Acute chorionitis	Acute chorioamnionitis Amniotic or chorionic polymorphonuclear leukocytes	Necrotising chorioamnionitis Karyorrhexis of polymorphonuclear leukocytes Amniotic necrosis	No specific terminology/ not severe	Acute chorioamnionitis Subchorionic abscesses	Chronic chorioamnionitis Subacute chorioamnionitis
Fetal (FIR)	Chorionic vasculitis Umbilical phlebitis	Umbilical vasculitis (umbilical vein only and/or involvement of one umbilical artery) Umbilical panvasculitis	Necrotising funisitis Concentric umbilical perivasculitis	No specific terminology/not severe	Intense chorionic/ umbilical vasculitis Confluent intramural polymorphonuclear leukocytes	Fetal thrombi Peripheral funisitis Acute villitis Acute intervillositis Intervillous abscesses Decidual plasma cells

Adapted from Redline *et al.,* (2003) (141), Khong *et al.,* (2016) (103) and Parris *et al.,* (2021) (72).

1.4.2 Infection-associated placental inflammatory responses

Acute histologically diagnosed chorioamnionitis (HCA), the hallmark MIR, is categorised by neutrophilic infiltration to the amniotic epithelium, and in some cases to the amniotic fluid. In placentas of all gestations, it is the most frequently diagnosed pathology (142). HCA is considered to be an ascending infection with an associated MIAC, demonstrated by gross placental pathological findings including opaque, yellow, diffluent membranes and/or fetal surface and ease of separating the chorion from the amnion (103, 128, 143, 144). In severe cases of HCA, where a robust FIR also occurs, yellowing and discolouration is seen in the umbilical cord in combination with surface microabscesses and halos of inflammatory cells around the umbilical vessels. A noticeable, foul odour may also be present (128). A vast array of pathogens has been linked to acute HCA in both preterm and term placentas, including, but not limited to *Streptococcus agalactiae* (Group B streptococcus; GBS), *Fusobacterium nucleatum*, *Escherichia coli* (*E. coli*), *Ureaplasma* spp. and *Mycoplasma* spp. Organisms associated with PTB are often isolated from placentas delivered preterm or following PPROM, both in the presence and absence of a diagnosed chorioamnionitis (145-149). The prevalence of acute chorioamnionitis is inversely correlated with gestational age; 94% of placentas delivered between 21-24 weeks' gestation have HCA compared to only 3-5% of placentas delivered at term (142); furthermore, at term, acute HCA tends to be a consequence of non-infectious, sterile inflammation (150, 151).

Villitis is defined as chorionic villi inflammation, specifically of the villous stroma. Diagnosis of acute villitis is most commonly from histologic examination due to the general lack of macroscopic findings in placental villitis. Acute villitis often spreads haematogenously, potentially following damage to and subsequent passage through the trophoblast layer or with assistance across this protective barrier, yet the mechanism of infection remains elusive (152). Whilst uncommon, the finding of acute villitis is alarming given the high prevalence of concomitant congenital infection and fetal sepsis, which often proves fatal (153, 154). Villitis often presents with no obvious gross histopathological features but is frequently accompanied by severe HCA and the hallmark opaque, yellow or green membranes (153, 155). Inflammatory responses and neutrophilic infiltration in cases of acute villitis are primarily found under the trophoblastic basement membrane of villi and can be destructive to the villous stroma. Abscesses may also be observed within the intervillous spaces,

indicative of a placental infection with *Listeria monocytogenes* (156, 157); infectious acute villitis has additionally been linked to GBS (154). As stated previously, these organisms are associated with PTB and fetal and neonatal morbidity and mortality (154, 157, 158). For the most part, chronic villitis shows no evidence of infectious agents within the villous tissue but is characterised by an infiltration of macrophages and lymphocytes in the villous stroma. Pathogen-associated, infectious chronic villitis generally affects the whole placenta with clusters of infected villi spread diffusely throughout. Trophoblastic necrosis is observed in clusters of affected villi, possibly as a result of direct infection of these cells or a breach of the villous syncytiotrophoblast outer layer (155). Histological examination may reveal increased perivillous fibrin deposition, a finding which shows an association with recurrent PTB (143, 159). Correlating histological findings to macroscopic findings is often difficult in chronic villitis due to a lack of clinical evidence beyond decreased placental mass. Occasionally, however, firm, white or pale lesions are seen within and across the placenta (158). To further complicate diagnosis, definitive macroscopic pathologies contradictory to those seen in chronic villitis are known to correlate to the presence of specific pathogens. In contrast to the decrease in placental mass generally observed in cases of chronic villitis, infection with *Treponema pallidum* (syphilis) and cytomegalovirus (CMV) result in very large and very small but thick placentas, respectively, and neither present with an obvious reduction in overall mass (158). From here on in placental mass is referred to as placental weight consistent with the inaccurate but accepted usage colloquially and clinically (103, 136). Likewise, the conventional terminology of birthweight is used throughout this thesis, however, it is noted this refers to the mass of the neonate at birth (160). All measurements of placental and neonatal mass are accurately recorded in grams, irrespective of the terminology presented.

Prior to ~21 weeks' gestation, the fetus does not have the capacity to respond to bacterial colonisation, beyond this period, the FIR manifests as funisitis or inflammation of the umbilical cord and is characterised by neutrophils first infiltrating the umbilical vein (phlebitis). Progression to the umbilical arteries (arteritis) and into the Wharton's jelly (funisitis) may occur and is indicative of the pathological severity of the FIR (142, 161). Fetal plasma IL-6 concentrations also correlate with the severity of funisitis (161). Although an uncommon inflammatory lesion, the histologic hallmarks of a severe, stage 3 necrotising funisitis are calcification, neutrophil debris and concentric umbilical perivasculitis (142, 162),

which indicate an increased risk for adverse neonatal outcome (163). Preterm FIRs are more intense than term FIRs and it is postulated this results in MIAC at term originating from repeated digital vaginal examinations during labour in contrast to preterm MIAC which is likely to have been established prior to PPROM or preterm labour (142). Gross placental pathological findings are uncommon in cases of funisitis; however, maternal fever is a more frequent clinical correlation with acute funisitis subsequently diagnosed histopathologically than placentas without this lesion (15.2% vs. 2.2%). Additionally, acute funisitis is associated with an increased prevalence of membrane inflammation (8.7% vs. 2.2%) (164). Although rare, funisitis and vasculitis represent a severe FIR and are associated with high grade and stage of chorioamnionitis, therefore, early interventions are vital to reduce the risk of adverse neonatal outcomes (164).

1.4.3 Non-infectious placental inflammatory responses

The presence of permeating lymphocytes in the chorion or amnion, plus scant inflammatory cells, are diagnostic features of chronic chorioamnionitis (CCA), although this is a rare inflammatory lesion and is not considered to be from an infectious agent (143, 165). An additional characteristic observed in CCA but not HCA is the presence of a thickened chorioamniotic membrane and defined fibrosis, both of which are observed on gross and microscopic assessment. In cases of subacute chorioamnionitis, necrosis is present, and a combination of neutrophils and mononuclear cells are observed to infiltrate the subamniotic tissue in a zonal manner. Unlike in HCA, it is not possible to separate the amniotic membranes due to adhesions forming between the layers (143). Often, chorioamnionitis is subclinical with no defining features; both PPROM and PTB are reported as frequent consequences of a subclinical chorioamnionitis. However, it should be noted that PPROM itself can also lead to an increased risk of chorioamnionitis, thereby adding complexity to determining whether the chorioamnionitis is the cause or consequence of PPROM (145). Due to the infrequency of CCA, scant recent evidence of prevalence is available, the same applies for the overlapping lesions of subacute and subclinical chorioamnionitis (166, 167). Reports from 2010 show more than a four-fold increase in the presence of CCA in placentas from preterm labouring women compared to those who delivered at term following labour (34% vs. 8%) (165), corroborated more recently by Lee and colleagues who demonstrated the frequency of CCA in PTBs was

almost double that in term births (20.8% vs. 10.5%) (168). Of interest, Lee's study also revealed CCA to be the most frequently diagnosed lesion in late PTBs (168).

Villitis in the absence of identifiable pathogens is termed villitis of unknown aetiology (VUE). Characteristically for VUE, there is sporadic placental villi infiltration of inflammatory maternal $CD3^+$ T cells, $CD8^+$ T cells and activated maternal and fetal $CD68^+$ macrophages in the absence of any infectious cause (155). Placentas are often small for gestational age with diffuse inflammatory lesions or, alternatively, have vast perivillous fibrin deposition (PVFD) and are large for gestational age. Most frequently lymphocytes are observed histologically as infiltrating the villi. Where subchorionic VUE is found, the effects are generally more severe and can result in obliterative fetal vasculopathy, defined as luminal destruction and avascular villi (103, 169). Notably, VUE occurs more frequently at term; reports suggest it occurs in up to 15% of births >37 weeks, and, for the most part, it does not appear to result in obstetric complications in these cases (155, 170), instead, it is believed the inflammatory process is a maternally-driven immune response to the developing fetus (170). Further evidence to support this has been described by Kim *et al*., (2015) (171) who noted in previous studies that mothers receiving donated eggs experienced VUE more frequently. Ovum donation represents a complete fetal allograft as opposed to the normal semiallogenic fetus; increased prevalence of VUE in these cases strengthens the hypothesis that VUE is a negative maternal immunological response to the fetus akin to graft versus host response (72, 143, 155). Nonetheless, in cases of reported VUE, evidence shows an increased risk of spontaneous abortion, preterm labour, IUGR and a small for gestational age infant, with adverse outcomes positively correlated to disease severity (155). However, conflicting data are presented as to the prevalence of VUE in PTBs; in 2015, Iskender *et al.,* reported similar VUE rates between preterm and control groups (172), contrary to findings of a study of over 1600 placentas conducted in Bangalore in 2020 which observed villitis in 10% of the entire cohort. Of this villitis-reported cohort (163 placentas), 59.5% were delivered preterm and from those, the authors describe over 64% being diagnosed with VUE, albeit predominantly of low-grade (173). Whilst much is to be determined about the pathology of VUE, its contribution to negative obstetric outcomes remains unchallenged.

1.5 Viral infection of the placenta and preterm birth

Studies have shown viral pathogens to be associated with PTB, including adenovirus, cytomegalovirus (CMV), enterovirus, herpesviruses, human immunodeficiency virus (HIV), influenza A (H1N1), parvovirus B_{19}, respiratory syncytial virus (RSV), rubella virus and, most recently, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (174-177). Predominantly, however, viral DNA or RNA has been located in amniotic fluid samples rather than the placenta (175, 176), or the virus-associated PTB has been iatrogenic due to maternal or fetal disease (177). Of the listed viruses, CMV, herpesviruses, HIV, rubella virus and SARS-CoV-2 have been shown to have the potential to infect the placenta and cause spontaneous PTB (35, 155, 177-180). Infection with rubella virus is rare in the UK and other developed countries due to robust vaccination programmes virtually eradicating the disease (179). Placental pathology where rubella infection is observed is unique in that rather than the normal chronic villitis features, cytoplasmic or intra-nuclear viral inclusions of round eosinophilic cells are seen across the placenta in decidual cells, trophoblasts, the amnion and in endothelial cells (181). Histopathological examination of placentas from women who are HIV-positive shows chronic inflammatory lesions including CCA and VUE and, on occasion, acute villitis suggestive of fetal septicaemia (155). Considering the scarcity of rubella infection in the UK, this thesis does not examine this virus further. Likewise, as the prevalence of HIV in the UK is low, reported as 0.08% in December 2019 (182), it is unlikely to be a major contributory factor in the cohort studied for this thesis. Therefore, neither HIV or rubella virus were included for review or analysis. CMV and the herpes simplex viruses 1 and 2 were initially selected to ascertain their prevalence and location in preterm and term placentas via immunohistochemical (IHC) staining and, following the outbreak of SARS-CoV-2 in December 2019 and the subsequent links between maternal Covid-19 disease and PTB, IHC staining for SARS-CoV-2 was retrospectively included.

1.5.1 Histopathological findings in placental CMV, HSV-1/2 and SARS-CoV-2 infection

A detailed review of CMV, HSV-1/2 and SARS-CoV-2 is provided in *Chapter 5*. Briefly, gross pathology following chronic CMV infection of the placenta reveals a pale and hydropic-appearing placenta with decreased weight, fibrosis and a shrunken appearance (54). Microscopically, placental CMV infection shows a distinct observable immune cell infiltration of lymphocytes and histiocytes, haemosiderin deposition, chorionic plate thrombi and

marked trophoblastic necrosis, however, in many cases of CMV infection, no placental pathology is seen (183-185). CMV inclusions, although patchy, are evident in Hofbauer and trophoblastic epithelial cells on haematoxylin and eosin (H&E) staining and present with an 'owl-eye' or 'halo' appearance (54). Placental HSV-1/2 infections are rare, however, in many cases, placentas are not examined as HSV-infected neonates do not present with complications at or close to delivery. Villous necrosis, chronic intervillositis and necrotising chorioamnionitis are characteristic findings in HSV-1/2 placental infection (186). Comparable to CMV, necrotic Hofbauer and trophoblastic epithelial cells may be detected and HSV inclusion bodies, known as Cowdry type B, are observed with a 'ground-glass' appearance in the nucleus of these cells (187). Placental infection with SARS-CoV-2 presents with an atypical viral pattern in that inflammation is less prevalent than maternal and fetal vascular malperfusion lesions (MVM and FVM, respectively) and almost one-third of infected placentas have increased PVFD (188, 189).

1.6 Placental macrophages

The placenta harbours a unique suite of immune cells which alters dynamically in both composition and function throughout gestation in order to maintain immune tolerance of the semi-allogenic fetus and respond to pathogenic insults (190, 191). Within the compartments of the placenta, variations in these immune cell configurations ensure protection of the fetus from invading pathogens, congenital infections, or a breakdown in maternal immunotolerance (190). Leukocytes are the most abundant cell in the decidua in early pregnancy, comprising of up to 40% of all cells, with ~70% of these being natural killer (NK) cells (192). This complement of B cells, dendritic cells (DC), macrophages, NK cells and T cells contributes to early pregnancy establishment, placentation, spiral artery remodelling and modulation of immune tolerance (190, 192). The placental villous tissue differs in immune composition to that of the decidua in that its primary immune cells are macrophages, specifically fetally-derived macrophages termed Hofbauer cells (HBC). These are considered to be crucial for placental development and immune tolerance and are diffusely spread throughout placental villi and into the chorionic villi (192-194). In contrast to HBCs, decidual macrophages and those found within the intervillous space are generally considered to be maternally-derived (190). Despite advances in cell sorting techniques, the exact function, location and specific composition of HBCs throughout pregnancy remains elusive (192, 194).

First identified by Metchkinoff in 1882 and usually derived from circulating monocytes, macrophages are critical contributors to immuno-protective and pathogenic environments and are involved in a diverse range of processes such as wound-healing, phagocytosis of pathogens and autoimmunity (195-197). Tissue-resident macrophages are found in organs throughout the human body including Kupffer cells in the liver, alveolar macrophages in the lungs, osteoclasts in bone and, as stated, Hofbauer cells in the placenta; commonly each is involved in homeostasis within their respective organ (197, 198). Macrophages are highly plastic which results in the ability to respond rapidly to inflammatory and environmental stimuli and to alter their intracellular metabolism (197). Circulating macrophages infiltrate tissues in response to these chemotactic cues and are activated according to one of two pathways, either *classically* to a proinflammatory M1 phenotype following LPS or interferon-gamma (IFN-γ) stimulation, or *alternatively* to an anti-inflammatory M2 phenotype via interleukin-4 (IL-4) or interleukin-10 (IL-10) stimulation. Nonetheless, it has been demonstrated that tissue-specific combinations of signalling factors beyond this linear spectrum are involved in activation of more diverse, stimulus-dependent phenotypes (195, 199, 200). Macrophage polarisation to either M1 or M2 phenotypes results from antagonistic pathways of arginine metabolism (201), with LPS stimulation driving inducible nitric oxide synthase (iNOS) to produce nitric oxide (NO) and citrulline to shift macrophages to an M1 phenotype. Subsequently, proliferation is inhibited and microbiocidal functions are promoted. Conversely, IL-4 or IL-10 stimulation induces the metabolism of arginine to ornithine and urea through hydrolysis of arginase which promotes proliferation and wound healing (198, 201, 202).

Placental macrophages are comprised of two discrete populations, that is, maternal decidual macrophages and HBCs, considered to be of M1 and M2 phenotypes, respectively (196). Aberrant polarisation of macrophages in the placenta and at the maternal-fetal interface has been implicated in pregnancy pathologies including spontaneous abortion, preeclampsia and PTB (201). In mouse models of PTB, macrophages contribute to inflammation by polarisation to an M1 phenotype and induction of MMPs which subsequently degrade collagen in the cervix. In humans, elevated numbers of macrophages are seen in the cervix of women delivering prematurely, thus supporting the involvement in some capacity of maternal macrophages in preterm labour processes (201). Altered functional activity of the monocyte-

macrophage system manifests as dysregulated or abnormal maternal immune responses including fetal-allograft rejection and dysfunctional initiation of labour which may also contribute to PTB (203-205). Alongside phenotypic differences between maternal macrophages and HBCs, fetally-derived macrophages are localised close to trophoblast cells and fetal vessels, a finding described by Castelluci *et al*. in 1980 following a three-dimensional study of the villous stroma core (206). Although little has changed in the understanding of the role of HBCs, development of visualisation techniques has resulted in a clearer description of their location within the placenta which extends to HBCs now shown in the amnion, chorionic laeve and within the placental villous core (207). Observed as early as day 18 post-fertilisation (192), functionally and morphologically, HBCs most closely resemble homeostatic, anti-inflammatory M2 phenotype macrophages (194, 207), yet, despite extensive research into macrophage plasticity, HBCs do not appear to polarise to an M1 phenotype in response to microbial infection (208). They do, however, release proinflammatory cytokines, induce fibrosis and contribute to chronic inflammation as well as sequestering HIV-1 without replication (196, 207). The role of HBCs in placental physiology is understudied, but evidence indicates they contribute to maternal-fetal antibody transmission through fragment crystallisable receptors (FcR) expressed on their surface and are involved in nutrient transport (194). Their early presentation also suggests placentation and immunotolerance are central functions and that HBCs may, like other tissue-resident macrophages, regulate angiogenesis, specifically in early pregnancy villi (209, 210). Although a proinflammatory role of HBCs via a shift to an M1 phenotype has not been defined, Tang *et al.* (191) showed a two- to three-fold increase in placental macrophages in the villous stroma in the presence of HCA when compared to gestational age-matched controls. Preceding studies had been unable to definitively show similar results with some reporting HCA to elevate macrophage numbers (211) and others reporting the converse (212). Nonetheless, it is agreed that numbers and functions of HBCs, and indeed decidual macrophages, is altered in the presence of placental inflammation (194, 213), but research on how this affects maternofetal tolerance and PTB continues to be neglected (194).

1.7 Placental inflammatory mediators of pregnancy and parturition

Pregnancy and labour are tightly controlled inflammatory events mediated by a plethora of immunomodulatory proteins, yet the role of those produced by the placenta and fetal membranes is underappreciated and seldom studied (214, 215). Throughout gestation, the assortment of antimicrobial proteins, cytokines, chemokines, matrix metalloproteinases and other proteins capable of regulating immune processes fluctuates. Gestational homeostasis and maternofetal tolerance are established through a carefully choreographed set of interactions between cells, stimuli and their receptors. Diverse cell types secrete these proteins, and the cells and tissues which respond to individual and collective stimuli vary as pregnancy progresses (214, 216). Characteristics of selected fundamental placental mediators concerned with pregnancy, parturition and PTB are described below.

1.7.1 Antimicrobial proteins

There is minimal knowledge of natural antimicrobial proteins (AMP) during pregnancy and parturition. AMPs are a diverse class of peptides and proteins produced as a first line of defence in multicellular organisms. Also known as host defence peptides (HDPs), they form an essential component of the innate immune system in higher organisms and are produced by bacteria to kill competing bacteria (217). AMPs exhibit extensive antimicrobial activity, which includes Gram-negative and Gram-positive bacteria, mycobacteria, fungi and viruses. The ability of AMPs to bind and neutralise bacterial endotoxins, coupled with their anti-inflammatory, immunomodulatory and wound-healing properties, makes them promising therapeutic agents (217, 218). AMPs display structural and functional commonalities. They are small, amphipathic and generally cationic proteins consisting of 10-60 amino acids with an excess of basic and hydrophobic arginine, histidine and lysine residues (219, 220). The structural class, source, activity and amino acid-rich species determines each of the 3,240 identified AMPs with the secondary structure classifying each protein as either an α-helix, β-sheet or extended peptide (220). Through upregulation of inflammatory cytokines and chemokines, such as IL-6 and IL-8, AMPs recruit leukocytes to sites of inflammation and aid in bacterial clearance. Additionally, AMPs are able to modulate the intracellular signalling pathway Nuclear Factor kappa B (NFκB) via ligand binding, activation and oligomerisation of their molecular target, epidermal growth factor receptor B2 (ErbB2), which is expressed on a variety of cell types, including endothelial and epithelial cells (221, 222).

1.7.1.1 Human cathelicidin antimicrobial protein 18

The amphipathic, α-helical LL-37, so termed due to the two 2 leucines and 37 amino acids at the N-terminal, is the only AMP derived from human cathelicidin antimicrobial protein 18 (hCAP18), itself the only human cathelicidin identified to date (223, 224). This active component of hCAP18, LL-37, is found in abundance in granules of neutrophils but can also be detected in epithelial cells, lymphocytes and monocytes, as well as showing high plasma concentrations (221, 223). Gestational tissues contain hCAP18 and concentrations increase synergistically with proinflammatory cytokines including IL-6, IL-8 and PGE_2 in the myometrium and fetal membranes of labouring women at term (214, 225). High LL-37 RNA and protein concentrations have been detected on fetal skin and within the vernix caseosa, further highlighting the protective nature of this AMP (226). Inflammatory pathways control LL-37 expression and, alongside its antimicrobial role, it demonstrates immunomodulatory properties including macrophage polarisation to an M1 phenotype (227), potentially assisting in both implantation and parturition processes. Crucially, LL-37 and its precursor protein hCAP18 are inhibitors of an array of ErbB-dependent viral infections, including CMV, which persist through host cell survival and immune evasion. SARS coronaviruses exploit ErbB to enter host cells by inducing epidermal growth factor receptor-dependent micropinocytosis; more recently, studies have shown LL-37 to bind to the angiotensin converting enzyme-2 (ACE2) receptor necessary for SARS-CoV-2 viral entry to host cells, thus conferring protection from infection (222, 228). LL-37 also exhibits specific antibacterial activity against Group B Streptococcus, a bacterium where maternal colonisation has been associated with PTB (226, 229). As such, hCAP18 and its active component LL-37 present a potential therapeutic agent to attenuate the effects of bacterial and viral infections in pregnancy.

1.7.1.2 Secretory leukocyte protease inhibitor

Secretory leukocyte protease inhibitor (SLPI) is a 107-amino acid, 11.7 kDa nonglycosylated antimicrobial protein with distinct HDP properties (230-232). As a member of the whey acidic protein (WAP) family, SLPI is the dominant inhibitor of neutrophil elastases (NE), enzymes stored in the azur granules of neutrophils which are involved in the degradation of a broad range of extracellular matrix components, immunoglobulins and coagulation proteins (233-235). SLPI has three main functions, to act as an antiviral protein, suppress inflammatory responses via modulation of NFκB and limit protease activity; it also has the ability to

neutralise the endotoxic effects of Gram-negative bacterial cell wall components such as LPS (231, 232). Primarily a secretory product of epithelial cells, SLPI is present in first trimester and term decidua, as well as term amnion epithelium (226, 231, 236). Nonetheless, transient SLPI expression is reported in myeloid cells including granulocytes such as neutrophils, dendritic cells and macrophages (231, 232). As for hCAP18 and LL-37, high concentrations of SLPI protein have been detected on fetal skin and within the vernix caseosa (226). Additionally, SLPI protein expression has been reported from chorion and placental trophoblast cells (237) and from the cervix and cervical mucus plug (238); however, more recent studies have been unable to consistently replicate these findings. From the second trimester towards term, amniotic fluid SLPI protein expression levels increase, likely due to stimulation from normal upregulation of inflammatory cytokines IL-1β, IL-6 and TNF-α at parturition when placental, amniotic membrane and uterine cells are in an inflammatory state (226, 233, 236, 239). In contrast, SLPI protein expression is decreased when associated with PPROM, but PTL with accompanying infection elicits a greater increase in inflammatory cytokine production and subsequently elevated amniotic fluid SLPI concentrations (236, 239).

Despite the evidence that AMPs may contribute to pregnancy maintenance and infection-associated pregnancy pathologies, including PPROM and PTB, and that elevated levels of α-defensins such as hCAP18 and SLPI correlate with an increased risk of PTB prior to 32 weeks' gestation, there is a dearth of research, especially recent studies, considering the influence of placental hCAP18 and SLPI on PTB (226, 240). This study, therefore, sought to examine the association between placental hCAP18 and SLPI protein levels, placental inflammation and preterm birth.

1.7.2 Cytokines

Cytokines are small, enzymatic glycoproteins with specific and redundant functions, i.e., they are both diverse and overlapping (241). Their function as inter- and intracellular signalling molecules, combined with pleiotropic activities and synergistic activity in various complex networks, influences the production, release and action of multiple other cytokines, chemokines and AMPs (242). Cytokines range in molecular mass from 6kDa (IL-8) to 70kDa (IFN-γ) (241, 243), but, in spite of their harmony in function and molecular mass, their structure is heterogeneous (244). Several cytokines act systematically, affecting biological

processes such as inflammation and wound healing. Although this behaviour is akin to hormones, cytokines differ in that they are not produced by specialist cells and the breadth of their target cells is more extensive (241). Cytokines are divided into six sub-families, chemokines (discussed below), haematopoietins, interferons, interleukins, transforming growth factor betas and tumour necrosis factors, each with distinct modes of action. Pro- and anti-inflammatory cytokines have been identified, however, due to the synergistic and antagonistic influences of cytokines, this distinction is often distorted (245). Cytokine production during pregnancy is pivotal to successful implantation, establishment and maintenance of immunological tolerance and initiation of parturition processes, as described in further detail below (216), however, in a study conducted by Pavlov *et al.* (2020) it was justifiably noted that since these regulatory processes are often locally controlled, it is challenging to ascertain the engagement of specific placental cell types (246). Pavlov and colleagues' study analysed macrophages and villous tissue isolated from first and third trimester placentas and revealed significantly increased expression levels of a panel of cytokines involved in control of inflammatory responses previously associated with spontaneous labour at term and preterm, IL-1β, IL-6, IL-8, IL-10 and TNF-α, between the first and third trimesters. Furthermore, stimulation (23 hours) with LPS significantly increased intracellular expression of IL-1β, IL-6, IL-8, IL-10 and TNF-α in placental macrophages indicating a contribution from pro-inflammatory macrophages to global placental cytokine production and extraplacental levels (246). Placental macrophages were also shown to constitutively express cytokines impervious to LPS stimulation, including IL-11, IL-17A, IL-17F, TGF-β and VEGF, which the authors suggest primarily exert local effects with responsibility for modulation of morphogenic rather than immunogenic processes (246). These inflammatory mediators, amongst others, are also reported to be secreted from non-macrophage cell types in the placenta, each with physiological and pathological roles. Table 1.3 below summarises the cell populations known to secrete these essential inflammatory mediators plus their major physiological and pathological functions during pregnancy.

Table 1. 3 Cytokine secretion by cell type and physiological and pathological roles in pregnancy

Cytokine	Cell Type	Physiological Role	Pathological Role
IFN-γ	$CD8^+$ T, Mac, NK, STb (constitutively)	Provides STb and neighbouring cells with resistance to viruses and TORCH pathogens (248) Aids in clearance of viruses from infected tissues (249)	Contributes to placental damage through inadequate spiral artery remodelling (216) Dysregulation leads to ineffective viral clearance and potential viral transmission to fetus (249)
IL-1β	Mac, Tb	Induces muscle contraction and activates MMPs to degrade amniotic membrane ECM at parturition (248, 250)	Dysregulation may induce PTB (251), placental inflammation and impaired fetal growth (252)
IL-6	$CD8^+$ T, CTb, dNK, DSC, EVT, Mac, STb	Promotes implantation success via stimulation of trophoblastic invasion and migration. Regulates essential placental hormone synthesis (253) Stimulates oxytocin secretion and expression of oxytocin receptors (253)	Increased expression in response to inflammatory stimuli may lead to recurrent implantation failure, miscarriage, maternal immune activation, acute placental inflammation and PTB (253)
IL-8	$CD8^+$ T, CTb, dNK, DSC, EVT, GEC, Mac, STb	Promotes monocyte-macrophage proliferation and differentiation, spiral artery remodelling and regulates trophoblastic invasion. Mediates parturition-associated cervical remodelling and membrane rupture (253)	Increased expression in response to inflammatory stimuli leading to maternal immune activation, acute placental inflammation, PPROM and PTB (253)
IL-10	CTb, dNK, Mac, T_{Reg}	Downregulates inflammatory response of PTL through attenuation of IL-1β, MMPs and TNF-α (254, 255)	Deficiency induces fetal resorption or PTB in mouse model through activation of cytotoxic dNK cells, T cells or macrophages coupled with production of TNF-α (255)
TNF-α	CTb, Mac, STb, VSC	Regulates trophoblastic and vascular smooth muscle cell apoptosis. Promotes EVT differentiation, trophoblast syncytialisation and hCG expression (250) Induces muscle contraction and activates MMPs to degrade amniotic ECM at parturition (248, 250)	Induces PPROM and PTB (250, 251, 254) Overexpression may lead to placental inflammation and excessive destruction of syncytiotrophoblast (250)

$CD8^+$ T; cytotoxic T cell*, CTb;* cytotrophoblasts, *dNK;* decidual natural killer cell, *DSC;* decidual stromal cell, *ECM;* extracellular matrix*, EVT;* extravillous trophoblast, *GEC;* glandular epithelial cell*, hCG;* human chorionic gonadotrophin*, Mac*, macrophage*, MMP;* matrix metalloproteinase*, NK;* natural killer cell*, PPROM;* preterm prelabour rupture of membranes*, PTB;* preterm birth, *PTL;* preterm labour*, STb;* syncytiotrophoblast*, Tb;* trophoblast, *T_{Reg};* regulatory T cell*, VSC*; vascular stromal cell. The acronym TORCH relates to toxoplasmosis, other (syphilis), rubella, cytomegalovirus, and herpes simplex virus.

1.7.3 Chemokines

Considerably smaller than cytokines at only 70-100 amino acids in length and 8 to 12 kDa in molecular mass, chemokines are the largest sub-family of cytokines with chemotactic properties and an ability to activate leukocytes (256, 257). Structurally homologous, chemokines comprise of an α-helix at the C-terminal, a three-stranded β-sheet and four cysteines which characteristically form intrachain disulphide bridges (256-258). The chemokine family is divided into subgroups dependent on the positioning of the first two cysteines; in the CXC and CX3C groups these amino-terminal cysteines are separated by one and three amino acids, respectively, whereas in the CC group the cysteine residues are adjacent (257, 259). Primarily, chemokines regulate leukocyte trafficking, a crucial process in homeostasis and immune response to infiltrating pathogens (257). Chemokine activity is controlled by their binding to heterotrimeric Gi proteins, a gamma-subfamily group of G-protein-coupled receptors (GPCR) (257, 258). Although approximately fifty chemokines have been identified, only nineteen receptors bind these proteins resulting in an indiscriminate and divergent response by the immune system dependent upon the activating chemokine (257). Proinflammatory cytokines and chemokines such as IL-6 and IL-8 (CXCL8) have been associated with labour onset with elevated expression levels in all gestational tissues in women experiencing spontaneous term labour (214, 260), yet contradictory results have been reported in respect of their expression levels specifically in the placenta when associated with labour (214, 260, 261). For example, a systematic review conducted by Hadley *et al.* (2018) described study findings of no change in placental IL-6 or IL-8 in laboured versus non-laboured tissue (262, 263). Additionally, having postulated that increased IL-6 expression would reduce expression of the oxytocinase, placental leucine aminopeptidase, subsequently increasing functional oxytocin concentration and initiating uterine contractions, Ikoma *et al.* (2003) found there to be no response in BeWo cells following 72 hours of incubation with 10ng/ml IL-6 (264). Unlike the numerous studies focused on proinflammatory cytokine expression, and despite some anti-inflammatory and antiviral cytokines having also been linked to maintenance of pregnancy and labour onset, there is a lack of research into these cytokines, specifically IL-10 and IFN-γ (214, 265, 266). Extending this further, consideration into the role of both pro- and anti-inflammatory cytokines in unison or in preterm labour onset, PPROM and where chorioamnionitis is histologically-diagnosed requires significant attention (267).

1.7.4 Matrix metalloproteinases

MMPs are a family of zinc-dependent endopeptidases with a range of biological functions including wound repair and inflammation, predominantly through their activities against extracellular matrix (ECM) components, especially collagen and elastin (268, 269). Almost all MMPs contain a COOH-terminal hemopexin domain (PEX), a structural feature vital for interactions with inhibitors and cell surface receptors, as well as substrate specificity (270). MMPs are classified according to this substrate specificity and their primary amino acid sequences and are termed as collagenases (MMP-1 to MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MMP-14 to MMP-17 and MMP-24 to MMP-25) and stromelysins (MMP-3, MMP-10 and MMP-11) (271). Multiple MMPs have been implicated in the pathophysiology of PTB, and polymorphisms that increase promoter activity in MMP-encoding genes have been associated with PPROM through candidate gene studies (75). Although stromelysins and collagenases have the same domain arrangement, collagenases cleave interstitial collagen but stromelysins preferentially degrade elastin, laminin and fibronectin (272). Subsequently, their roles in PTB differ, with MMP-8, or neutrophil collagenase, having been shown to be a valuable biomarker of clinical chorioamnionitis and fetal inflammatory response syndrome (273) whereas MMP-3 and MMP-10 are known to contribute to preterm labour through remodelling of the myometrial extracellular matrix (ECM) (274). Previous studies have shown elevated amniotic fluid, myometrial tissue and serum concentrations of the gelatinases MMP-2 and MMP-9 in women delivering preterm (275) and that increased gelatinase activity results in the degradation of ECM and a cascade leading to rupture of membranes (276). A study conducted by Xu, Alfaidy and Challis in 2002 revealed significantly elevated MMP-9 levels in placentas from women who had laboured compared to non-labouring women. Having localised MMP-9 to the syncytiotrophoblasts, the authors suggested placental villi were the source of the elevated MMP-9 following labour induction and that circulating MMP-9 may be a predictive marker of PTB (276). Since Xu *et al*.'s study two decades ago, minimal further investigation of the role of placental MMP-9 in PTB has been undertaken, although it has been shown that MMP-9 mRNA is increased in placentas from spontaneous preterm vaginal deliveries compared to those at term (269). For this reason, MMP-9 was chosen for analysis in the present study. Furthermore, MMP-9 has been shown to cleave both the C-terminal and N-terminal functional domains of SLPI resulting in reduced antiproteolytic activity against

neutrophil elastase and a decreased ability to bind LPS. SLPI is expressed by inflammatory cells, including activated macrophages and neutrophils, and is able to disrupt IκBα and IκBβ degradation which results in inhibition of NF-κB (277), therefore, it was hypothesised that any identified alterations to the complement of macrophages, or expression of SLPI would correlate to altered MMP-9 expression and this was a second factor for inclusion of MMP-9 in the study.

1.7.4.1 MMP-9 overview

MMP-9, or gelatinase B, is responsible for direct proteolysis of type 1 and type IV collagen (268). It is a 92kDa proenzyme first detected in neutrophils in 1974; oxidative stress, other MMPs or removal of the prodomain by serine proteases such as NE activates MMP-9 to a mature 83kDa enzyme capable of performing proteolytic functions (268). Unique to MMP-9, a 64 amino acid flexible O-glycosylated domain links the PEX domain to the catalytic domain which is formed from the Zn^{2+}-binding and active sites. Without this, MMP-9 is unable to perform its proteolytic functions (270). MMPs are critical for normal tissue remodelling and MMP-9 has a central role in endometrial tissue remodelling in healthy pregnancy (271), from the first trimester when it is required for remodelling of the spiral arteries and early placenta (268), through to the latter stages of parturition, membrane rupture (271) and placental detachment (269). Gene expression for MMPs, including those for MMP-9, is regulated transcriptionally by extracellular agonists including IFN-γ, IL-6, IL-8 and IL-10 (278); this combined with its ability to degrade vascular basement membrane proteins makes MMP-9 a key potential biomarker of PPROM and preterm labour (268).

1.7.5 Revisiting the Th1/Th2 dichotomy of pregnancy

As previously highlighted, pregnancy is an immunologically intricate state requiring subtle interactions between immune cell subsets to ensure maintenance of maternofetal tolerance whilst avoiding compromise to the mother (279). The T helper 1/T helper 2 (Th1/Th2) paradigm has long been established as characterising $CD4^+$ T cell clones by their nonoverlapping cytokine secretion patterns, specifically IL-2 and IFN-γ secretion from Th1 cells and IL-4 and IL-5 from Th2 cells. When first proposed by Mossman and colleagues in 1986, it was considered that Th1 cells were responsible for mediating cellular immunity essential for protection against intracellular pathogens while Th2 cells supported humoral

immunity through secretion of Immunoglobulin (Ig) G and IgE and promotion of immediate-type hypersensitivity reactions (280). In the context of pregnancy, the Th1/Th2 paradigm explained, in part, the ability of the maternal immune system to strike the delicate balance required to protect the developing fetus whilst simultaneously evading potential maternal threats (281). The widely-accepted theory of a shift towards a Th2 bias resulting in maintenance of pregnancy was supported with studies detailing how an aberrant Th1/Th2 balance led to early pregnancy loss, preeclampsia, and preterm labour, as reviewed by Sykes *et al*. (279). However, the field of reproductive immunology has rapidly evolved over the last ten years, and it is now understood that a greater complexity in the establishment of maternal tolerance towards the semi-allogeneic fetus exists, as discussed in a comprehensive review published in 2020 (216). Rather than the simplistic, almost binary, switch from Th1 to Th2 dominance previously suggested, Wang and colleagues present a compelling array of research findings to illustrate the dynamic temporal fluctuations between Th1/Th2 dominance required to maintain the pregnancy, each of which is supported by other T cell subsets including Th9, Th17, Th22, T cytotoxic (Tc) and T regulatory (T_{Reg}) cells (216). The authors highlight recent evidence describing the dissonance between the originally proposed gestational Th2 bias and the necessity for a Th1 dominance during the peri-implantation period and placentation to ensure adequate pro-inflammatory cytokine secretion and regulated trophoblastic invasion (216, 278). Early polarisation to a Th1 predisposition is achieved primarily through a defined Th1 secretion pattern of IL-2, TNF-α and IFN-γ (216, 282). Post-early placentation, an anti-inflammatory Th2 dominance is seemingly the prerequisite for establishing maternofetal tolerance, driven by infiltration of Th2 cells into the decidua basalis, potentially mediated by prostaglandin D2, and trophoblastic activation of Th2 cells at the maternofetal junction (283). However, it is unclear whether Th2 dominance arises solely from enhanced Th2 cytokine expression, repressed Th1 immunity or a combination of the two (216). What is evident, though, is that increased levels of Th2 cytokines is a local phenomenon and not an intrinsic characteristic of Th2 cells (284). Previous studies have reported the Th2 cytokine, IL-10, to downregulate cytokine production by Th1 cells, attenuate lipopolysaccharide-induced proinflammatory responses such as uterine TNF-α expression, nitric oxide release and uterine epithelial cell apoptosis, and to inhibit multiple macrophage functions (284, 285). It is therefore suggested that the primary function of the shift to Th2 bias, in association with other T cell subtypes, is not to dampen the Th1 response *per se*, but

to strictly regulate and maintain expression levels required to provide angiogenic, vascularisation and antiviral functions by moderately raising Th2 expression levels. The noted return to Th1 dominance in preparation for parturition is thus explained by a withdrawal or decrease of Th2 cytokine expression and activation of proinflammatory pathways (216).

1.7.6 M1/M2 macrophage dichotomy of pregnancy

Interesting parallels have been made between the Th1/Th2 dichotomy and M1/M2 macrophage polarisation throughout gestation; dysregulation of M1/M2 proportions has similarly been linked to inadequate trophoblastic invasion and spiral artery remodelling resulting in subsequent adverse pregnancy outcomes (201, 286, 287). However, in contrast to the previously discussed gestational Th1/Th2 cell biases, there is no clear consensus on the polarisation state or relative abundance of macrophages throughout key pregnancy stages. Shynlova *et al*. reported macrophages to account for approximately 25% of $CD45^+$ leukocytes in the human uterus, a relative percentage the authors state does not significantly change throughout pregnancy, thus suggesting no alterations to the inflammatory state (287). Further complicating this debate is that there is no agreement on whether CD45 is a phenotypic marker of classically-activated M1 macrophages (288) or alternatively-activated M2 macrophages (289), therefore studies utilising this common leukocyte marker rather than more defined M1 and M2 markers may not be reporting the true macrophage polarisation state throughout gestation. Nonetheless, comparable to the early Th1 bias, peri-implantation and placentation appear to be characterised by a predominance of M1 proinflammatory macrophages, but, unlike the reported discrete and rapid shift to Th2 immunity on completion of implantation (216), the post-implantation macrophage polarisation is reported to be to a heterogeneous M1/M2 population until the early second trimester (201, 289). It is suggested that tissue-resident HBCs then skew towards an M2 phenotype, yet it is not certain whether any polarisation occurs in decidual macrophages (289). Known for their tissue remodelling properties and ability to modulate proinflammatory M1-like decidual macrophages, it is postulated that this M2 dominance promoted by HBCs is an essential component of pregnancy maintenance and successful outcome (201, 289, 290). Prior to initiation of labour at term, and analogous to that of Th1 immunity, macrophage dominance reverts to M1 phenotypes stimulated by increased uterine IL-6, IFN-γ and TNF-α secretion and classical activation of decidual macrophages (287, 289). Table 1.4 summarises the purported dynamic

changes of T helper cells and macrophages during pregnancy, along with the expressed mediators primarily understood to initiate and regulate these changes. Whilst there are a number of similarities in the T cell and macrophage inflammatory states and their mediators, it is evident that these immune cell populations are also influenced separately from each other.

Table 1. 4 T cell and macrophage dynamics throughout pregnancy

Gestational Timepoint	T Cell Bias	Predominant T Cell Secretions	Macrophage Bias	Predominant Macrophage Secretions
Peri-implantation	Th1	IFN-γ, IL-2, TNF-α	M1	IL-1β, IL-6, IL-12, NO, TNF-α
Placentation	Th1	IFN-γ, IL-2, TNF-α	M1	IFN-γ, IL-6
First Trimester	Th1	IFN-γ, IL-2, TNF-α	M1/M2	IL-10, VEGF
Early Second Trimester	Th2	IL-1β, IL-6, IL-8, IL-10, TNF-α	M1/M2	IL-10, VEGF
Second Trimester	Th2	IL-4, IL-10	M2	Arg, IL-10, TGF-β
Early Third Trimester	Th1	IL-8, TNF-α	M2	Arg, IL-10, TGF-β
Parturition	Th1	IL-8, TNF-α	M1	IL-1β, IL-6, IFN-γ, TNF-α

Arg; arginine*, IL-;* interleukin, *IFN-γ;* interferon-gamma*, NO;* nitric oxide*, TGF-β;* transforming growth factor-beta, *TNF-α;* tumour necrosis factor-alpha, *VEGF;* vascular endothelial growth factor.

Although evidence alludes to Th2 immunity and M2 polarisation moderating untimely inflammation, and promoting positive obstetric outcomes, it would be naïve to accept such a binary and oversimplified view and no work has been done to firm this link up. Recent studies favour *timing* of the dominance shift, be it of T helper cells or macrophages, to be the critical feature of establishing maternofetal tolerance (216, 289), yet, defining these key timepoints beyond early, mid and late gestation remains a neglected research area. Additionally, in respect of macrophages, there is a paucity in studies which consider the effects of absolute numbers, polarisation and proportions of M1 and M2 phenotypes throughout gestation. Further appraisal of Th1/Th2 profiles throughout pregnancy is beyond the scope of this thesis but can be found in the aforementioned review by Wang *et al.,* 2020 (216).

1.8 Current methods of predicting preterm birth

Histopathological examination of the placenta, viral load, macrophages and inflammatory proteins is only feasible postpartum. At present, a shortage of accurate diagnostic tools makes prediction of PTB problematic for clinicians, particularly when women present with threatened preterm labour (thPTL). The ability to accurately predict PTB, either imminently or in early gestation, is a long-standing issue and, despite a plethora of research, no single test or assessment has proved fruitful (291, 292). The importance of accurate diagnostic technologies being available is three-fold; firstly, to administer individual treatment to at-risk women, secondly, to identify high-risk populations and finally, to better understand the distinct biological pathways and mechanisms associated with the multifactorial aetiology of PTB (292). A significant challenge in determining a useful predictive test for PTB lies in the two populations involved; a test to predict an imminent preterm delivery in symptomatic women may be of no value in predicting the risk of PTB in asymptomatic women early in their pregnancy (293). This disparity in PTB prediction tools can lead to unnecessary, and costly, hospital admissions, inappropriate interventions, or the exclusion of women who subsequently deliver preterm (291, 293, 294). Current methods of assessing risk to predict preterm labour include standard clinical assessment (SCA), where a clinical history is taken, observations relating to both mother and fetus are made, and cervical dilation is assessed through a speculum examination (294, 295). Where it is not possible to determine cervical dilation, a digital vaginal examination (VE) is performed, however, this does not reduce the incidence of PTB, increases the risk of chorioamnionitis and has been shown to shorten the latent period (296, 297).

1.8.1 Biomarkers as predictors of preterm birth

Biochemical markers are commonly utilised in combination with transvaginal ultrasound (TVU) and cervical length (CL) measurement (291) but, despite over one hundred individual biomarkers being identified in the last four decades, no agreement exists on which, if any, of these biomarker(s) are effective predictors of PTB (298). Consensus exists, however, that the probability of there being a single predictive biomarker of PTB is exceptionally low (298). Unlike SCA, TVU CL measurement or VE, biomarkers generally look at the final pathways leading to spontaneous PTB, often those indicative of intrauterine infection or inflammation such as serum c-reactive protein (CRP), or cervical IL-1β, IL-2, IL-8 and TNF-α (299, 300).

Markers of extracellular matrix degradation, such as fetal fibronectin (fFN), phosphorylated insulin-like growth factor-binding protein-1 (phIGFBP-1), placental alpha microglobulin-1 (PAMG-1) and pregnancy associated plasma protein-A (PAPP-A) have also been described in the literature (294, 301). These four main biomarkers currently in use are discussed briefly below.

1.8.1.1 Fetal fibronectin

Fetal fibronectin values have been shown to be a useful diagnostic test for determining the likelihood of delivery within forty-eight hours in women > 30 weeks' gestation, as described in section 1.7.5 of the National Institute for Health and Care Excellence (NICE) Guideline NG25: Preterm labour and birth (295). A threshold of ≥50ng/ml is considered positive on a qualitative fFN test (QuikCheck/Rapid fFN™, Hologic, USA) (295, 302). Negative prediction of PTB is strong, fFN testing can be carried out at the bedside, and results are rapidly available (302), attributes which have led to fFN tests being routinely employed in determining PTB risk in symptomatic women (295, 302). fFN is most informative when a result is negative and the best strategy overall is considered to be a combination of fFN testing and TVU CL measurement (303, 304). Despite the fact that positive results are less effective at predicting PTB, fFN remains a useful tool in identifying women unlikely to deliver preterm on account of the subsequent reduction in unnecessary interventions and hospital admission, thus reducing the use of healthcare resources (302, 305).

1.8.1.2 Phosphorylated insulin-like growth factor-binding protein-1

As found with fFN, measurements of phIGFBP-1 (phIGFBP-1 test: Actim Partus™, Oy Medix Biochemica Ab, Finland) add limited value to assessment of CL alone and, therefore, it is not recommended as routine. Reported results from a prospective cohort study carried out at a Macedonian tertiary care centre between 2014 and 2015 identified phIGFBP-1 had a 100% negative predictive value (NPV) ≤7 days to delivery. However, this was only demonstrated in high-risk women presenting with a short cervix (<30 mm) and, in the total cohort, only 29% of women who attended the centre with symptoms of preterm labour and a TVU-diagnosed short CL went on to deliver preterm in ≤7 days (306). More recently, though, Melchor, Wing and Surbek (2018) concluded a phIGFBP-1 pooled NPV from a systematic review of the

available evidence of 98.7% (95% CI, 98–99%) but a pooled PPV of only 35.2% (95% CI, 31–40%), adding further credence to its restricted value in clinical practice (307).

1.8.1.3 Placental alpha microglobulin-1

Found to be more specific than phlGFB-1, the PAMG-1 test (PartoSure™, Parsagen Diagnostics, USA) has a higher PPV than phlGFB-1, but only in women with CL measurements between 15 and 30 mm (308). PAMG-1 has also been shown to have a greater PPV than quantitative fFN, is rapidly available as a bedside test which does not require a speculum, and can be carried out after digital VE, thus making it less invasive for patients and a more appealing option than fFN (307, 309). Strong pooled PPV of 76.3% (95% CI, 69–84%) and NPV of 96.6% (95% CI, 94–99%) support the use of PAMG-1 testing, in some cases in place of TVU CL. Furthermore, the reduced pressure on healthcare resources from a high NPV and unnecessary administration of tocolytics and/or corticosteroids makes this a promising future bedside test (307-310).

1.8.1.4 Pregnancy associated plasma protein-A

Considering PAPP-A's role in placental trophoblast development and function, low levels (≤1 percentile) have been linked to a variety of adverse pregnancy outcomes, including IUGR, preeclampsia, PTB and stillbirth (311, 312). Low PAPP-A suggests placental dysfunction, yet evidence is lacking regarding a significant link between low first trimester PAPP-A values (≤0.4 Multiples of Medium) and immediate adverse neonatal outcomes, maternal post-partum haemorrhage (PPH) or an increased need for emergency Caesarean (CS) delivery, as described by Turner and Kumar (2020) (313). However, the same study showed a significantly increased likelihood of delivering preterm when first trimester PAPP-A was low (13.0% vs. 5.3%, aOR 2.51, 95% CI 1.76–3.56) (313). Although studies appear to agree that PAPP-A is a predictor of PTB, for most studies it is not the primary outcome (312, 314) and, as such, it is challenging to accurately quantify the predictive value of first trimester PAPP-A or define its potential as an effective biomarker of PTB.

As described above, aside from taking a clinical history, current prediction of PTB relies heavily on the use of transvaginal ultrasound to assess cervical length and/or analysis of biomarkers in cervical secretions. Although assessing proteins and hormones as biomarkers derived from

other bodily fluids, such as plasma/serum, amniotic fluid and saliva is feasible, each is with varying degrees of success. In addition, histopathologically determining placental dysfunction and identifying clinicopathological phenotypes which reflect the underlying pathophysiology of PTB remain challenging. As such, continued research to identify histopathological markers and relevant panels of molecular biomarkers is required, as is evaluation of biomarkers which may predict the severity or location of inflammation in the placenta. In the future this may assist in preventing unnecessary or untimely administration of antibiotics or corticosteroids, unwarranted hospital admissions and relieve the burden on maternity healthcare systems.

1.9 Hypothesis and aims

1.9.1 Hypothesis

This thesis is derived from the primary hypothesis that an increased risk of PTB is associated with activated inflammatory processes in the placenta, either in response to pathogens or initiation by locally produced inflammatory mediators, and that these subsequently adversely affect placental development and function or lead to a breakdown in maternofetal tolerance. It is further hypothesised that these altered inflammatory signatures in preterm placentas result from an increased prevalence of viral pathogens and/or aberrant activation and localisation of macrophages, the tissue-resident and vital subset of immune cells in the placenta. Finally, it is suggested that the expression levels of key inflammatory mediators in placentas of women delivering preterm will vary compared to their term counterparts even in the absence of histologically-diagnosed placental inflammation.

1.9.2 Aims

The aims of this PhD project were to compare the contribution of placental infections and inflammation on sPTB and investigate specific and non-specific markers of inflammation in women delivering preterm and at term. Primary aims of each aspect of the project were to:

a) characterise inflammatory and non-inflammatory features associated with PTB, compare these to term placentas following current standard histopathological and clinical protocols and elucidate PTB-specific histopathological markers,
b) identify, quantify and localise CMV, HSV-1/2 and SARS-CoV-2 in the placenta and investigate whether the quantity or location of viral pathogens varied between preterm and term placentas and in those with and without a histologically-diagnosed inflammatory response,
c) quantify and localise placental macrophages and determine subpopulations by phenotype to examine whether these are altered in preterm versus term placentas and between those with and without a histologically-diagnosed inflammatory response, and,
d) examine levels of target AMPs, cytokines, chemokines and MMPs, specifically hCAP18, SLPI, IFN-γ, IL-6, IL-8, IL-10 and MMP-9, in the placenta and investigate whether expression levels varied at preterm and term gestations or between placentas with and without a histologically-diagnosed inflammatory response.

Chapter 2: Materials and Methods

2.1 Ethical approval

All aspects of the study were granted ethical approval as part of the wider cross-sectional study conducted by the Preterm Birth Prevention and Management (PRIME) research group under protocol number STH20635. Ethical approval was granted by the London-Fulham Research Ethics Committee on 12 December 2018 (REC Number 18/LO/2044). Confirmation and approval of the study and of sponsorship by Sheffield Teaching Hospitals NHS Trust (STH) was granted by the Health and Research Authority (HRA) and Health and Care Research Wales (HCRW) on 12 December 2018 (IRAS 256135). Copies of approval documents can be found in Appendix I (Figures AI.Ia, AI.Ib, AI.II, AI.IIIa-c and AIV.4).

2.2 Participant recruitment

All participants were recruited from antenatal wards or labour ward (LW) of the Jessop Wing (JW), Sheffield, UK, a tertiary referral unit where approximately 8000 births per annum occur and which houses the regional neonatal intensive care unit with high dependency facilities equipped to manage extremely preterm births, including neonates born at gestational ages considered borderline for viability (23-26 weeks). Participants comprised of two clinical categories, women delivering preterm and women delivering at term. No restrictions on parity, upper age limit, mode of delivery or presence of obstetric complications such as gestational diabetes mellitus or preeclampsia were applied. Inclusion and exclusion criteria and target recruitment numbers for this project were established as per those for the PRIME study with no flexibility or deviations permitted.

Given the pilot nature of the PRIME studies, no formal sample size calculation was performed; one aim of the wider studies was to generate sufficient data to inform sample size for future placental histopathological analyses. Likewise, the preliminary nature of the experimental studies included in this thesis meant it was justified not to perform formal sample size calculations. A typographical error in the originally approved protocol was highlighted to the Principal Investigator and STH Clinical Research and Innovation Office in early 2021 as this excluded women undergoing current antibiotic treatment. Given that many women delivering preterm, especially those with PPROM, would likely be receiving antibiotic treatment, it was deemed necessary to rectify this error and a non-substantial amendment

(AM01-Category C) was submitted and granted by STH on 25 March 2021. Due to pandemic restrictions and the protracted pause on recruitment, the effect of this error prior to the granting of the non-substantial amendment had been minimal. Subsequently, all women who met the criteria below were approached to take part in the study.

2.2.1 Inclusion criteria

Pregnant women who:

- gave written (personally signed and dated) informed consent
- were aged over 16 years (inclusive)
- were able to understand and were willing to comply with requirements of the protocol

2.2.2 Exclusion criteria

Pregnant women who:

- had uncertain gestation by last menstrual period (LMP)/ultrasound scan (USS)
- had a multiple pregnancy
- had an elective Caesarean section without prior labour
- were unable to understand and/or were unwilling to comply with requirements of the protocol

2.2.3 Effect of Covid-19 on participant recruitment and placenta collection and sampling

Participant recruitment was paused on account of the Covid-19 pandemic and subsequent access and contact restrictions between 23 March 2020 and 01 October 2020. Once resumed, recruitment remained tightly regulated with no access to labour ward for the author (KMP) and any non-clinical members of the PRIME research team; screening, obtaining consent and enrolment of participants was managed by KMP through liaison with members of the Jessop Wing Research Team and staff in the Department of Histopathology at Sheffield Children's Hospital (SCH). Access to JW laboratories was restricted to 08:00-18:00 hours Monday to Friday in short, pre-bookable slots which led to challenges with recruitment and sample collection where deliveries occurred out of hours or when JW laboratories were unavailable. Risk assessments completed by KMP with support from SCH for handling and sampling placentas stated no fresh placenta should be processed in the JW laboratories on account of the hoods being biohazard Category 2 (at a time when SARS-CoV-2 was considered a Category

3 pathogen), and due to the risk of touch transmission or aerosolisation of viral particles through placental sampling procedures. Consequently, all placentas collected from women delivering at term post-October 2020 were immediately fixed in 10% neutral buffered formalin (NBF) before any samples were taken. All preterm placentas were sent directly from labour ward to the Department of Histopathology at SCH as per standard protocol. Samples were then taken by KMP once placentas were fully formalin-fixed.

2.2.4 Case selection

Notwithstanding the amendments described above, placentas included in the study were collected at or soon after delivery from a cohort of women screened as part of the wider PRIME study between April 2019 and August 2021. Briefly, 167 women booked into LW or antenatal wards of the JW, Sheffield were screened for eligibility at which time thirty-seven were excluded. Three women did not meet one or more inclusion criteria, five declined to participate, two were returned to antenatal ward due to no progress with their labour and twelve women were either unwell following delivery or had progressed to a stage in their labour where it was inappropriate to approach them to consent to the study. Covid-19 restrictions on LW and in the JW laboratories further hampered enrolment; in nine cases of women being identified as eligible from the Jessop Wing Maternity Information System (JMIS), no researcher or research team member was available to take consent and in three cases there was no laboratory availability. All women booked to wards of the JW were screened for Covid-19 via a lateral flow test (LFT) on arrival, however, in the case of emergencies or rapid deliveries this was not always performed and, in this study, this lack of LFT excluded a further three women. Of the 130 enrolled participants, 70 delivered ≥37 weeks completed gestation and were assigned to the term group; the remaining 60 delivered <37 weeks' completed gestation and were allocated to the preterm group. No minimum gestational age was set for the study but only livebirths were included. In the term group, three women were excluded from further analysis when clinical notes showed their labour had been induced and one woman was excluded pre-delivery due to a refusal to take a Covid-19 LFT. Following consent and delivery, a further 15 cases were excluded in the term group, seven due to a delay in receiving Covid-19 LFT results, six who delivered outside of permitted laboratory access hours and two where difficulties in obtaining deliveries of 10% NBF prevented immediate fixation meaning it was not possible to sample the placenta for health

and safety reasons. In the preterm group, 13 cases were lost to follow up; one Covid-19 LFT result was pending preventing inclusion of the participant, and nine women consented whilst at a preterm gestation but later delivered at term. Whilst this in itself would not exclude participants, in these cases the placentas were discarded in accordance with Covid-19 protocols in place at the time of delivery. Finally, three women consented on presenting or being transferred to the Jessop Wing with threatened preterm labour (thPTL) but were later discharged and delivered out of area. Subgroup analyses of PTB was conducted following the WHO-defined descriptors, as comparisons between preterm and term gestations do not take into account the significant differences in placental and fetal development at extremely (EPT), very (VPT) and moderate to late (MPT) preterm gestations. Births occurring <28 weeks were grouped as EPT, between 28 and 32 weeks as VPT and ≥32 to <37 weeks to MPT as described in Quinn *et al.* (2016) (5). The flow-chart in Figure 2.1 summarises screening, inclusion and exclusion for the study. No neonatal follow-up was performed for either the term or preterm groups as per the approved PRIME protocol STH20635.

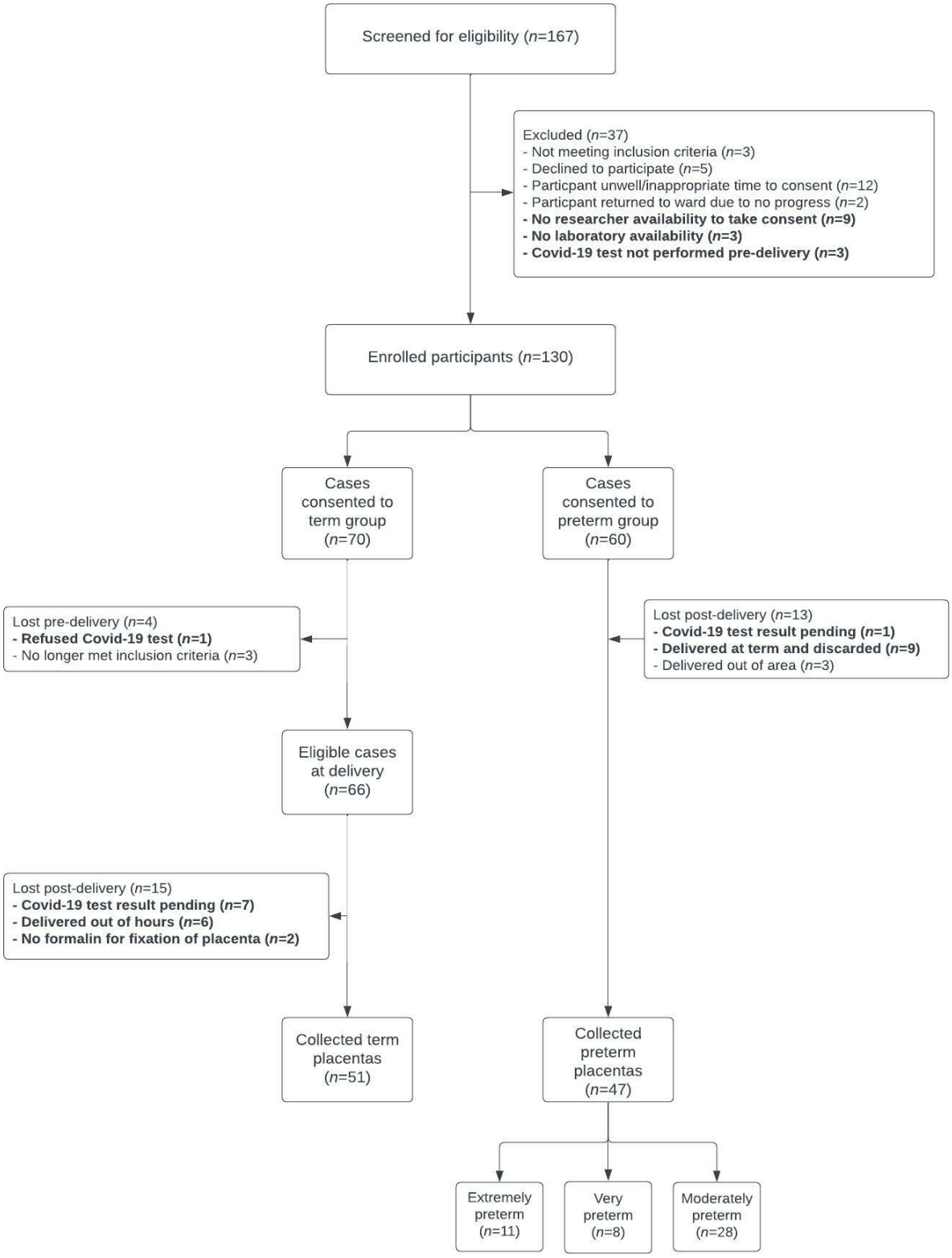

Figure 2. 1 Flowchart outlining participant screening, inclusion and exclusion to the study.

A total of 98 placentas delivered between April 2019 and August 2021 were included in the study after exclusions were applied. **Bold** text denotes exclusion, loss or withdrawal due to reasons connected to Covid-19 restrictions or safety precautions.

2.3 Fresh placenta collection and tissue isolation

2.3.1 Placenta collection

All samples included in the studies described in this thesis were taken from a total of 56 term (≥37 weeks of gestation) and 50 preterm (<37 weeks of gestation) placentas collected between 15 April 2019 and 24 March 2022 as part of the PRIME studies. Placentas with attached fetal membranes and umbilical cord were collected from the delivery room or theatre anteroom on labour ward as soon as possible after delivery, with collection and sampling to be completed within 6 hours (see rationale below). Once standard clinical assessment by the attending midwife or obstetrician was complete, term placentas were transferred by nitrile-gloved hand to a yellow clinical waste bag; preterm placentas were placed directly into a pre-labelled white 5 litre plastic bucket as per standard STH procedures. Sealed specimens were collected by a member of the PRIME research team (pre-Covid restrictions) or the dedicated Jessop Wing Research Team (during and post-Covid restrictions) and transferred to the Jessop Wing level 4 laboratories for processing.

2.3.2 Placenta grading

Initially all placentas obtained from recruited participants were to be graded according to their structure, completeness of membranes and freshness from the time of delivery to the time of receipt in the laboratory, as described in Table 2.1. Placental grading and ideal collection and sampling limits for the purpose of the varied PRIME studies were developed by the PRIME research teams at the University of Sheffield (UoS) and the University of Cape Town (UCT) based on published literature describing placental morphology and RNA, DNA and overall cellular stability in placental tissue at various time points post collection (315-317).

Table 2. 1 Placenta grading criteria

Grade	Description	Membranes	Time Received in Laboratory
1a	Good	Complete	≤ 6 hours from delivery
1b	Good	Incomplete	≤ 6 hours from delivery
2a	Good	Complete	> 6 hours, ≤ 12 hours from delivery
2b	Good	Incomplete	> 6 hours, ≤ 12 hours from delivery
3	Variable	Complete/Incomplete	> 12 hours, ≤ 24 hours from delivery
4	Variable	Complete/Incomplete	> 12 hours, ≤ 36 hours from delivery
5	Variable	Complete/Incomplete	> 36 hours from delivery
6	Bad	Incomplete	> 36 hours from delivery

Following extensive discussion with the Histopathology Department at SCH, particularly in light of Covid-19 pandemic restrictions preventing the author from conducting participant recruitment and placental collection in person, it was established that experimental procedures and assessments conducted as described in this thesis in *Chapters 3 – 6* would not be negatively affected by placental collection and sampling exceeding 6 hours given that placentas were appropriately fixed in 10% neutral buffered formalin (Section 2.4.1, below). Upon resuming recruitment on 27 October 2020, all placentas, irrespective of gestational age at delivery, were immediately deposited into a pre-labelled white 5 litre plastic bucket and either formalin fixed in JW laboratories or transferred to SCH for fixation, dependent on reagent availability. Only samples extracted from placentas collected pre-pandemic (date range 21 May 2019 to 10 March 2020) where it could be ascertained that collection and sampling had occurred within 6 hours were selected for inclusion in the study described in *Chapter 7* to ensure optimal protein integrity.

2.3.3 Tissue isolation and storage process

In accordance with sampling procedures for PRIME studies, the mass of fresh, whole placentas, including membranes and the umbilical cord, was recorded in grams using a Fisherbrand™ portable balance (Cat. No. 15385103, Fisher Scientific, Pittsburgh, PA, USA). After removing excess maternal blood by dabbing the surface with a paper towel, a single swab sample was taken from each of the chorionic plate (fetal surface) and the decidua basalis (maternal surface) using a Fisherbrand™ ethylene oxide-sterilised Dacron-tip swab (Cat. No. 11532483, Fisher Scientific, Pittsburgh, PA, USA) which was swept and twisted over the entire surface, replaced in the plastic housing tube and stored at -80°C until analysis. Tissue samples were collected from a pericentral location clear of the umbilical cord insertion point by excising a 2cm x 2cm square section of the full thickness of the placental disc using a Swann Morton No.22 blade sterile disposable scalpel (Cat. No. 11758353, Fisher Scientific, Pittsburgh, PA, USA) before this portion was sectioned into five pieces for PRIME RNA-sequencing, RNA-microbiome and DNA and RNA viral studies, plus protein studies described in this thesis (PhD study samples are highlighted in grey on Table 2.2)**.** Fetal membrane samples of 5cm x 5cm incorporating a portion of the rupture site, where evident, were excised using 10cm straight micro-dissecting scissors (Cat. No. 14393, World Precision Instruments, Hitchin, UK). No standardised mass of placental tissue was collected for PRIME studies nor was the collected 2cm x 2cm square section equally portioned for further study at the point of sampling and storage. For PRIME RNA studies, samples were dissected to separate placental compartment tissues, that is amniotic (fetal) membranes, decidua basalis and villous tissue, all other samples were stored as full thickness intact tissue as detailed in Table 2.2. Media and containers used for sample storage are also noted.

Table 2. 2 Tissue isolation and storage strategy

	Metabolome	RNA-Sequencing	RNA-Microbiome	Protein Extraction - ELISA	RNA-virus	DNA-virus	Histology
Medium	N/A	RNA-Later	FBS/DMSO	Snap-freeze (Liquid N_2)	Snap-freeze (Liquid N_2)	Snap-freeze (Liquid N_2)	10% formalin
Media Volume	N/A	1.5 ml	1.5 ml	n/a	n/a	n/a	3L
Container	Dacron Swab Housing	2 ml Cryovial	2 ml Cryovial	2 ml Cryovial	2 ml Cryovial	2 ml Cryovial	5L White Plastic Bucket
Tissue Type	Maternal	AM	AM	Full thickness	Full thickness	Full thickness	Whole disc, membranes and cord
		DB	DB				
	Fetal	DP	DP				
		VT	VT		X	X	
Storage Temperature	-80^{0}C	-80^{0}C	-80^{0}C	-80^{0}C	-80^{0}C	-80^{0}C	Room Temperature

AM; amniotic membranes, *DB;* decidua basalis, *DNA;* deoxyribonucleic acid, *DP;* decidua parietalis, *ELISA;* enzyme-linked immunosorbent assay, *FBS/DMSO;* fetal bovine serum/dimethyl sulphoxide, *N_2;* nitrogen, *RNA;* ribonucleic acid, *VT;* villous tissue. N.B. FBS/DMSO is 10% DMSO in heat inactivated FBS.

2.4 Formalin-fixed placental examination and sample processing

2.4.1 Gross morphological examination

Following fresh sample collection, preterm placentas were transferred to SCH according to standard clinical practice and fixed in 10% NBF (Cat. No. HT501640-19L, Sigma-Aldrich, St. Louis, MO, USA) in the Department of Histopathology. The following description of gross morphological sampling refers to term placentas conducted by the author. All preterm placentas were fixed, sampled, analysed and reported at SCH by a consultant histopathologist according to standard clinical practice. The contribution made by staff at SCH is noted and acknowledged. Term placentas were fixed whole in 10% NBF in the Jessop Wing Level 4 laboratories for a minimum of 48 hours on account of the thickness and robustness of

placental tissue and to ensure the tissue was fully fixed (Table 2.2). Gross morphological examination and sampling was carried out in accordance with standard clinical protocols in use at SCH, Department of Histopathology, as outlined by RCPath (318) and in the Amsterdam Placental Workshop Group Consensus Statement (103) and, for research purposes, in line with previously described methods (316, 319-322). Data were recorded on SCH Document No. 326.4.34 Singleton Placenta Dissection Reporting Form (amended for PRIME); an example form is found in Appendix I (Figure AII.IIa-c).

2.4.2 Fixed placenta morphological assessment and sampling methodology

2.4.2.1 Placental disc

Examination recorded the colour of the fetal and maternal surfaces of the placental disc; fetal surfaces were considered to be translucent, opaque or green, whereas maternal surfaces were normal or pale. Whether fetal surfaces displayed normal chorionic vessels was recorded, as was the presence of prominent calcification or attached blood clots on maternal surfaces and whether this surface was intact or ragged. Dimensions were measured in three planes: the maximum length, the maximal diameter of the axis perpendicular to the length measurement (width) and the maximum mural thickness. Shape of the disc was recorded as discoid, succenturiate if bilobate or an accessory lobe was present, or irregular if any other shape. Placental mass was recorded in grams (g) once extraplacental membranes and umbilical cord had been removed (*vide infra*) (103).

A Swann Morton No.22 blade sterile disposable scalpel (Cat. No. 11758353, Fisher Scientific, Pittsburgh, PA, USA) and a FEATHER® F130P trimming handle (Cat. No. 205530001, pfm medical, Cologne, Germany) mounted with a 130mm FEATHER® trimming blade (Cat. No. 206600130, pfm medical, Cologne, Germany) were used to uniformly section placentas to obtain tissue samples. Placental discs were sliced vertically into 2-4 cm transverse sections (referred to as the cut surface) and each cut surface was examined with a record made of the colour and presence of any lesions. Then, one 4 cm-wide sample was collected from a central representative area of three of the cut surfaces, selected as described in Figure 2.2, before being placed individually into Histosette® (Cat. No. Z672122, Fisher Scientific, Pittsburgh, PA, USA) tissue embedding cassettes. No samples were taken from the placental margins or cord insertion site as these areas are typically non-representative which increases the possibility

of inaccurately identifying non-pathological lesions (103). Where abnormal features were observed, e.g. firm, white lesions, a sample including the lesion and a sample of adjacent normal-looking parenchyma were collected, with samples again placed individually into tissue embedding cassettes. A record of volume of lesions as a percentage of total parenchyma volume was made to indicate severity and effect of lesions (103) prior to samples being transferred to SCH for automated tissue processing.

2.4.2.2 Umbilical cord

Umbilical cord features were recorded including length, average diameter, direction of coiling (handedness), as determined by diagonal seams running from upper left to lower right in the case of left twists, and depth of indentations (shallow or deep). The number of coils were counted, and record made of whether the cord was hypocoiled or hypercoiled and if this was segmental. Umbilical coiling indices (UCI) were calculated by dividing the number of coils by the cord length in centimetres (normal range 0.07 – 0.30). The presence of thrombi, true or pseudo knots and strictures was recorded. Umbilical cord insertion was recorded as central, para-central, eccentric - into the chorionic plate but not centrally, velamentous - insertion into the fetal membranes before entering the disc (323), or marginal which was calculated by measuring the distance from the insertion site to the nearest placental margin (103). The presence of three umbilical vessels was confirmed and documented. With a Swann Morton No.22 blade sterile disposable scalpel (Cat. No. 11758353, Fisher Scientific, Pittsburgh, PA, USA), umbilical cords were removed from the placental disc at the insertion site. Two 2–4 cm transverse sections of umbilical cord were taken, one from the cut (distal) end and the second taken ~5cm from the insertion point in order to avoid the Hyrtl's anastomosis which is unrepresentative of the remainder of the cord. Samples were placed together in a Histosette® (Cat. No. Z672122, Fisher Scientific, Pittsburgh, PA, USA) tissue embedding cassette along with the membrane roll (see 2.4.2.3).

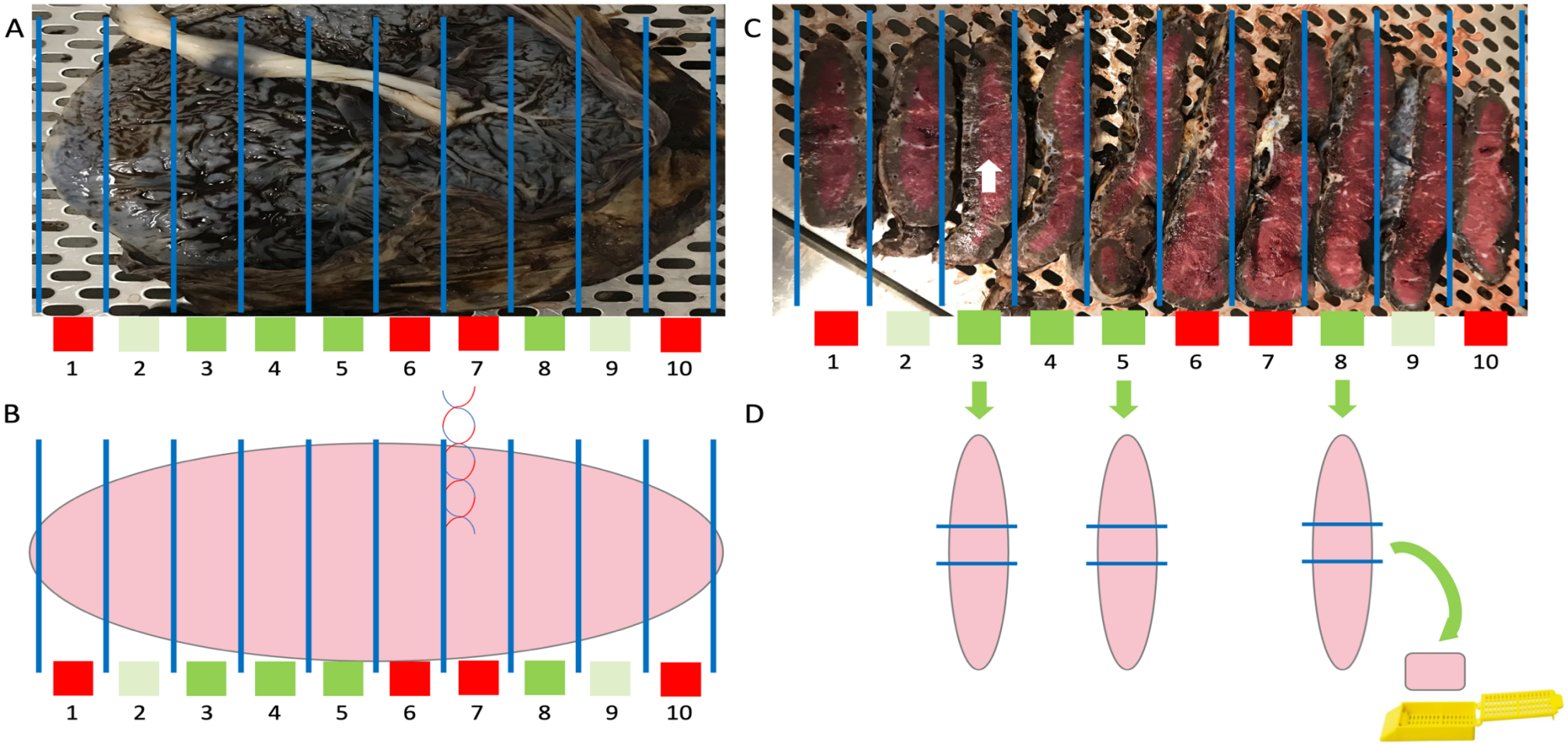

Figure 2. 2 Photographic images and schematics of a placental disc and cut surfaces to demonstrate sectioning for sampling.

Placentas were sliced into sections of 2-4 cm thickness (A and B) to expose cut surfaces (C). In images A-C, red squares denote sections which were excluded from sampling, specifically margins and the cord insertion site. Bright green squares identify optimum sections for sampling; however, samples may be taken from sections with lighter green squares (numbered as 2 and 9 in this representative example) dependent on the placental length and distance from the margins. A minimum of three 4cm wide cross-sectional samples were sliced from three representative transverse cut surfaces and placed into histology cassettes for processing (D). The white block arrow at (C) highlights a haemorrhagic lesion which would be of interest to sample. In this instance this is in an optimal sampling section; if not, this would be taken as an additional sample.

2.4.2.3 Peripheral membranes

Peripheral membranes were observed for completeness, which refers to whether the membranes are able to be pulled closed together as *in utero*. Of note, complete or incomplete membranes is a Department of Histopathology reference to the submission of membranes rather than a pathological observation. Membrane colour, that is if membranes were translucent, opaque or green (discoloured), rupture site location, additional tears or holes, extra vessels or lobes and the presence of both amnion and chorion were recorded. The insertion point of the membranes was recorded as marginal, circumvallate or circummarginate. Schematics depicting circumvallate, circummarginate and normal marginal membrane insertion are found in Figure 2.3. Circumvallation may be clinically significant, however, circummargination generally is not. Nonetheless, a record of any abnormality and, in the case of circumvallation and circummargination, the percentage of the circumference involved, was documented (103).

For sampling, a roll of peripheral membranes extending from the rupture point to the placental margin was collected by rolling the membranes around sterile, blocked end forceps (Cat. No. 12730046, Fisher Scientific, Pittsburgh, PA, USA), pinning the roll in place and sectioning at the margin with a scalpel. A 3 cm wide section was then cut from the membrane roll and this was placed into a Histosette® (Cat. No. Z672122, Fisher Scientific, Pittsburgh, PA, USA) tissue embedding cassette together with the two umbilical cord samples for histological processing as described below. The remainder of the peripheral membranes were then removed from the placental disc using 10 cm straight micro-dissecting scissors (Cat. No. 14393, World Precision Instruments, Hitchin, UK) before being discarded. Tissue embedding cassettes containing full thickness placental samples and umbilical cord/membrane rolls were stored in 10% NBF until processing to prevent the tissue drying out. As standard, for each placenta in the study, samples for examination and assessment comprised of three representative full thickness placental disc sections with the blocks for these labelled as A.1 – A.3. Block A.4 contained the two umbilical cord samples (insertion and cut end) and one section of the membrane roll unless either of these samples were too large to be contained in one cassette. On these rare occasions, the umbilical cord and membrane roll would be placed in separate cassettes and labelled consecutively (A.5, A.6, etc.) as were any additional samples taken, such as those for placental lesions.

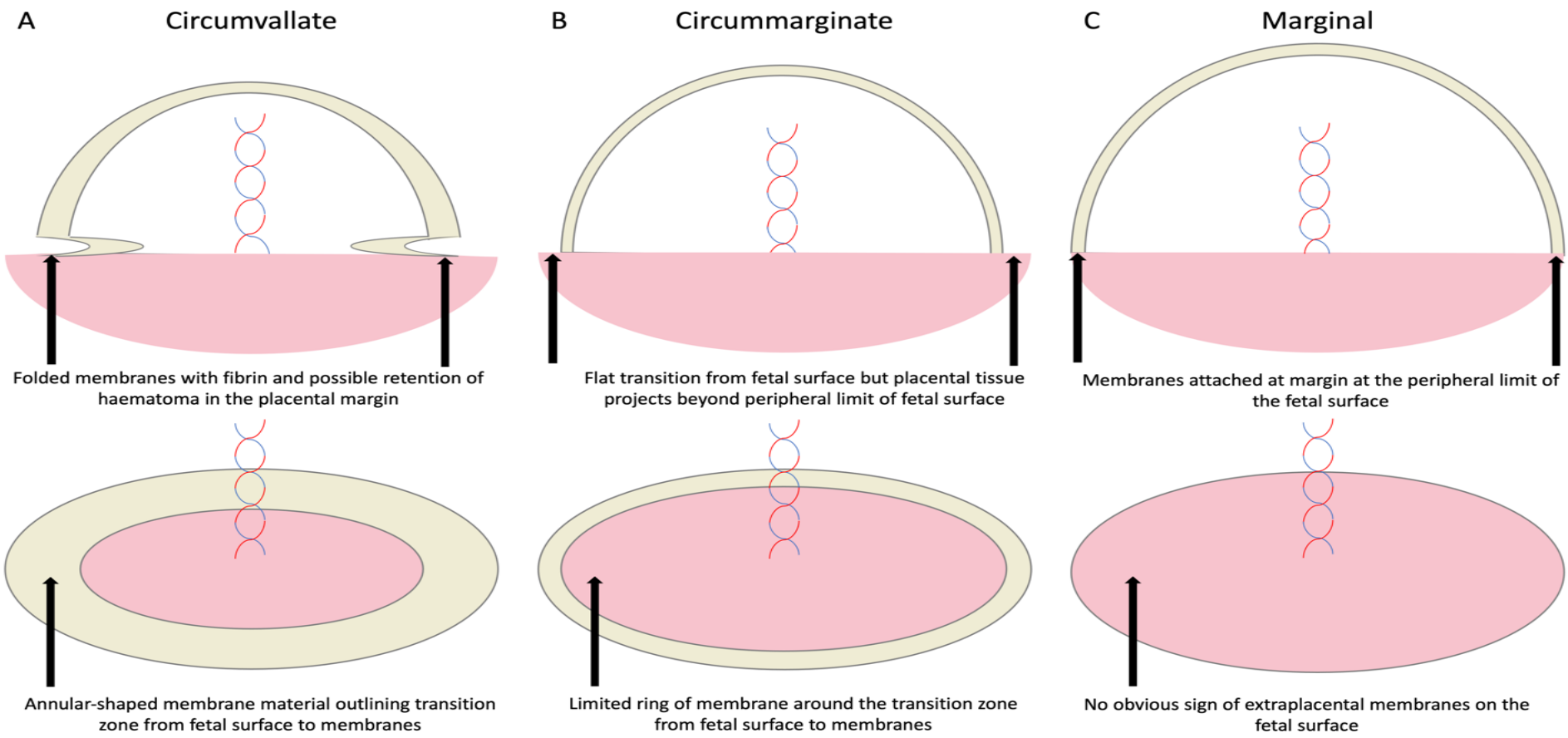

Figure 2. 3 Schematics depicting circumvallate, circummarginate and normal marginal peripheral membrane insertion.

A Circumvallate placentas are identified by an annular raised placental margin which may be present on a portion of the fetal surface or covering up to 100% of it. The basal plate is larger than the chorionic plate resulting in misalignment of the surfaces, membranes reflecting back upon themselves and the possibility of haematomas and fibrin becoming trapped in the placental margin (324). **B** The second recognised type of placenta extrachorialis, circummarginate, tends not to cause any clinical problems but presents as exposed placental disc tissue where the membranes project beyond the peripheral limit of the fetal surface. Unlike in circumvallation, the membranes do not reflect or fold back upon themselves, instead they insert in a normal, flat manner. **C** Normal insertion of membranes is within the peripheral limit of the fetal surface, that is, the margin. The membranes lay flat and are not reflected (325).

2.4.3 Tissue embedding and sectioning

Processing, embedding and sectioning of term placentas was carried out by the author pre-Covid restrictions. From April 2020 onwards, processing, embedding, microtomy and haematoxylin and eosin (H&E) staining of all preterm and term placenta samples was carried out by staff of the Sheffield Children's Hospital Department of Histopathology in accordance with standard clinical protocol. To enable histopathological analyses, formalin-fixed, sectioned placental, umbilical cord and membrane roll samples were processed through fixation, dehydration, clearing and wax infiltration steps using an automated Shandon Excelsior processor (Thermo Scientific, Waltham, Massachusetts, USA) on an extended routine due to placental tissues being haemorrhagic and rich in fat. Stages, reagents and timings of this automated extended routine are shown in Table 2.3.

Table 2. 3 Stages, reagents and timing schedule of tissue processing for histopathological analyses

Stage	Process Step	Reagent	Time (hour:minute)
1	Fixation	Formalin	00:05
2	Dehydration	Industrial Methylated Spirit	01:00
3		Industrial Methylated Spirit	01:00
4		Industrial Methylated Spirit	01:00
5		Industrial Methylated Spirit	01:00
6		Industrial Methylated Spirit	01:30
7		Industrial Methylated Spirit	01:30
8	Clearing	Xylene	01:20
9		Xylene	01:20
10		Xylene	01:30
11	Wax Infiltration	Wax	01:30
12		Wax	01:30
13		Wax	01:30
Total			**15:57**

Following processing, excised placenta samples were embedded in molten paraffin wax on a Shandon HistoCentre 2 (Thermo Scientific, Waltham, Massachusetts, USA) then left to cool and solidify for 30 minutes. According to standard SCH procedure, membrane rolls, and umbilical cord samples were embedded on edge as a composite block, tissue sections were orientated in blocks with the hardest part of the tissue (chorionic plate) away from the leading edge of the knife. A HistoCore Biocut (Leica Biosystems, Wetzlar, Germany) manual microtome was used to cut placental samples to 4 µm thick sections, according to standard procedures. Individual sections were lifted to Corning® frosted one side/one end microscope slides (Cat. No. CLS294875X25-72EA, Merck, Darmstadt, Germany) for histopathological evaluation and to Dako FLEX adhesive-coated glass slides (Cat. No. K802021-2, Agilent Dako, Santa Clara, CA, USA) for immunohistochemistry (IHC) and immunofluorescence (IF) experiments.

2.5 Histopathological techniques

2.5.1 Haematoxylin and Eosin staining

Placental sections were stained with H&E to allow visualisation, assessment, and reporting of inflammatory status and non-inflammatory features. H&E staining was performed at SCH Department of Histopathology on an ST5010 Autostainer XL (Leica Biosystems, Wetzlar, Germany). Details of the H&E staining process are provided in Table 2.4 with steps described briefly below. Following staining, slides were automatically cover-slipped using a CV5030 Fully Automated Glass Cover-slipper (Leica Biosystems, Wetzlar, Germany).

Steps 1-2 were completed using xylene as a clearing and deparaffinising agent, specifically to displace alcohol and other dehydrants from the tissue and to make the tissue transparent. Steps 3-4 ensured water in the tissue was replaced by graded alcohol before this was removed in Wash 1 and acidic haematoxylin was added at step 6. Excess haematoxylin was removed at Wash 2 and 1% acid/alcohol added for 25 seconds to remove any non-specific haematoxylin stain. As this was an automated process, Scott's tap water (Cat. No. 3802900, Leica Biosystems, Wetzlar, Germany) was added after Wash 3 as a bluing agent to haematoxylin before Wash 4 and the addition of eosin. Final wash steps, dehydration with alcohol and clearing with xylene took place before embedding of the tissue (326).

Table 2. 4 Haematoxylin and eosin staining process

Reagent Station	Bath Number (Autostainer XL)	Time (minute:second)
Xylene	4	1:00
Xylene	5	1:00
Alcohol	6	1:00
Alcohol	7	1:00
Wash 1	Wash 1	0:30
Haematoxylin	11	2:30
Wash 2	Wash 2	0:30
1% Acid/Alcohol	10	0:25
Wash 3	Wash 3	0:30
Scott's Tap Water	9	0:25
Wash 4	Wash 4	0:30
Eosin	8	1:15
Wash 5	Wash 5	0:25
Alcohol	12	0:30
Alcohol	13	0:30
Xylene	14	1:00
Xylene	15	1:00

2.5.2 Placental sample visualisation and histopathological reporting

Placental features and inflammatory status were evaluated by visualisation of full thickness tissue, umbilical cord sections and the membrane roll using an Eclipse 80i Y-THS light microscope (Nikon, Minato City, Tokyo, Japan) located at SCH Department of Histopathology. One slide per block, i.e., three slides of full thickness placental tissue samples and one slide comprising of the two umbilical cord samples and the membrane roll, was analysed for the

placenta from each participant enrolled as part of the study. Where additional blocks had been collected due to lesions or areas of interest, e.g. firm, white lesions, thrombi or umbilical cord knots, this separate slide(s) was sectioned and reviewed in addition to the four standard slides. Inflammatory and non-inflammatory features were recorded and reported on SCH Document No. 326.4.34 according to the Amsterdam Placental Workshop Group Consensus Statement (103) and SCH standard protocols. All preterm placentas were reported by a consultant paediatric and perinatal pathologist employed in the department; all term placentas were reported by the author (KMP) and reviewed by Professor Marta Cohen (MCC) or, in her absence, Dr Sophie Stenton (SRS; Consultant Paediatric and Perinatal Pathologist). A detailed process of histological review and reporting is described in *Chapter 4*.

2.6 Immunohistochemical staining

Full thickness placental tissue samples were placed on Dako FLEX adhesive-coated glass slides (Cat. No. K802021-2, Agilent Dako, Santa Clara, CA, USA) and incubated at 37°C overnight to ensure sections were adhered to the slides before being used in immunohistochemistry (IHC) experiments. All IHC staining was performed on an Autostainer Link 48 (Dako, California, US) in the SCH Department of Histopathology. Positive CMV, HSV-1/2 and SARS-CoV-2 control slides, kindly provided by SCH, were stained alongside each individual batch of viral pathogen IHC staining.

2.6.1 Cytomegalovirus (CMV) staining

To optimise staining efficiency, slides were first pre-treated in a 3-in-1 specimen preparation procedure of deparaffinisation, rehydration and epitope retrieval on a Dako PT Link (Dako Agilent, California, US) at pH 6.0 and a constant temperature of 95°C for 20 minutes. Using a pre-diluted mouse monoclonal anti-CMV antibody cocktail (Cat. No. ab17073; Abcam, Cambridge, MA, USA) (1:40) against immediate early antigen clone DDG9 and early CMV antigen CCH2, sections were stained according to the protocol in Table 2.5.

Table 2. 5 CMV staining protocol

Protocol Step	Reagent	Volume (µl)	Incubation Time (minutes)
Rinse	Buffer		
Endogenous Enzyme Block	EnVision FLEX Peroxidase-Blocking Reagent	200	5
Rinse	Buffer		
Primary Antibody	CMV (prediluted) (1:40)	200	30
Rinse	Buffer		
Labelled Polymer	EnVision FLEX/HRP	200	30
Rinse	Buffer		
Rinse	Buffer		5
Substrate-Chromogen	Substrate Working Solution (mix)	200	10
Rinse	Buffer		
Counterstain	EnVision FLEX Haematoxylin	200	5
Rinse	Buffer		
Rinse	Buffer		5
Rinse	Deionised Water		

CMV; cytomegalovirus, *HRP;* horseradish peroxidase.

2.6.2 Herpes Simplex Virus-1/2 (HSV-1/2) staining

As for CMV, slides were first pre-treated in a 3-in-1 specimen preparation procedure of deparaffinisation, rehydration and epitope retrieval on a Dako PT Link (Dako Agilent, California, US) at pH 6.0 and a constant temperature of 95°C for 20 minutes. Using a ready-to-use rabbit polyclonal anti-herpes simplex virus type 1 and 2 (Cat. No. IR521; Dako Agilent, California, US) sections were stained according to the protocol in Table 2.6.

Table 2. 6 HSV-1/2 staining protocol

Protocol Step	Reagent	Volume (μl)	Incubation Time (minutes)
Rinse	Buffer		
Endogenous Enzyme Block	EnVision FLEX Peroxidase-Blocking Reagent	150	5
Rinse	Buffer		
Primary Antibody	Herpes Simplex Virus 1/2	150	20
Rinse	Buffer		
Labelled Polymer	EnVision FLEX/HRP	150	20
Rinse	Buffer		
Rinse	Buffer		5
Substrate-Chromogen	FLEX DAB + Substrate-Chromogen	150	5
Substrate-Chromogen	FLEX DAB + Substrate-Chromogen	150	5
Rinse	Buffer		
Counterstain	EnVision FLEX Haematoxylin	150	5
Rinse	Deionised Water		
Rinse	Buffer		5
Rinse	Deionised Water		

DAB; 3,3'-Diaminobenzidine, *HRP;* horseradish peroxidase.

2.6.3 Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) staining

As with CMV and HSV-1/2, slides were first pre-treated in a 3-in-1 specimen preparation procedure of deparaffinisation, rehydration and epitope retrieval on a Dako PT Link (Dako Agilent, California, US) at pH 6.0 and a constant temperature of 95°C for 20 minutes. Using a mouse monoclonal anti-SARS-CoV-2 nucleocapsid antibody clone B46F (Cat. No. MA1-7404; Thermo Fisher Scientific, Waltham, Massachusetts, USA) at a manufacturer's recommended dilution of 1:100, sections were stained according to the protocol in Table 2.7.

Table 2. 7 SARS-CoV-2 staining protocol

Protocol Step	Reagent	Volume (μl)	Incubation Time (minutes)
Rinse	Buffer		
Endogenous Enzyme Block	EnVision FLEX Peroxidase-Blocking Reagent	150	5
Rinse	Buffer		
Primary Antibody	SARS-CoV-2 Nucleocapsid	150	20
Rinse	Buffer		
Labelled Polymer	EnVision FLEX/HRP	150	20
Rinse	Buffer		
Rinse	Buffer		5
Substrate-Chromogen	FLEX DAB + Substrate-Chromogen	150	5
Substrate-Chromogen	FLEX DAB + Substrate-Chromogen	150	5
Rinse	Buffer		
Counterstain	EnVision FLEX Haematoxylin	150	5
Rinse	Deionised Water		
Rinse	Buffer		5
Rinse	Deionised Water		

DAB; 3,3'-Diaminobenzidine, *HRP;* horseradish peroxidase.

2.6.4 Immunohistochemical analyses

All samples stained for CMV, HSV 1/2 and SARS-CoV-2 were visualised and imaged using an Eclipse 80i Y-THS light microscope (Nikon, Minato City, Tokyo, Japan) located at SCH Department of Histopathology. All placenta samples from IHC experiments were evaluated by KMP and reviewed by MCC (CMV and SARS-CoV-2) and SRS (HSV 1/2) to determine presence of immunopositive staining.

2.7 Immunofluorescence techniques

2.7.1 Double-label immunohistochemistry of CD68 and CD163

Immunofluorescence staining was performed to visualise CD68 and CD163 protein expression and localisation in full thickness placenta samples. For each sample, 5 µm sections were cut and mounted on Dako EnVision FLEX coated glass slides (Cat. No. K802021-2, Agilent Dako, Santa Clara, California, USA), deparaffinised in Histo-Clear (Cat. No. A2-0101, Geneflow, Lichfield, Staffordshire, UK) for 2 x 10 minutes and rehydrated in a graded series of Industrial Methylated Spirit (IMS) solutions (Cat. No. 10552904, Fisher Scientific, Loughborough, UK): 100% v/v, 95% v/v, 70% v/v and 50% v/v for 5 minutes each, before being rinsed in distilled water and washed in 1X PBS (pH 7.4) (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 5 minutes. For antigen retrieval, rehydrated sections were microwaved on full power in preheated 0.01M w/v Citrate Buffer (pH 6.0) for 4 x 5 minutes, with distilled water added between each heating step to maintain a consistent volume of solution without altering pH. Slides were cooled to room temperature (RT) under running water and washed in 0.5% Tween-1X PBS (pH 7.4) v/v (Tween-20 Cat. No. P1379, Sigma, St. Louis, Missouri, USA and pH 7.4 1X PBS Cat. No. 10010056, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 2 x 5 minutes with shaking. Non-specific antibody binding was prevented by incubating sections with CAS-Block (Life Technologies, Carlsbad, CA, USA) at RT for 30 minutes, followed by washing in 0.5% Tween-1X PBS (pH 7.4) v/v for 5 minutes. Sections were incubated with permeabilisation buffer consisting of 0.2% Triton X-100 (Cat. No. T8787, Sigma, St. Louis, Missouri, USA) in 1X PBS (pH 7.4) v/v (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 10 minutes to facilitate antibody access to cytoplasmic CD68. Primary antibodies against CD68 and CD163 were diluted 1:100 in CAS-Block (Cat. No. 10282343, Fisher Scientific, Loughborough, UK), as detailed in Table 2.8, added to the sections and incubated overnight at 4°C in humidified chambers. Recipes for buffers used can be found in Appendix V.

Table 2. 8 Primary, secondary and IgG non-immune antibodies for immunostaining and isotype controls

Antibody	Type	Target	Host Species	Concentration (µg/µl)	Manufacturer
ab201340	Monoclonal Primary	CD68	Mouse	0.002	Abcam, Cambridge, Massachusetts, USA
ab106162	Monoclonal Primary	CD163	Rabbit	0.010	Abcam, Cambridge, Massachusetts, USA
Alexa Fluor® 680 ab175775	Polyclonal Secondary	Mouse IgG	Goat	0.002	Abcam, Cambridge, Massachusetts, USA
Alexa Fluor® 488 ab150073	Polyclonal Secondary	Rabbit IgG	Donkey	0.002	Abcam, Cambridge, Massachusetts, USA
sc-2025	IgG non-immune	Mouse IgG	n/a	0.002	Santa Cruz Biotechnology, Dallas, Texas, USA
2729S	IgG non-immune	Rabbit IgG	n/a	0.010	Cell Signaling Technology, Danvers, Massachusetts, USA

IgG; Immunoglobulin G

Following overnight incubation, slides were washed in 0.5% Tween-1X PBS (pH 7.4) for 3 x 15 minutes with shaking to remove primary antibodies before sections were incubated with species-matched Alexa Fluor®-conjugated secondary antibodies (diluted 1:1000 in CAS-Block (Cat. No. 10282343, Fisher Scientific, Loughborough, UK); Table 2.8) for 45 minutes at RT in the dark. Secondary antibodies were removed by 3 x 15-minute washes in 0.5% Tween-1X PBS (pH 7.4) with shaking. Autofluorescence of red blood cells was quenched with Vector® TrueVIEW® Autofluorescence Quenching reagents (Cat. No. SP-8400-15, Vector Laboratories, Peterborough, Cambridgeshire, UK) which were added to each section in accordance with the manufacturer's instructions and incubated for 2 minutes at RT before being washed off in 1X PBS (pH 7.4) for 5 minutes with shaking. Prolong® Gold Antifade Reagent with DAPI (Cat. No. 15898391, Invitrogen, Waltham, Massachusetts, USA) was added to each of the sections to stain nuclei and prolong fluorescence, mounted with a glass coverslip and dried at RT in the dark. Following this, coverslips were sealed with nail polish to prevent drying of the tissue sections and movement under the microscope. Slides were stored in the dark at -20°C until imaging.

2.7.2 Immunofluorescence staining of positive and negative controls

Placental samples were stained in a batch which included three full thickness sections from each of the four study groups, one tonsil section as a positive control and a further five placental sections as negative controls. The negative controls included were as follows:

a) **Isotype control** to check observed staining is not caused by non-specific interactions of the primary antibody with the tissue. One section was incubated with species-matched non-immune IgG antibodies in place of primary antibodies. IgG antibodies were diluted in CAS-Block to the same concentration as the primary antibodies, as detailed in Table 2.8,
b) **Mouse anti-CD68** primary antibody with goat anti-rabbit IgG secondary antibody to check species specificity of the secondary antibody,
c) **Rabbit anti-CD163** primary antibody with goat anti-mouse IgG secondary antibody to check species specificity of the secondary antibody,
d) **CAS-Block** in place of primary antibodies with secondary antibodies to check for non-specific secondary antibody binding, and,
e) **Primary antibodies** with CAS-Block in place of secondary antibodies to confirm primary antibodies are not fluorescing.

2.7.3 Image acquisition and immunolabelling of macrophages

Stained sections were blind-coded to limit bias before being visualised and imaged with an EVOS FL Auto fluorescence microscope (Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts, USA) using a 40X objective. Representative images were acquired from all three placental compartments, chorionic plate, villous tissue and decidua basalis. Using built-in software on the EVOS microscope, gridlines were placed over the slide and six uniformly randomly sampled sections per slide, two from the chorionic plate, two from the villous tissue and two from the decidua basalis, were imaged. The number of $CD68^{+}$ and $CD163^{+}$ macrophages were then counted within each of these selected areas as demonstrated in Figure 2.4 where blue boxes represent selected sections from the chorionic plate, green boxes from the villous tissue and red boxes from the decidua basalis. Cell counts were performed on unenhanced images and only cells with a clearly stained nucleus were counted. To eliminate inter-operator error all slides were read by a single investigator (KMP).

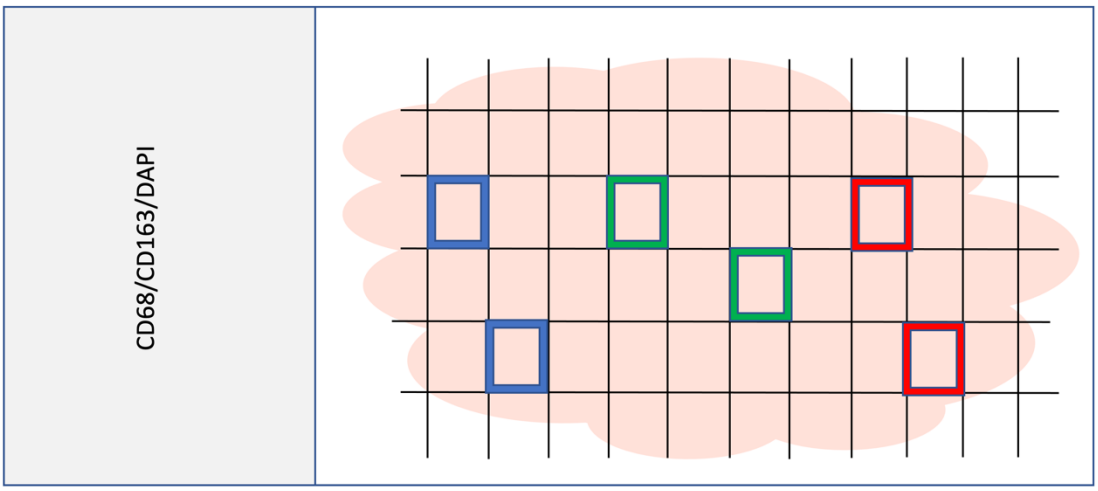

Figure 2. 4 Schematic diagram of immunofluorescent stained slide to identify macrophage phenotype and location.

Slides were overlaid with a grid built into the EVOS FL Auto microscope and the number of M1 and M2 phenotype macrophages were counted in two fields of view of the chorionic plate (represented by blue boxes), villous tissue (green boxes) and decidua basalis (red boxes).

2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were performed to quantify the amounts of target proteins in placental full thickness tissue samples as described below.

2.8.1 Protein extraction and total protein quantitation

The mass of snap-frozen full thickness placental tissues was measured before these were divided into 200 mg sections. Each section was placed in a microfuge tube containing two 5 mm stainless steel beads (Qiagen, Hilden, Germany) and 50 µl of pH 7.4 lysis buffer comprised of 0.1% Igepal CA-630 non-ionic detergent (Sigma, St. Louis, MO, USA) in 1X PBS (pH 7.4) (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and one cOmplete™ Mini Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, Ind., USA) per 10mg of tissue. Placenta tissue samples were homogenised using a TissueLyser (Qiagen, Hilden, Germany) at 50 Hz for 4 minutes. Homogenates were incubated at 4°C with constant agitation for 30 minutes then centrifuged at 13,000 x *g* at 4°C for 20 minutes. Total protein in each sample was determined using the QuBit® Protein Assay Kit (Life Technologies, California, USA) and QuBit® Fluorometer (Life Technologies, California, USA) in accordance with the manufacturer's instructions. Total protein values for all samples measured in micrograms per millilitre (µg/ml) and conversions to picograms per millilitre (pg/ml) are shown in Appendix IV (Figure AIV.V).

2.8.2 Enzyme-linked immunosorbent assay (ELISA)

Through comparison with a standard curve generated from samples containing known concentrations, commercial 96T sandwich ELISA kits were used to quantify amounts of target protein in placental homogenates using antibody-coated plates containing an epitope of the protein of interest. Briefly, homogenate solution extracted from full-thickness placental tissue samples was added to each well where the protein of interest binds to the antibody for analysis. Next a detection antibody raised against a second epitope of the target protein and labelled with biotin was added. Then, streptavidin peroxidase was added which has a high affinity for biotin and was detected by the addition of tetramethylbenzidine (TMB) before the reaction was stopped by adding sulphuric acid solution (Figure 2.5). Details of ELISA kits utilised in this chapter and their corresponding detection ranges and specificity are detailed in Table 2.9.

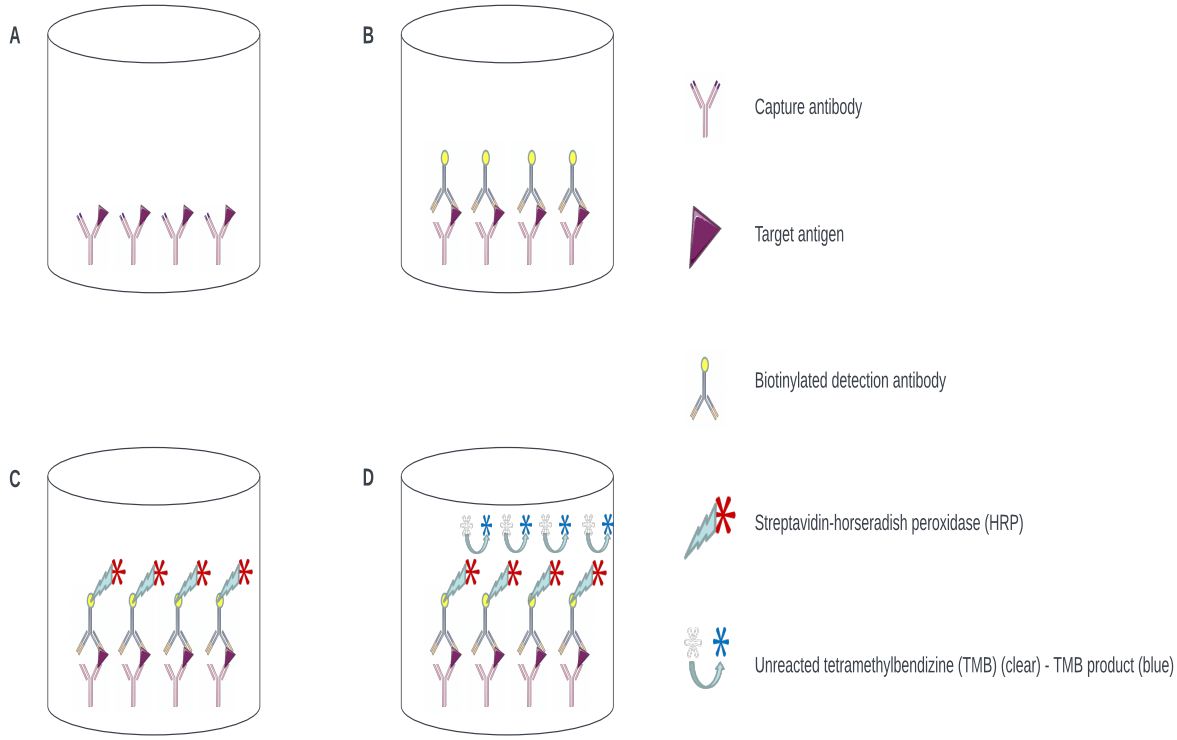

Figure 2. 5 Sandwich ELISA protocol overview.

Plates were coated with capture antibody against target protein. When placental homogenates were added, the target antigen bound to the capture antibody. Biotinylated detection antibody was then added which also bound to the target antigen using a second epitope. Streptavidin-horseradish peroxidase complexed with the biotinylated detection antibody before tetramethylbenzidine (TMB) was added. Clear, unreacted TMB differentially shifted in colour to a blue product before an acidic stop solution was added. Optical density (OD) of blue TMB products were read spectrophotometrically at a wavelength of 450nm. Adapted from Omnikine™ ELISA Kit Instructions.

Table 2. 9 Commercial 96T sandwich ELISA kits utilised to evaluate placental protein levels

Antibody	Abbreviation	Product Code	Manufacturer	Detection Range (pg/ml)	Sensitivity (pg/ml)
Human Cationic Antimicrobial Peptide 18	hCAP18	DL-CAMP-Hu	DLdevelop, Wuxi, China	156-10000	62
Interferon-Gamma	IFN-γ	OK-0122	OmniKine, Assay Biotechnology, San Francisco, CA, USA	46-3000	Not Stated
Interleukin-6	IL-6	DL-IL6-Hu	DLdevelop, Wuxi, China	7.812-500	3.11
Interleukin-8	IL-8	OK-0142	OmniKine, Assay Biotechnology, San Francisco, CA, USA	16-1000	Not Stated
Interleukin-10	IL-10	OK-0123	OmniKine, Assay Biotechnology, San Francisco, CA, USA	32-2000	Not Stated
Matrix Metalloproteinase 9	MMP9	DL-MMP9-Hu	DLdevelop, Wuxi, China	156-10000	63
Secretory Leukocyte Peptidase Inhibitor	SLPI	DL-SLPI-Hu	DLdevelop, Wuxi, China	62.5-4000	25.8

2.8.3 ELISAs for quantification of concentrations of proteins of interest

Taking into consideration results of the validation ELISAs (described in *Chapter 7*) and placental homogenate volumes, hCAP18, IFN-γ, IL-8, MMP9 and SLPI assay samples were diluted 1:2 in assay diluent (Assay Biotechnology). IL-6 assay samples were diluted 1:10 in 1X PBS (pH 7.4) (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and IL-10 assay samples were analysed undiluted. Standards, blank solutions and homogenate samples were run in triplicate for each assay. To reduce variations in results due to delays in adding samples and/or reagents to wells across the plate, samples from each of the four categorised groups were distributed across the plate, as shown in Figure 2.6. The optical density (OD) of each well was measured spectrophotometrically at a wavelength of 450 nm on an Accuris Instruments SmartReader MR-9600 (Benchmark Scientific, Sayreville, NJ, USA) before raw data was exported for analysis.

2.9 Statistical analyses

All demographic, clinical and experimental data were entered into GraphPad Prism 9.1.1 (GraphPad Software Inc., San Diego, CA, USA) and analysed employing descriptive and inferential statistics. Categorical data were presented as frequencies and analysed with Chi-square tests to establish relationships or, where greater than 20% of values had an expected frequency of less than 5 and/or zero values, Fisher's exact test were employed. For *r* x *c* data tables greater than 2 x 2, the Fisher-Freeman-Halton Statistic was applied. The distribution of the data was assessed with Shapiro-Wilk normality tests. If the assumption of normality was met, continuous data were compared using parametric tests, such as Student's *t*-test or ANOVA and presented as mean ± standard deviation (SD). Conversely, if data were found to be non-normally distributed, non-parametric tests, such as Mann-Whitney *U* and Kruskal Wallis tests were performed, and data were summarised using the median as a measure of centrality and the interquartile range (IQR) as a measure of data dispersion. Post-hoc tests including Tukey's and Dunn's multiple comparisons or Chi-square test for trends were employed for analysis between groups and to determine linearity and trends with advancing gestational age. Results were considered statistically significant at a *P*-value <0.05. Specific statistical tests performed on data collected for studies described within each chapter are described further in the materials and methods section of the chapter to which they relate.

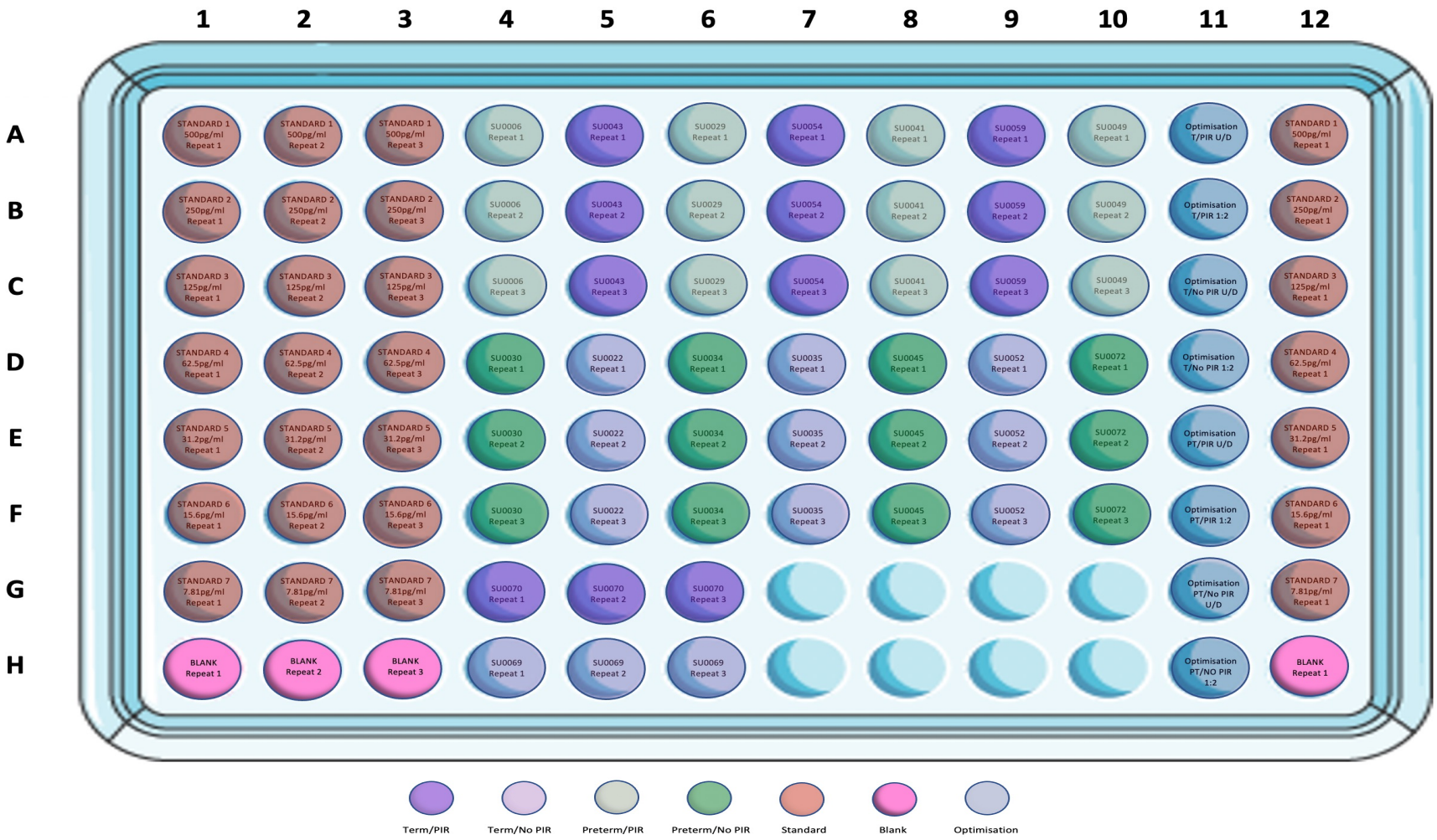

Figure 2. 6 ELISA plate layout for quantification of hCAP18, IFN-γ, IL-6, IL-8, IL-10, MMP-9 and SLPI levels in preterm and term placentas.

Standards, blanks and samples were run in triplicate and distributed across the plate identically for each assay to reduce variations due to delays in loading samples and reagents to wells and any subsequent fluctuations in temperature.

Chapter 3: Characterisation of Placental Histopathological and Morphological Features Associated with Preterm Birth and Development of a Risk Prediction Model for Preterm Birth Using Maternal and Obstetric Characteristics

3.1 Introduction

In developed countries, placental evaluation is now commonplace where there are diagnosed maternal or fetal complications (140, 328). The Royal College of Pathologists (RCPath) in the United Kingdom (UK) advises that all placentas from preterm births occurring <32 weeks' gestation should be submitted for pathological assessment (136). Unless clinically indicated, term placentas are not routinely assessed. Whilst this breadth of data collected across the preterm spectrum adds substantial value to clinical care and research studies, there is a concomitant paucity in data characterising comparative gross morphological features of term placentas which may provide important information for interpretation of preterm histopathological findings. Furthermore, correlations between maternal clinical and demographic characteristics, maternal and fetal indications for placental examination and placental histopathological evaluations are frequently overlooked.

3.1.1 Placental evaluation and preterm birth

Placental gross morphological evaluation documents the pregnancy and elucidates invaluable detail about maternal and fetal health (103, 329). Placental disorders or dysfunction may be revealed through morphological examination (330), yet challenges remain in interpretation since morphological findings often do not correlate directly to clinical conditions or severity (140). Research into better understanding of placental morphology and the associations with preterm birth frequently focuses on specific pathologies such as stillbirth (331), low birthweight (330), or PTB recurrence risk (332). The overarching aim of this chapter was to identify morphological features of preterm birth in the placenta, uniquely in an unselected cohort. Term placentas were collected and examined as controls. Clinical details, which were provided as recommended in the RCPath guidance (136) and documented on the Request for Placental Examination Form, SCH Doc. No. 322.4 (Appendix AII.Ia-b) for the information of the placental pathologist, were reviewed as supplementary data.

3.1.2 Maternal and obstetric characteristics as predictors of preterm birth

As discussed in detail in section 1.8, timely identification of women at risk of PTB is essential to ensure appropriate monitoring or treatment strategies are employed for high-risk groups. However, challenges arise since complete histopathological, morphological, immunological, and molecular analyses of placentas are only feasible postpartum and, realistically, are

impracticable due to the high economical and personnel resources these comprehensive placental assessments would demand. Although biochemical and clinical tests may provide some indication of PTB risk, assessing cervical length or measuring biomarkers such as fFN, IL-6, phlGFBP-1, PAMG-1 or PAPP-A is by no means a panacea of PTB prediction or prevention of costly and potentially unnecessary hospital admissions or treatments (see section 1.8 for further discussion). Moreover, many of these laboratory tests are inaccessible in low-resource settings and are only considered useful predictors in the second and third trimesters (333). Adding to the complexity is that many risk factors have been attributed to PTB, including advanced maternal age, obesity, use of tobacco products or a previous preterm birth, yet, most PTBs are to women with no evident risk factors and those who are nulliparous (45). Consequently, developing a model for prediction of PTB risk based on clinical and non-clinical maternal and obstetric characteristics, incorporating those collected throughout pregnancy, is paramount to allow effective monitoring and early interventions of women identified of being at high-risk of delivering preterm.

3.1.3 Hypothesis and aims

Initially, the aim of this chapter was to characterise gross morphological placental features and correlate anomalies to PTB, based on the hypothesis that an increased prevalence of abnormal placental morphology is associated with PTB. However, as the research progressed it seemed pragmatic to analyse available maternal and obstetric clinical and non-clinical characteristics since collection of these data was feasible and PTB is known to be syndromic (162). Often, where APH, IUGR, GDM or PE are present, delivery is indicated to improve maternal or fetal health and an iatrogenic PTB will result; this has been suggested to account for up to 35% of all PTBs (334, 335). As all participants enrolled in the study experienced a spontaneous delivery, it was of interest to analyse the *additional* indications for placental examinations in these cases to determine whether the same trend of complications and placental features were presenting in spontaneous PTBs and/or term births.

The revised hypotheses stated, first, that maternal and obstetric characteristics known to be risk factors for preterm birth predominate in the preterm cohort. Secondly, it was hypothesised that reports of abnormal gross morphology are increased in placentas delivered preterm compared to those delivered at term and that this inversely correlates with

gestational age. Finally, the hypothesis that women experiencing antepartum haemorrhage are at greater risk of histologically-diagnosed chorioamnionitis leading to preterm prelabour rupture of membranes and subsequent preterm birth was investigated. The main aims of the chapter were to characterise the gross morphology of preterm and term placentas and correlate these to birth outcome as well as describe maternal clinical and non-clinical characteristics of the total cohort and identify relationships with birth outcome. The final aim was to develop a risk prediction model for preterm birth using maternal and obstetric characteristics marked as indicators for placental examination *in addition* to prematurity.

3.2 Materials and Methods

3.2.1 Participant recruitment

Full descriptions of ethical approval, participant recruitment, study design, collection, processing and methods used in this chapter are set out in *Chapter 2: Materials and Methods*.

3.2.2 Placental morphological examination

Placentas were placed directly into a pre-labelled white 5 litre plastic bucket for fixation in 10% neutral buffered formalin (NBF) (Cat. No. HT501640-19L, Sigma-Aldrich, St. Louis, MO, USA). Gross morphological examination was performed on fixed placentas with findings documented on SCH Doc. No. 326.4.34 Singleton Placenta Dissection Reporting Form (Appendix II.11a-c) as described in detail in *Chapter 2: Section 2.4*.

3.2.3 Data collection

For the purposes of this retrospective study, indications for placental examination *in addition to prematurity* as marked on the receiving *Request for Placental Examination* Form (Doc. No. 322.4; Appendix II, Figure AII.Ia), were included in the models described. Any corresponding detail for term placentas was collected from clinical notes. Maternal clinical and non-clinical and basic neonatal data were collated following thorough examination of clinical obstetric notes, where available.

3.2.4 Statistical analyses

Statistical analyses were conducted as described in *Chapter 2: Materials and Methods*. Additionally, multiple linear regression models were applied to estimate the association

between gestational age at delivery (in weeks) and eight key indicators of placental examination (confounders). Dichotomous variables entered in the multivariate model were: fetal distress (FD), intrauterine growth restriction (IUGR), maternal pyrexia (MP), clinical chorioamnionitis (CCA), preterm prelabour rupture of membranes (PPROM), antepartum haemorrhage (APH), preeclampsia (PE) and gestational diabetes mellitus (GDM). Subsequent multivariate analyses were performed after backward selection of statistically significant variables (confounders), of which three were identified - APH, PE and PPROM. Individual-level slopes were used as the gestational age variable in the regression models, and regression coefficients were considered to represent a 1-unit (week) change in the linear slope. In composite models of confounding variables, regression coefficients represent the change in confounder variable presence for a 1-unit (week) change in composite value. Results were considered statistically significant at a *P*-value <0.05.

3.3 Results

3.3.1 Maternal characteristics of the total study population

Maternal characteristics may confound correlations between placental morphology and PTB, as described in *Chapter 1*. Mean maternal age at delivery was 29.15 (± 5.72) years of age. Body Mass Index (BMI) calculated at antenatal booking revealed a mean value within the overweight range (26.66 ± 7.23kg/m^2) (336). Most women (84.3%) enrolled in the study were nulli- or primiparous (parity range: 0 – 6, median 1), twenty women had experienced previous losses in the first trimester and three reported a mid-trimester loss. Previous preterm delivery was reported in 15 cases (Table 3.1). There was a White British predominance in women enrolled in the study (65.5%), although eight separate ethnicities were recorded. White Other was the second most reported ethnicity (9.2%) followed by Asian British/Pakistani and Asian British/Other (each representing 4.60% of the cohort). Only 12 women (13.8%) included in the study reported being a smoker at antenatal booking. Data were collected from antenatal booking forms regarding pre-pregnancy conditions which may have an effect on pregnancy outcome, including diabetes mellitus (DM) and hypertension, however, only one woman in the cohort reported DM while hypertension was noted on booking forms of two participants (Table 3.1).

3.3.2 Maternal characteristics by gestational age category

Deliveries were categorised to extremely preterm (EPT), very preterm (VPT), moderate to late preterm (MPT) and term, and maternal characteristics were evaluated, with data presented in Table 3.1. Women delivering VPT were youngest with a mean maternal age of 25.56 ± 5.37 years, and oldest in the term group (29.78 ± 5.79 years); however, all mean maternal ages were within the normal range and not considered to be extreme, that is teenagers and/or women aged ≥35 years (24), factors shown to be linked to adverse obstetric outcomes (24, 337). Evaluation by one-way ANOVA found no statistically significant difference in mean maternal age between gestational age groups ($F(3,87)=0.3024$, $P=0.2970$) (Table 3.1). Only women delivering in the VPT group had a mean BMI within the normal range (336) (24.70 ± 9.77kg/m^2); all other group means were within the overweight range, but there was no significant difference in mean BMI and gestational age ($P=0.3955$). Parity ranged from zero to six. Median parity and range were highest in the MPT and term cohorts (parity range: 0 – 6, median 1) and lowest in the VPT category (parity range: 0 – 1, median 0) although this did not differ significantly between the groups ($P=0.7490$) (Table 3.1). There was a significant difference in the number of first- and mid-trimester losses between preterm and term cohorts ($P=0.0116$ and $P=0.0106$, respectively) and, although a greater proportion of women delivering in the MPT group reported a previous PTB, this was not significant ($P=0.0562$). Since preterm birth was the primary outcome, term cohorts were excluded and analysis repeated for these data which revealed a statistically significant relationship between first trimester loss and preterm gestational age category ($P=0.0045$), but not mid-trimester loss or previous preterm birth ($P=0.1081$ and $P=0.1169$, respectively). Nonetheless, first trimester loss and MPT were significantly associated in a linear manner ($\chi^2_{df=1} = 13.19$; $P=0.0003$), and a previous PTB had been a significantly more frequent occurrence in women subsequently delivering at MPT gestations ($\chi^2_{df=1} = 5.525$; $P=0.0187$). Women were no more commonly smokers at preterm gestations than those at term ($P=0.3110$). A history of DM or hypertension was uncommon and not reported by any women in the EPT or VPT groups; no difference in the presence of either condition was shown between groups ($P=0.5455$ and $P=0.7000$, respectively) (Table 3.1). There was a significant difference in maternal ethnicity ($P=0.0002$) with most women self-reporting as White British (57 cases; 65.5%).

Table 3. 1 Maternal characteristics by gestational age category

	Extremely Preterm <28 weeks (*n* = 9)	Very Preterm 28-<32 weeks (*n* = 7)	Moderately Preterm 32-<37 weeks (*n* = 27)	Term ≥37 weeks (*n* = 44)	*P Value*	Total Cohort (*n* = 87)
Maternal age (years)*	(*n* = 11) 29.49 ± 5.49	(*n* = 8) 25.56 ± 5.37	(*n* = 28) 29.10 ± 5.72	29.78 ± 5.79	0.2970 ns	(*n* = 91) 29.17 ± 5.72
BMI (kg/m^2)†	(*n* = 2) 29.69 ± 0.41	(*n* = 3) 24.70 ± 9.77	(*n* = 17) 27.94 ± 8.70	(*n* = 36) 26.05 ± 6.54	0.3955 ns	(*n* = 58) 26.66 ± 7.23
Parity (median: range)†	0.5 (0-2)	0 (0-1)	1 (0-6)	1 (0-6)	0.7490 ns	1 (0-6)
First trimester loss (*n*)^	0	5	6	9	0.0116	20
Mid-trimester loss (*n*)^	0	2	1	0	0.0106	3
Previous preterm birth (*n*)^	0	1	9	5	0.0562 ns	15
Smoker (*n*) (%)^	0 (0)	0 (0)	5 (17.9)	7 (13.7)	0.3110 ns	12 (13.8)
Ethnicity (*n*)^						
Asian British/Other	0	1	0	3		4
Asian British/Pakistani	0	0	1	3		4
Black African	0	0	0	2		2
Black Caribbean	0	0	0	1	0.0002	1
Mixed	0	0	0	1		1
White British	4	4	19	30		57
White/Black Mixed	0	0	0	2		2
White Other	4	0	3	1		8
Not recorded	1	2	4	1		8
Diabetes mellitus (*n*)^	0	0	1	0	0.5455 ns	1
Hypertension (*n*)^	0	0	0	2	0.7000 ns	2

BMI; body mass index. Values are presented as mean ± SD unless stated otherwise. *One-way ANOVA with Tukey's multiple comparison, †Kruskall-Wallis with Dunn's multiple comparison, ^Fisher's exact test with Freeman Halton extension. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

3.3.3 Obstetric and neonatal characteristics of total study population

The mean gestational age at delivery in the total cohort was 35.9 ± 4.9 weeks with a range of 23.9 – 42.3 weeks; the mean birthweight for all neonates delivered of women in the study was 2530.0 ± 1008.0 grams. Most women delivered via standard vaginal delivery (*n*=76; 80.0%), eleven (11.6%) delivered by Caesarean section and the remaining eight (8.6%) required an instrumental vaginal delivery. Forty women (46.0%) experienced PPROM compared to twenty-three (27.6%) who had spontaneous membrane rupture (SROM) and twenty-four (27.6%) where artificial rupture of membranes (AROM) was necessary (Table 3.2). Labour lasted, on average, 9.43 ± 14.32 hours, and the mean duration of membrane rupture prior to delivery was 114.9 ± 359.5 hours. Antibiotic treatment during labour and delivery was recorded in fourteen (14.3%) cases and two doses of corticosteroids 24 hours apart were administered to 31 women (31.6%), all at preterm gestations. Male neonates slightly outweighed females in the total cohort (55.8% vs. 44.2%) (Table 3.2).

3.3.4 Obstetric and neonatal characteristics by gestational age category

The mean gestational age of term deliveries was 39.6 ± 1.4 weeks and for the total unstratified preterm cohort it was 31.9 ± 4.1 weeks. Preterm gestations ranged from 23.9 – 36.4 weeks compared to term gestations of 37.3 – 42.3 weeks (Table 3.2). Fisher's exact tests demonstrated significant relationships between gestational age category and method of rupture of membranes ($P<0.0001$). Nonetheless, excluding the two term cases of PPROM and applying a Chi-square test for trend showed there was no linear relationship and no increased prevalence of PPROM in earlier preterm gestations ($\chi^2_{df=1} = 2.757$; $P=0.0968$). There were no Caesarean section or instrumental vaginal deliveries in the EPT gestational age group; in the VPT group, three women delivered by Caesarean section with the remaining five experiencing a standard vaginal delivery. Most Caesarean section deliveries were in the MPT group (7 cases) and only one was reported in the term group. Instrumental vaginal deliveries were recorded in both the MPT (3 cases) and term (5 cases) groups. A statistically significant relationship was found between gestational age and method of delivery ($P=0.0055$) (Table 3.2).

Duration of labour (DoL) was determined as being from the time of ≥4cm cervical dilation as recorded in clinical notes (established labour) or from the point of labour ward admission if more dilated on arrival. There was no difference in duration of labour regardless of whether women delivered at gestations in the preterm groups or at term (*P*=0.1705). In addition, analysis of only the preterm groups found women did not labour significantly longer at any preterm gestation when assessed by a Kruskal-Wallis test with Dunn's multiple post-hoc tests (data shown Appendix AIII.III) (Table 3.2). Conversely, the duration of rupture of membranes (ROM) differed significantly between the four groups (*P*=0.0054) with the longest period of ROM, but also the largest standard deviation from the mean, observed in the VPT group (1560.0 ± 644.9 hours). Dunn's post hoc-analyses found the duration of ROM in VPT births to be significantly longer than in term births (*P*=0.0388) but there was no difference between only the preterm gestational age groups (Table 3.2) (Appendix III.IV).

Women were significantly more likely to have been administered antepartum corticosteroids (betamethasone) in preterm groups than at term (*P*<0.0001), unsurprising since the Royal College of Obstetricians and Gynaecologists recommend these be offered to women between 24 and 34+6 weeks' gestations in whom imminent preterm birth is anticipated (338). Chi square tests for trend analysis revealed a linear relationship between corticosteroid administration across the four groups ($\chi^2_{df=1}$ = 18.8272; *P*<0.0001) and, when term gestations were excluded, a similar linear relationship was revealed between the three preterm gestational categories ($\chi^2_{df=1}$ = 4.976; *P*=0.0257) demonstrating an anticipated increased prevalence of corticosteroid administration at earlier gestations (Table 3.2). Likewise, more women were administered intrapartum antibiotic prophylaxis in the EPT, VPT and MPT groups than at term (36.3%, 25.0%, 28.6% vs. 0%, respectively; *P*<0.0001) and this significant relationship was confirmed to be linear by a Chi square test for trend ($\chi^2_{df=1}$ = 6.2282; *P*=0.0126). No such linear relationship was seen when comparing only preterm gestations ($\chi^2_{df=1}$ = 0.1647; *P*=0.6848) (Table 3.2). More male neonates were born in all groups except at EPT gestations where there were half as many male neonates as female (1:2), however, Fisher's exact test with a Freeman-Halton extension found no significant relationship between neonatal sex and gestational age (*P*=0.4785) (Table 3.2).

Table 3. 2 Obstetric and neonatal characteristics by gestational age category

	Extremely Preterm (*n* = 11)	Very Preterm (*n* = 8)	Moderately Preterm (*n* = 28)	Term (*n* = 51)	*P V*alue	Total (*n* = 98)
Gestational age (weeks)*†	25.8 ± 1.6	29.9 ± 1.1	34.9 ± 1.2	39.6 ± 1.4	n/a	35.9 ± 4.9
Membrane Rupture				(*n*=40)		(*n*=87)
AROM	1	0	8	15	<0.0001	24
PPROM	10	8	20	2		40
SROM	0	0	0	23		23
Mode of delivery				(*n*=48)		(*n*=95)
CS	0	3	7	1	0.0055	11
IVD	0	0	3	5		8
SVD	11	5	18	42		76
Duration of labour (hours)*†	(*n*=3) 44.86 ± 49.01	(*n*=2) 2.34 ± 1.54	(*n*=9) 6.26 ± 5.01	8.07 ± 6.99	0.1705 ns	9.43 ± 14.32
Duration of ROM (hours)*†	(*n*=3) 49.04 ± 46.67	(*n*=2) 1560.0 ± 644.9	(*n*=9) 112.8 ± 175.6	15.96 ± 43.25	0.0054	114.9 ± 359.5
Antibiotics (intra-partum)	4	2	8	0	<0.0001	14
Corticosteroids (antenatal)	11	8	12	0	<0.0001	31
Birthweight (g)*†	799.3 ± 252.8	1387.0 ± 322.8	2316.0 ± 448.0	(*n*=41) 3363.0 ± 458.8	n/a	(*n*=88) 2530.0 ± 1008.0
Sex (*n*)	(*n*=9)			(*n*=41)		(*n*=86)
Male	3	5	15	25	0.4785 ns	48
Female	6	3	13	16		38

AROM; artificial rupture of membranes, *CS*; Caesarean section*, IVD*; instrumental vaginal, *PPROM*; preterm prelabour rupture of membranes, *ROM*; rupture of membranes, *SROM*; spontaneous rupture of membranes, *SVD*; standard vaginal delivery. Values are presented as counts (*n*), statistical analysis was by Fisher's exact test with Freeman Halton extension, unless stated. *Mean ± SD, †Kruskall-Wallis with Dunn's multiple comparison. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *n/a*; not applicable, *ns*; not significant.

Birthweight ranged from 455g (25.8 weeks' gestation) to 4020g (39.4 weeks' gestation). Mean birthweight in the EPT group was 799.3 ± 252.8g, increasing to 1387.0 ± 322.8g in the VPT group, 2316.0 ± 448.0g in the MPT group and 3363.0 ± 458.8g at term (Table 3.2). As birthweight alone provides little information about *appropriateness* for gestational age, birthweight centiles were calculated for each neonate where birthweight was recorded (*n*=88) using the World Health Organisation's (WHO) online Fetal Growth Calculator (https://srhr.org/fetalgrowthcalculator/#/) (339). Median birthweight centiles were lowest for VPT and MPT groups at 30.5 (IQR: 21.1) and 33.0 (IQR: 60.3), respectively. The median birthweight centile in the EPT group was 37.0 (IQR: 46.3) and at term this was calculated to be 47.0 (IQR: 52.0), but there was no statistically significant difference between birthweight centiles across the four gestational age categories (*P*=0.1367) demonstrating overall that birthweight was appropriate for gestational age within the entire cohort (Figure 3.1). Neonatal birthweights and birthweight centiles are shown in Appendix AIII.V.

Notwithstanding these non-significant findings, use of the WHO calculator revealed a small for gestational age (SGA) birthweight centile, specifically those ≤10th centile, in over one-quarter of cases (*n*=24; 27.3%), only seven of which had previously been identified as IUGR at or prior to delivery. Of the SGA cases, four (16.7%) were delivered EPT, two (8.3%) VPT, eleven (45.8%) MPT and seven (29.2%) at term, however, no significant relationship was found between these cases (*P*=0.1872).

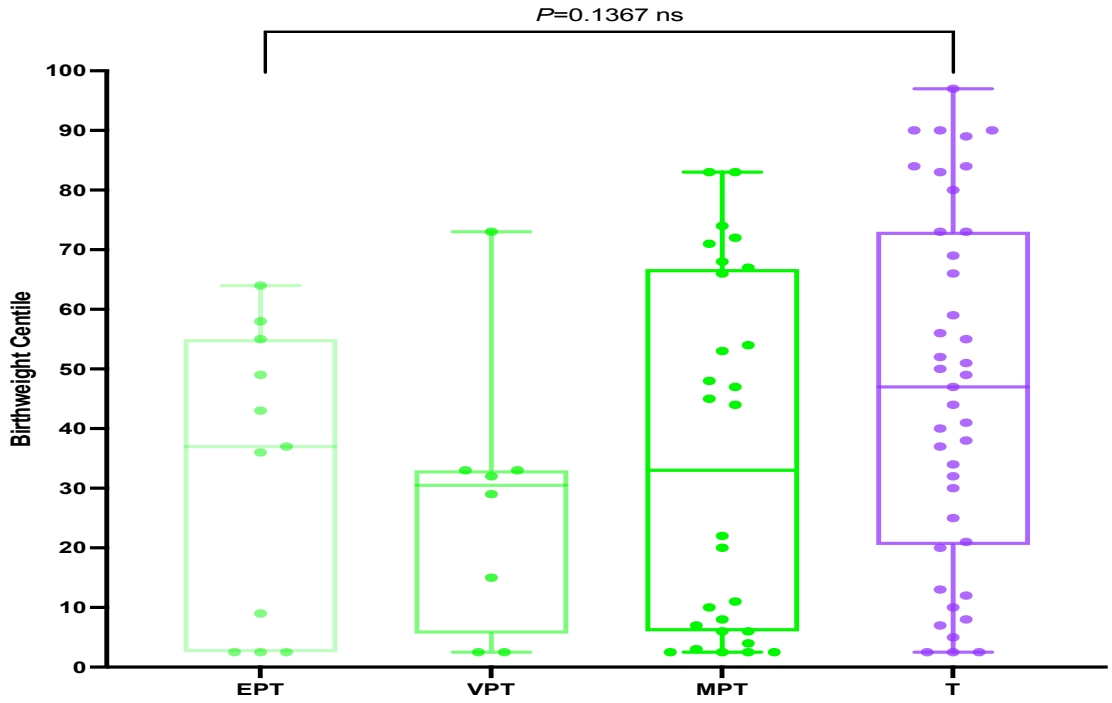

Figure 3. 1 Median birthweight centile by gestational age category.

The World Health Organisation's online Fetal Growth Calculator (https://srhr.org/fetalgrowthcalculator/#/) was used to calculate birthweight centile for each neonate where birthweight and sex were recorded in clinical notes. Kruskal-Wallis tests with Dunn's multiple comparisons were conducted to compare centiles between and within groups. No significant difference (*P*=0.1367) was found across the gestational age categories, nor within any of the groups thus demonstrating birthweights appropriate for gestational age in each preterm category and at term (data presented in Appendix AIII.VI).

3.3.5 Appraisal of obstetric, fetal and neonatal confounders in the total study population

The histopathological examination of all preterm placentas included in this chapter of the study was principally due to prematurity; however, to meet required clinical standards, a complete histopathological assessment was conducted, and a routine report was produced for each preterm placenta. For completeness, and as outlined in *Chapter 2*, term placentas were examined and assessed by the author to the same standard as for preterm placentas although no report was issued to participants as per study and clinical protocols. Obstetric complications which were opportunistically revealed during the study and were associated with a request for placental histopathological examination were evaluated and compared across the four gestational age groups. Table 3.3 details coexisting conditions indicated on placental examination request documentation sent to SCH with preterm placentas (*n*=47), as per Appendix AII.Ia. For term placentas (*n*=40), any findings recorded were incidental and unveiled due to enrolment in this research study when data were retrospectively collected from clinical notes.

Within the total study population there were ninety-three obstetric, fetal or neonatal complications indicated for placental assessment reported from fifty-three participants. Only eight confounders which may ordinarily justify a placental examination were reported from clinical notes of term participants. The most frequent complications were PPROM (40 incidences; 43.0%), APH (10 incidences; 10.8%), preeclampsia (7 incidences; 7.5%) and IUGR (7 incidences; 7.5%). The prevalence of gestational diabetes was low at 6.5% (6 incidences) and placental evaluation was infrequently indicated due to fetal distress (4 incidences; 4.6%) or clinical chorioamnionitis (4 incidences; 4.6%). Oligohydramnios was an uncommon observation (3 incidences; 3.2%), as were maternal pyrexia and meconium-stained liquor (each 2 incidences; 2.2%). Both morbidly-adherent placenta and polyhydramnios were each only reported once in the entire cohort (1.1%) (Table 3.3).

Of the forty-seven included preterm placentas received by SCH for placental evaluation, only six cases had no additional obstetric, fetal or neonatal condition indicated (6.5%). A significant relationship between PTB and prematurity as the only criterion for placental pathological evaluation was, expectedly, shown when a Chi-square test for independence was applied (P=0.0289) (data not shown). In the remaining forty-one preterm deliveries, at least one other condition was recorded. All term placentas were only assessed for research purposes so obstetric complications were incidental findings which would ordinarily have been unreported. PPROM was indicated on thirty-eight preterm placental examination request forms and in two term participants' notes. PPROM accounted for most (43.0%) of the associated conditions coexisting with PTB and a highly significant relationship was shown with prematurity (P<0.0001).

Table 3. 3 Obstetric, fetal and neonatal complications as confounders to prematurity by gestational age category

	Extremely Preterm (*n*)	Very Preterm (*n*)	Moderately Preterm (*n*)	Term (*n*)	*P V*alue	Total (*n*)
Antepartum haemorrhage	5	0	4	1	0.0022	10
Clinical chorioamnionitis	2	1	1	0	0.0282	4
Fetal Distress	0	1	2	1	0.4232 ns	4
Gestational diabetes	0	1	3	2	0.4754 ns	6
IUGR	2	0	5	0	0.0135	7
Maternal pyrexia	0	2	0	0	0.0075	2
Meconium-stained liquor	0	0	2	0	0.3745 ns	2
Morbidly adherent placenta	0	0	1	0	0.5402 ns	1
Oligohydramnios	1	2	0	0	0.0050	3
Polyhydramnios	0	0	0	1	0.7557 ns	1
PPROM	10	8	20	2	<0.0001	40
Preeclampsia	2	1	3	1	0.1563 ns	7
Prematurity only	0	1	5	n/a	0.3984 ns	6

Fisher's exact test with Freeman Halton extension applied to all data. *IUGR*; intrauterine growth restriction, *PPROM*; preterm prelabour rupture of membranes. Values are presented as counts (*n*) of condition (incidences). *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant, *n/a* not applicable.

3.3.6 Appraisal of obstetric, fetal and neonatal confounders by gestational age category

Antepartum haemorrhage was most common in extremely and moderately preterm births. Incidences in these groups combined accounted for 90% of reported APHs and this association was found to be statistically significant by Fisher's Exact test (*P*=0.0022). Clinical chorioamnionitis (CCA) and maternal pyrexia, conditions generally reported to occur together, were found to have a significant association with gestational age category (*P*=0.0282 and *P*=0.0075, respectively), although both were uncommon additional indications for placental examination. No neonate born in the term or VPT gestational age category had a recorded IUGR, but this was a confounder in two (18.2%) EPT and five (17.9%) MPT deliveries and a statistically significant relationship between gestational age group and IUGR was shown (*P*=0.0135). Oligohydramnios, a decreased amniotic fluid volume (340), was reported in three cases, one in the EPT category and two in the VPT category, a relationship found to be significant (*P*=0.0050). Over 80% of women in the preterm cohort had experienced PPROM; in the EPT group there was only one case without associated PPROM and none in the VPT group. PPROM occurred in almost three-quarters of MPT births (20 incidences; 71.4%) but was a feature in only two cases where women then delivered at term (5.0%). A highly significant relationship between gestational age category and PPROM was observed in this cohort (*P*<0.0001) (Table 3.3).

Conversely, incidences of fetal distress were infrequent (4 cases) and not significantly associated with gestational age category (*P*=0.4232), nor were cases of meconium-stained liquor (*P*=0.3745), which itself may indicate fetal distress (341). Polyhydramnios, an excessive accumulation of amniotic fluid (340), was observed in only one participant, who delivered at term and no significant relationship was shown (*P*>0.9999). There was also only one report of a morbidly adherent placenta in the entire cohort, recorded as placenta percreta with invasion through the myometrium and serosa into the bladder, and this occurred in an MPT birth; as such, no significant association between this obstetric complication and gestational age category was revealed (*P*=0.5402). Gestational diabetes mellitus (GDM) and preeclampsia, maternal complications which may contribute to preterm birth and are indications for placental assessment, were observed in all groups apart from GDM in the EPT category. However, no statistically significant relationship between gestational age group and either GDM (*P*=0.4754) or preeclampsia (*P*=0.1563) was shown. Additionally, it was noted that

both obstetric conditions were reported infrequently overall with only six (6.9%) women having diagnosed GDM and seven (8.0%) women experiencing preeclampsia in this cohort, potentially due to elective Caesarean section being a frequent outcome where either of these conditions are present, thus excluding women with GDM and preeclampsia from the study (Table 3.3).

The absence of confounding conditions was also assessed by performing a Fisher's exact test with a Freeman Halton extension on preterm cohorts where no confounders for placental examination were indicated on the request documentation, i.e., prematurity only. All cases (100%) in the EPT group had at least one additional indicator for placental examination, as did the majority of the VPT (7 cases; 87.5%) and MPT (23 cases; 82.1%) groups. There was no statistically significant association between preterm gestational age category and prematurity alone as an indicator for placental examination (*P*=0.3984) (Table 3.3).

3.3.7 A significant linear trend is observed between gestational age category and antepartum haemorrhage, clinical chorioamnionitis, and oligohydramnios

Having determined significant associations between gestational age category and obstetric or fetal confounders of APH, CCA, IUGR, maternal pyrexia and oligohydramnios, Chi square tests for trend were applied to these data to test the hypothesis of there being a linear relationship. More specifically, the hypothesis stated that the frequency of each confounder would be increased in preterm gestational age categories in a linear fashion, for example, APH would occur more frequently at EPT gestations than at term. Of all APH occurrences, 50% (5 cases) were reported in the EPT group, 40% (4 cases) in the MPT group and 10% (1 case) at term. A significant linear trend was demonstrated ($\chi^2_{df=1}$ = 11.1456; *P*=0.0008) with a propensity for a greater frequency of APH at EPT gestations. Clinical chorioamnionitis (CCA) was reported only in preterm gestations and was indicated as an additional factor for placental examination in just two EPT, one VPT and one MPT case in the entire cohort. A significant linear relationship was found on application of a Chi square test for trend ($\chi^2_{df=1}$ = 7.4802; *P*=0.0062) which demonstrated an increased prevalence of CCA with decreasing gestational age category. Despite being an infrequent additional indication for placental examination, whereby one third (1 case; 33.3%) of cases were in the EPT category and the remaining two-thirds (2 cases; 66.7%) in the VPT category, oligohydramnios demonstrated a significantly

linear trend with increased frequency observed at preterm gestations compared to term ($\chi^2_{df=1}$ = 6.2410; *P*=0.0125) (Table 3.4).

Although Fisher's exact test with Freeman Halton extension revealed a significant association between gestational age category and maternal pyrexia frequency, no cases of this confounding condition were reported in the EPT, MPT or term group and only two cases were recorded in the VPT group. Consequently, no linearity was shown by a Chi square test for trend ($\chi^2_{df=1}$ = 2.4368; *P*=0.1185). Similarly, IUGR cases were distributed across only two groups, EPT (2 cases; 28.6%) and MPT (5 cases; 71.4%), and no significant linear trend was observed in the data ($\chi^2_{df=1}$ = 3.4343; *P*=0.0639) (Table 3.4).

Table 3. 4 Trends in obstetric, fetal and neonatal complications as confounders to prematurity

	χ^2 Trend Value (*df*)	***P V*alue**
Antepartum haemorrhage	11.1456 (1)	0.0008
Clinical chorioamnionitis	7.4803 (1)	0.0062
Intrauterine growth restriction	3.4343 (1)	0.0639 ns
Maternal pyrexia	2.4368 (1)	0.1185 ns
Oligohydramnios	6.2410 (1)	0.0125

df; degrees of freedom, ns; not significant, *P*; probability. Two-Tailed *P*<0.05 considered to be statistically significant.

3.3.8 Key obstetric, fetal and neonatal indications for placental examination correlate to decreasing gestational age and are significantly associated with preterm categories

Multiple linear regressions were performed in order to estimate the effect of key obstetric, fetal and neonatal indicators for placental evaluation (independent variables) on gestational age at delivery in weeks (dependent variable). Results for all independent variables are presented in Table 3.5, including the estimate of gestational age (beta coefficient), magnitude of each association (t-statistic) and significance (*P* value).

An overall statistically significant regression equation was shown ($F(8,89) = 12.59$; $P<0.0001$). The estimated effect size (regression coefficient (R^2)) of 0.5309 demonstrated 53% of the variation in reported key obstetric, fetal and neonatal conditions indicative of a placental examination can be explained by the model containing gestational age. Significant associations were found between decreasing gestational age and an increased prevalence of APH, preeclampsia (PE) and PPROM. The greatest effect size was observed where PPROM occurred, then APH and finally PE, as demonstrated by the statistically significant t-statistics of 6.662 ($P<0.0001$), 3.232 ($P=0.0017$) and 3.043 ($P=0.0031$), respectively, for each of these variables (Table 3.5).

Table 3. 5 Multiple linear regression analysis, ANOVA, and overall model summary of obstetric, fetal and neonatal indications for placental examination as predictors of gestational age

Variable	ANOVA *F(DFn,DFd)*	Beta-Coefficient Estimate	Standard Error	95% Confidence Interval	t-Statistic	R^2	*P* Value
Intercept (GA)(β_0)	12.59(8, 89)	38.81	0.4680	37.88 to 39.74	82.93	0.1629	<0.0001
APH	10.44(1, 89)	-4.102	1.269	-6.623 to -1.580	3.232	0.1998	0.0017
CCA	3.148(1, 89)	-3.562	2.007	-7.550 to 0.4269	1.774	0.2839	0.0794 ns
Fetal distress	0.1903(1, 89)	0.8527	1.955	-3.032 to 4.737	0.4362	0.2662	0.6638 ns
GDM	1.055(1, 89)	-1.549	1.509	-4.547 to 1.448	1.027	0.1629	0.3072 ns
IUGR	1.794(1, 89)	2.149	1.604	-1.039 to 5.336	1.339	0.1461	0.2838 ns
Maternal pyrexia	0.0518(1, 89)	-0.6166	2.709	-5.999 to 4.765	0.2276	0.2061	0.8204 ns
Preeclampsia	9.262(1, 89)	-4.675	1.536	-7.727 to -1.623	3.043	0.0425	0.0031
PPROM	44.38(1, 89)	-5.814	0.8728	-7.549 to -4.080	6.662	0.1515	<0.0001

ANOVA; analysis of variance, *APH;* antepartum haemorrhage, *CCA;* clinical chorioamnionitis, *F*(*DFn,DFd);* ratio of the mean regression sum of squares divided by the mean error sum of squares*(*degrees of freedom in the numerator, degrees of freedom in the denominator), *GDM;* gestational diabetes mellitus, *IUGR*; intrauterine growth restriction, ns; not significant, P; probability value*, PPROM*; preterm prelabour rupture of membranes, R^2; coefficient of determination, *t-statistic;* magnitude of estimated association. Two-Tailed $P<0.05$ considered to be statistically significant.

Backward selection of variables was conducted with removal of statistically insignificant variables iterated until all dependent variables were removed without markedly worsening prediction of gestational age (independent variable). A statistically significant regression equation was shown in the selected model ($F(3.94) = 30.61$; $P<0.0001$) and the estimated R^2 value of 0.4941 demonstrated 49% of the variation in three obstetric conditions indicative of a placental examination to be explained by the model containing gestational age. Significant associations of selected variables are detailed in Table 3.6; as for the unselected model, effect size was greatest for PPROM ($P<0.0001$), PE ($P=0.0044$) and APH ($P=0.0057$).

Table 3. 6 Multiple linear regression analysis, ANOVA, and overall model summary of obstetric, fetal and neonatal indications for placental examination as predictors of gestational age

Variable	ANOVA *F(DFn,DFd)*	Beta-Coefficient Estimate	Standard Error	95% Confidence Interval	t-statistic	R^2	*P* Value
Intercept (GA)(β_0)	30.61(3, 94)	38.68	0.4597	37.77 to 39.59	84.14	0.4941	<0.0001
APH	7.993(3, 94)	-3.461	1.224	-5.892 to -1.031	2.827	0.0689	0.0057
Preeclampsia	8.532(3, 94)	-4.083	1.398	-6.858 to -1.308	2.921	0.0131	0.0044
PPROM	59.12(3, 94)	-5.926	0.7707	-7.456 to -4.396	7.689	0.623	<0.0001

ANOVA; analysis of variance, *APH;* antepartum haemorrhage, *F*(*DFn,DFd);* ratio of the mean regression sum of squares divided by the mean error sum of squares*(*degrees of freedom in the numerator, degrees of freedom in the denominator), ns; not significant, P; probability value*, PPROM*; preterm prelabour rupture of membranes, R^2; coefficient of determination, *t-statistic;* magnitude of estimated association. Two-Tailed $P<0.05$ considered to be statistically significant.

3.3.8.1 PPROM, antepartum haemorrhage and preeclampsia are significantly associated with moderate to late preterm birth when occurring independently

Considering each independent variable separately, significant relationships were revealed between earlier gestational ages and increased frequency of PPROM ($P<0.0001$), APH ($P=0.0057$) and PE ($P=0.0044$). As shown in Table 3.6, presence of PPROM was significantly associated with delivery 5.93 (± 0.77) weeks earlier than an absence of PPROM.

A significant relationship was found between PPROM and the MPT group (Ÿ = 32.75 weeks) when the linear regression model equation below was applied, where Ÿ is the estimated gestational age at delivery, β_0 is the intercept estimate and β_1 is the regression coefficient associated with the independent variable (in this case, PPROM):

PPROM Ÿ = intercept (β_0) ± β coefficient estimate (β_1)

PPROM Ÿ = 38.68 – 5.93

PPROM Ÿ = 33.75 weeks

APH was significantly associated with delivery at a gestational age 3.46 (± 1.22) weeks earlier than pregnancies without APH and, as such considered to be significantly associated with births in the MPT category when the linear regression model was applied (Ÿ = 35.22 weeks' gestation), as demonstrated below:

APH Ÿ = intercept (β_0) ± β coefficient estimate (β_1)

APH Ÿ = 38.68 – 3.46

APH Ÿ = 35.22 weeks

In cases of PE, a significant relationship was shown where delivery was 4.08 (± 1.40) weeks earlier than no PE. As for PPROM and APH, PE was significantly associated with delivery in the MPT category when the linear regression model was applied (Ÿ = 34.60 weeks' gestation), as shown:

PE Ÿ = intercept (β_0) ± β coefficient estimate (β_1)

PE Ÿ = 38.68 – 4.08

PE Ÿ = 34.60 weeks

3.3.8.2 Combined features of PPROM, APH and PE are significantly associated with extremely preterm birth

The multiple linear regression equation detailed below was applied to estimate gestational age as a function of the presence of PPROM, APH and PE in combination:

$$Ÿ = \beta_0 \pm \beta_{x1}(1) \pm \beta_{x2}(1) \pm \beta_{x3}(0)$$

Where Ÿ is the estimated gestational age, β_0 is the intercept estimate, β_{xn} represents the regression coefficient associated with each independent variable and (0) or (1) indicate absence or presence of the independent variable, respectively.

In cases of placental request documentation indicating PPROM, APH and PE in combination, multiple linear regression revealed a significant association between the three independent variables and gestational age at delivery (Ÿ) which was estimated to be 25.21 weeks. As such, a significant relationship between these conditions and EPT was shown.

$$\text{PPROM + APH + PE } \ddot{Y} = \beta_0 \pm \beta_{x1}(1) \pm \beta_{x2}(1) \pm \beta_{x3}(1)$$

$$\text{PPROM + APH + PE } \ddot{Y} = 38.68 - 5.93(1) - 3.46(1) - 4.08(1)$$

$$\text{PPROM + APH + PE } \ddot{Y} = 25.21 \text{ weeks}$$

3.3.8.3 Where PPROM and preeclampsia, PPROM and antepartum haemorrhage or preeclampsia, and preeclampsia and antepartum haemorrhage are observed in combination, they are significantly associated with very preterm birth

Applying the multiple linear regression equation to cases of placental request documentation indicating PPROM and PE in combination but an absence of APH (0), a significant association between the two independent variables and gestational age at delivery was shown. Gestational age (Ÿ) was estimated to be 28.67 weeks and a significant relationship between PPROM, PE and VPT was shown.

$$\text{PPROM – APH + PE } \ddot{Y} = \beta_0 \pm \beta_{x1}(1) \pm \beta_{x2}(0) \pm \beta_{x3}(1)$$

$$\text{PPROM - APH + PE } \ddot{Y} = 38.68 - 5.93(1) - 3.46(0) - 4.08(1)$$

$$\text{PPROM - APH + PE } \ddot{Y} = 28.67 \text{ weeks}$$

The presence of PPROM and APH but an absence of PE was significantly associated with very preterm birth. Application of the multiple linear regression equation revealed an estimated gestational age (Ÿ) of 29.29 weeks in the presence of PPROM and APH.

$$\text{PPROM + APH - PE } \ddot{Y} = \beta_0 \pm \beta_{x1}(1) \pm \beta_{x2}(1) \pm \beta_{x3}(0)$$

$$\text{PPROM + APH - PE } \ddot{Y} = 38.68 - 5.93(1) - 3.46(1) - 4.08(0)$$

$$\text{PPROM + APH - PE } \ddot{Y} = 29.29 \text{ weeks}$$

There was a significant relationship between PE and APH combined and VPT birth in the absence of PPROM. Applying the multiple linear regression equation to cases of placental request documentation indicating PE and APH presence and PPROM absence estimated gestational age (Ÿ) at delivery to be 31.14 weeks.

$$\text{- PPROM + APH + PE } \ddot{Y} = \beta_0 \pm \beta_{x1}(1) \pm \beta_{x2}(1) \pm \beta_{x3}(0)$$

$$\text{- PPROM + APH + PE } \ddot{Y} = 38.68 - 5.93(0) - 3.46(1) - 4.08(1)$$

$$\text{- PPROM + APH + PE } \ddot{Y} = 31.14 \text{ weeks}$$

Multiple linear regression analyses demonstrated the combination of confounding obstetric factors to contribute the greatest estimated effect on gestational age. Where antepartum haemorrhage, preeclampsia and preterm prelabour rupture of membranes were present *in combination*, gestational age at delivery was estimated to occur at 25.21 weeks, within the EPT category. The presence of any two of these complications increased the estimated gestational age at delivery to within the VPT category, however, multiple linear regression analysis demonstrated significant trends of decreasing gestational age at delivery where these confounding factors were present.

3.3.9 Gross morphological evaluation of placentas

As described in detail in *Chapter 2: Materials and Methods,* formalin-fixed placentas were examined following the principles set out in the Amsterdam Criteria (103). Placental assessment included evaluation of the peripheral membranes, umbilical cord and placental disc with measurements and observations recorded for each. Measurements which relate to placental mass, length and width were recorded once the peripheral membranes and umbilical had been removed. Additionally, as noted in Section 1.4.1, clinical documentation and the Amsterdam Criteria inaccurately use terminology of weight when referring to mass. Results in this section are presented as mass (g) and not weight (mass (g) x gravity (m/s^2), except in Sections 3.3.13. and 3.3.14 which refer specifically to analysis of placental weight centiles (PWC) and fetoplacental weight centiles (PWC). Given that placental examination was performed in accordance with the Amsterdam Criteria and clinical practise, within these sections, terminology reflects that widely used in the field, as recorded on SCH documentation and as presented in the Amsterdam Criteria.

3.3.9.1 Gross morphological evaluation of placentas in the total study population

Mean (± SD) placental mass was 403.7 (± 139.0) grams in the entire cohort. Mean length, width and thickness were 17.3 (± 2.6) cm, 15.5 (± 2.8) cm and 2.2 (± 0.6) cm, respectively (Table 3.7). Predominantly, placentas were discoid in shape in the study population (*n*=78; 79.6%), peripheral membranes were primarily translucent in colour (*n*=73; 74.5%) and were inserted marginally (*n*=77; 81.9%), features considered to be normal. Circummarginate and circumvallate peripheral membrane insertions were uncommon and occurred in only eleven (11.7%) and six (66.7%) cases, respectively (Table 3.7).

Mean mass of preterm placentas was approximately two-thirds that of term placentas at 316.4 (± 112.1) grams vs. 490.8 (± 106.0) grams, and mean placental width was less than 3 cm greater at term than preterm, recorded as 16.9 (± 1.9) cm versus 14.1 (± 0.8) cm. Expectedly, when assessed by a parametric Student's *t*-test, highly significant differences in placental mass and width were found between preterm and term placentas (each $P<0.0001$). Similarly, placental length was significantly lower in preterm than term placentas ($P=0.0001$). Mean placental lengths of 16.2 (± 2.8) cm and 18.3 (± 2.1) cm, respectively, were recorded at preterm and term gestations. Conversely, placental thickness (depth) did not differ significantly between preterm and term gestations ($P=0.6249$). On average, both preterm and term placentas were reported to have a disc depth of 2.2 cm (± 0.4 preterm; ± 0.8 term) (Table 3.7). Minimum, maximum and mean (± SD) values for preterm and term placental disc measurements, total cohort mean placental values, plus recorded proportions of placental shape, membrane insertion and colour are detailed in Table 3.7.

Table 3. 7 Mean placental characteristics, counts of normal or irregular placental shape and peripheral membrane colour and insertion in term and preterm placentas

		Preterm (*n* = 47)			Term (*n* = 47)			Total (*n* = 94)
Placental disc		**Minimum**	**Maximum**	**Mean ± SD**	**Minimum**	**Maximum**	**Mean ± SD**	**Mean ± SD**
Mass (g)		87.0	550.0	316.4 ± 112.1 (*n* = 46)	280.7	734.9	490.8 ± 106.0 (*n* = 46)	403.7 ± 139.0 (*n* = 92)
Length (cm)		9.0	24.0	16.2 ± 2.8	13.8	25.0	18.3 ± 2.1	17.3 ± 2.7
Width (cm)		8.0	20.0	14.1 ± 2.9	11.0	20.0	16.9 ± 1.9	15.5 ± 2.8
Depth (cm)		1.5	3.0	2.2 ± 0.4	0.8	3.8	2.2 ± 0.8 (*n* = 46)	2.2 ± 0.6 (*n* = 93)
Shape (*n*)							(*n*=51)	(*n*=98)
	Discoid		34				44	78
	Irregular		13				7	20
Peripheral Membranes							**(*n* = 51)**	**(*n* = 98)**
Completeness (*n*)^	Complete		20				41	61
	Incomplete		27				10	37
Colour (*n*)	Translucent		40				33	73
	Opaque		7				18	25
Insertion (*n*)			(*n*=42)				(*n*=49)	(*n*=91)
	Circummarginate		4				7	11
	Circumvallate		3				3	6
	Marginal		35				39	74

cm; centimetres, *g;* grams, *n*; number (count), SD*;* standard deviation.

3.3.10 Mean placental mass, length, width and thickness do not increase uniformly as gestation progresses

Mean (± SD) placental mass of placentas delivered in the EPT group was 208.8 ± 73.2 grams compared to 291.8 ± 62.7 in the VPT group and 367.8 ± 101.7 grams in the MPT group (Table 3.8). An increase in mean placental mass of 39.8% (83.0 g) between the EPT and VPT categories was observed, which was greater than that shown between either the VPT to MPT category (+26.0%; 76.0 g) or the MPT to term categories (+33.4%; 123.0 g). Unsurprisingly, a highly significant difference was shown between placental mass within the four gestational age categories (P<0.0001). Mean placental length increased by 32.6% (4.5 cm) from an EPT gestation value of 13.8 ± 1.8 cm to the term value of 490.8 ± 106.0 cm with the greatest percentage growth (14.5%; 2.2 cm) seen between the VPT and MPT groups where mean placental lengths were measured as 15.2 ± 1.9 cm and 17.4 ± 2.5 cm, respectively (Table 3.8). Between EPT and VPT groups mean placental length increase was calculated as 10.1% (1.4 cm), but the slowest proportionate change in length was observed between the MPT and term categories where only a 5.2% (0.9 cm) increase in mean placental length was recorded, thereby suggesting overall placental length changes reduce in pace as gestation progresses. Expansion of placental width followed a similar pattern with term placentas recorded as 16.9 ± 1.9 cm. On average, this was found to be 52.3% wider than EPT placentas measured at 11.1 ±1.9 cm. Most of the increase in width occurred between EPT and VPT gestations (25.2%; 2.8 cm) which had an average placental width of 13.9 ±2.4 cm, this was seen to reduce to 9.4% (1.3 cm) between VPT and the MPT group mean value of 15.2 ± 2.5 cm, before a slight increase of 11.2% (1.7 cm) to term (Table 3.8). As for placental mass, both mean placental length and width were significantly greater at term than at earlier gestations (each P<0.0001). Conversely, placental depth only differed between the EPT group measurement of 2.0 ± 0.4 cm and all other gestational age categories, which were each recorded as a mean value of 2.2 cm (SDs shown on Table 3.8); as such, no significant statistical difference between placental thickness was demonstrated as gestational age progressed (P=0.7003). These results indicate that thickness of the placenta is established by very preterm gestations, at approximately 28 weeks, and is maintained in nonpathological pregnancies and that thick or thin placentas may be associated with placental insufficiencies.

Table 3. 8 Placental characteristics by gestational age category

		Extremely Preterm (*n* = 11)	Very Preterm (*n* = 8)	Moderately Preterm (*n* = 28)	Term (*n* = 47)	*P Value*
Placental Disc						
Mass (g)*		208.8 ± 73.2	291.8 ± 62.7	367.8 ± 101.7 (*n* = 27)	490.8 ± 106.0 (*n* = 46)	<0.0001
Length (cm) †		13.8 ± 1.8	15.2 ± 1.9	17.4 ± 2.5	18.3 ± 2.1	<0.0001
Width (cm)*		11.1 ± 1.9	13.9 ± 2.4	15.2 ± 2.5	16.9 ± 1.9	<0.0001
Depth (Thickness) (cm) †		2.0 ± 0.4	2.2 ± 0.3	2.2 ± 0.3	2.2 ± 0.8 (*n* = 46)	0.7003 ns
Shape (*n*)^					(*n* = 51)	
	Discoid	5	6	23	44	0.0288
	Irregular	6	2	5	7	
Peripheral Membranes					**(*n* = 51)**	
Completeness (*n*)^	Complete	3	3	14	41	0.0007
	Incomplete	8	5	14	10	
Colour (*n*) ^	Translucent	8	8	24	33	0.0621 ns
	Opaque	3	0	4	18	
Insertion (*n*) ^				*(n*=23)	*(n*=49)	
	Circummarginate	0	4	0	7	0.0013
	Circumvallate	3	0	0	3	
	Marginal	8	4	23	39	

Values are presented as mean ± SD unless stated otherwise. *One-way ANOVA with Tukey's multiple comparison, †Kruskall-Wallis with Dunn's multiple comparison, ^Fisher's exact test with Freeman Halton extension. *Mean ± SD. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

3.3.11 Succenturiate placentas are significantly associated with extremely preterm gestations

Discoid (normal) placentas predominated in both the preterm (*n*=34; 72.3%) and term (*n*=44; 86.3%) cohorts and no significant association was shown between placental shape and PTB (*P*=0.1314). However, preterm subcategory analysis revealed a preponderance of succenturiate (bilobate) placentas in the EPT group (6 cases; 54.5%). In all other groups, discoid placentas were more prevalent in increasing proportions, that is, VPT; 75.0% (6 cases), MPT; 82.1% (23 cases) and term; 86.3% (44 cases) (Table 3.8). Statistical analysis revealed a significant relationship between gestational age category and placental shape by application of a Fisher's exact test with Freeman Halton extension (*P*=0.0288). Further, conducting a Chi square test for trend and applying a suspected order of gestational age subcategory, demonstrated a linear relationship whereby succenturiate placentas were significantly more common at EPT gestations ($\chi^2_{df=1}$ = 7.961; *P*=0.0048) (data not shown). Representative examples of a succenturiate and a discoid placenta are shown in Figure 3.2.

Figure 3. 2 Succenturiate and discoid-shaped placentas.

Placentas may have two distinct lobes and are termed bilobate or succenturiate, as shown in the left image. However, normal placentas are predominantly discoid in shape as shown in the right image. Both placentas shown were delivered at term and have similar diameters (placental length). White block arrows labelled 1 and 2 indicate lobes 1 and 2, respectively; yellow arrows highlight sampling defects.

3.3.12 There is a significant trend for incomplete peripheral membranes, but not opacity or aberrant insertion, at extremely preterm gestations

Peripheral membrane characteristics were assessed in accordance with the Amsterdam Criteria and standard clinical protocols and record made of whether membranes were submitted complete or incomplete, if they were translucent or opaque and the insertion point. As noted in *Chapter 2: Materials and Methods*, in cases of circummarginate or circumvallate insertion, the percentage of aberrant insertion was recorded. For the purpose of statistical analysis, a binary measure of marginal vs. aberrant insertion was used. Incomplete peripheral membranes were a characteristic of the EPT and VPT groups with only 27.3% and 37.5% of placentas submitted being reported as having complete membranes in these categories. Conversely, in the MPT and term categories, the majority of placentas were submitted with complete membranes (85.7% and 80.4%, respectively) and this relationship was shown to be significant (P=0.0007) (Table 3.8). Translucent and marginally inserted peripheral membranes are considered normal and, in most placentas, these were the reported findings. Eight of the eleven EPT placentas (72.7%) had both translucent and marginally inserted membranes, in the remaining three cases (27.3%), membranes were opaque and a circumvallate insertion was noted (Table 3.8). Although no opacity was reported in VPT peripheral membranes, 50% were recorded as a circummarginate insertion (4 cases). Most MPT placentas had translucent (24 cases; 85.7%) and marginally inserted (23 cases; 82.1%) peripheral membranes, as did those at term where translucency was reported in 33 cases (64.7%) and marginal insertion in 39 cases (76.5%) (Table 3.8). Statistical analysis showed no significant association between gestational age category and peripheral membrane colour (P=0.0621); however, a Fisher's exact test with Freeman Halton extension revealed a statistically significant relationship between membrane insertion and gestational age category (P=0.0013). Subsequent analysis for trends in peripheral membrane characteristics across gestational age categories showed only a statistically significant linear relationship between gestational age group and peripheral membrane completeness ($\chi^2_{df=1}$ = 15.4782; P<0.0001) confirming membranes were more frequently incomplete at EPT gestations. No significant trends were observed across gestational age subcategories when evaluating peripheral membrane translucency (colour) or circummarginate and circumvallate insertions individually (Table 3.9).

Table 3. 9 Trends in peripheral membrane characteristics across gestational age categories

	χ^2 **Trend Value (*df*)**	***P V*alue**
Completeness	15.4782 (1)	<0.0001
Colour	2.3576 (1)	0.1247 ns
Circummarginate insertion	2.9294 (1)	0.0385 ns
Circumvallate insertion	0.0026 (1)	0.9594 ns

df; degrees of freedom, ns; not significant, *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant.

Representative examples of normal and pathological peripheral membrane characteristics, that is completeness, presence of unidentified additional tissue and translucency or opacity are shown in Figure 3.3. Marginal and circumvallate insertions are presented in Figure 3.4.

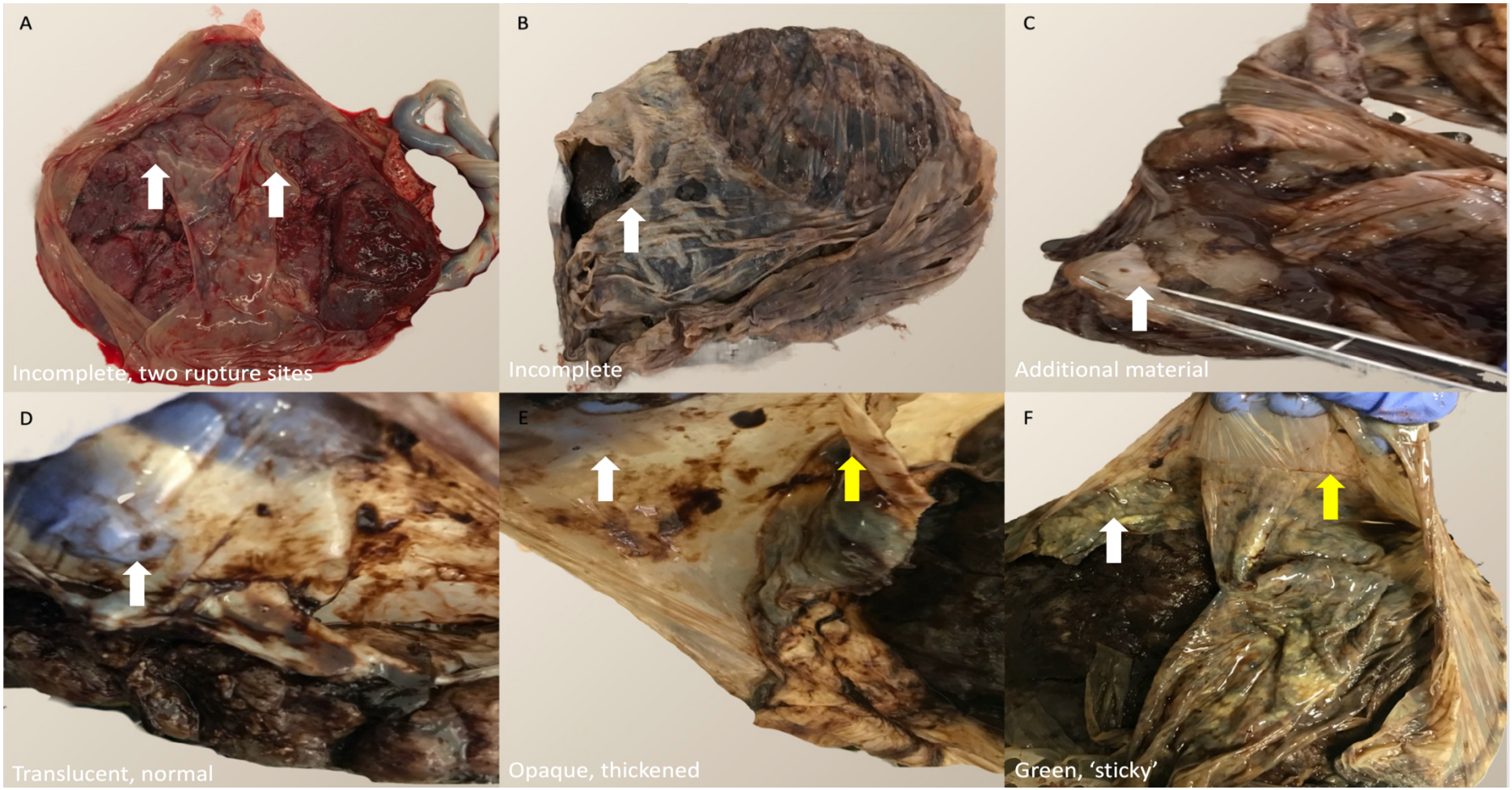

Figure 3. 3 Example pathological and normal findings of peripheral membranes as defined by the Amsterdam Criteria.

A fresh preterm placenta with incomplete membranes and two potential rupture sites as indicated by white arrows. **B** fixed term placenta with incomplete membranes denoted by the inability to enclose the full disc with the membranes (white arrow). **C** unidentified white gelatinous substance attached to the peripheral membranes (white arrow). **D** term translucent placental membranes considered to be normal. White arrow highlights visibility of gloved hand through membranes. **E** stiffened, opaque and separated peripheral membranes (yellow arrow) indicating chronic inflammation. White arrow highlights poor visibility of gloved hand through membranes **F** discoloured green-blue term placental membranes (white arrow) with easily separated amnion and chorion (yellow arrow). No clinically significant inflammation was noted in this placenta on histology.

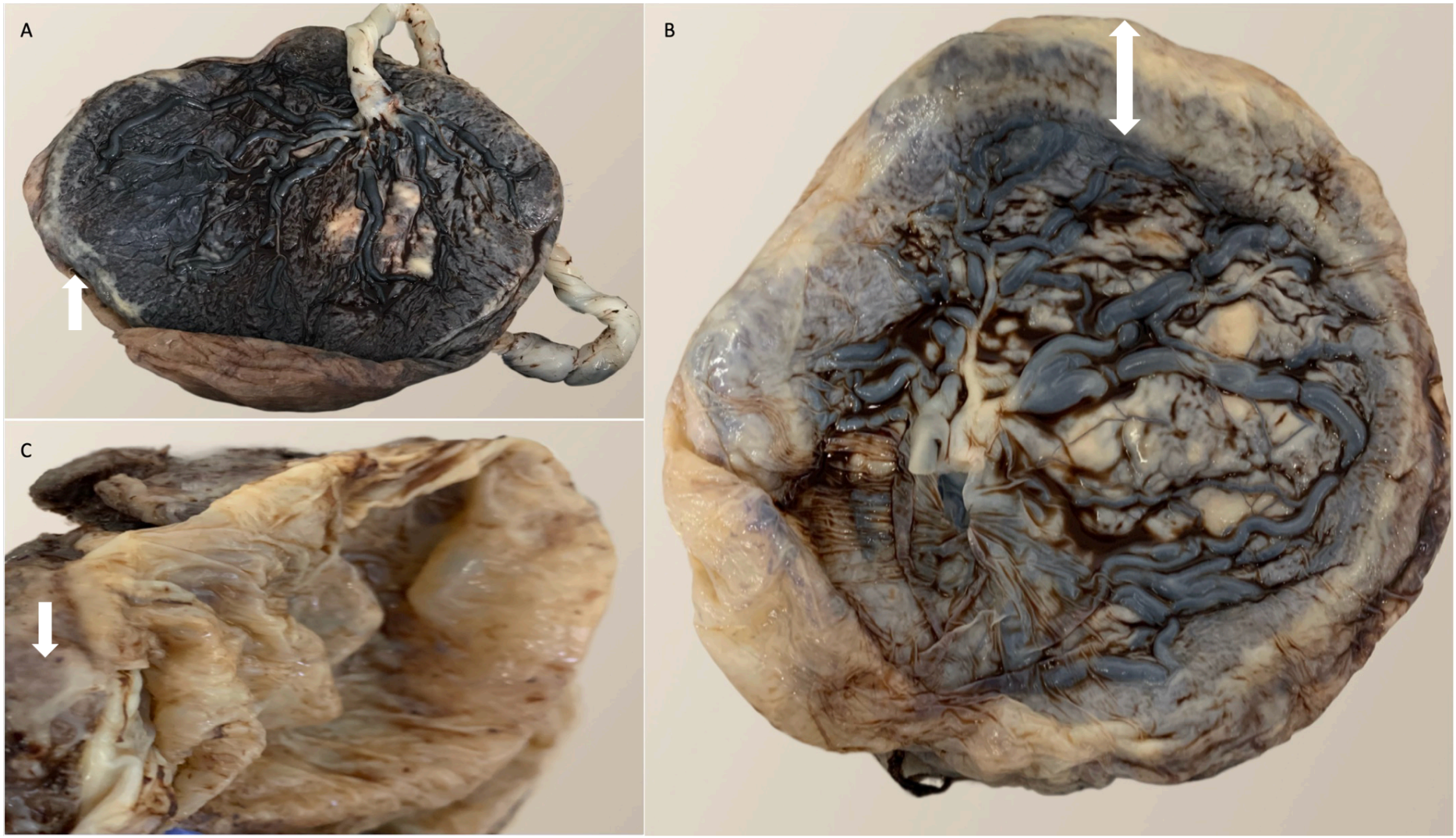

Figure 3. 4 Placental membrane insertions as defined by the Amsterdam Criteria.

A fixed term placenta with normal marginal insertion of peripheral membranes (white arrow) **B** 100% circumvallate peripheral membranes giving rise to the distinctive annular shape. Double-headed arrow denotes reflection of membranes back on themselves and a basal plate larger than the chorionic plate as described in Figure 2.3 (*Chapter 2*). **C** pulling back the peripheral membranes reveals the raised margin. This also gives the impression of a thickened placental disc as highlighted by the white arrow.

3.3.13 No preterm categories show an association with small (<10th centile) or large (>90th centile) for gestational age placental weight

Placental size, specifically the mass (hereafter in this section referred to as weight), correlates to fetal and neonatal health. Low weight placentas are associated with elevated maternal haemoglobin levels, reduced maternal and fetal weight (mass) gain during pregnancy, gestational hypertension and preeclampsia (342, 343). Conversely, neonates are at greater risk of respiratory distress syndrome, neurologic abnormalities, antenatal hypoxia and death where the placenta is measured as being of excess weight (342). To establish whether placentas in the study population were of appropriate weight for gestational ages, placental weight centiles (PWC) were calculated from published tables (Appendix AIII.I and (344)). Over three-quarters (77.4%) of placentas assessed as part of the study had a placental weight centile within the normal range (10th - 90th centile) (344). Ten (19.6%) term placentas were found to be outside the normal range; three >90th centile and seven <10th centile. In the preterm cohort, twelve (26.1%) placentas were considered to be either large (>90th centile; 5 cases) or small (<10th centile; 7 cases) for gestational age, however, statistical analysis did not find this relationship to be significant (*P*=0.6383). Upon classification of PTBs to gestational age categories, it was shown that there were no increased proportions of small or large for gestational age placentas within any group. Seven (63.6%) EPT, eight (100%) VPT, nineteen (70.4%) MPT, and forty-one (80.4%) term placentas were calculated to be within the normal range; statistical analysis revealed no significant relationship (*P*=0.5289) between PWC and gestational age category (Figure 3.5). All placentas in the study cohort were therefore deemed to be an appropriate weight for gestational age.

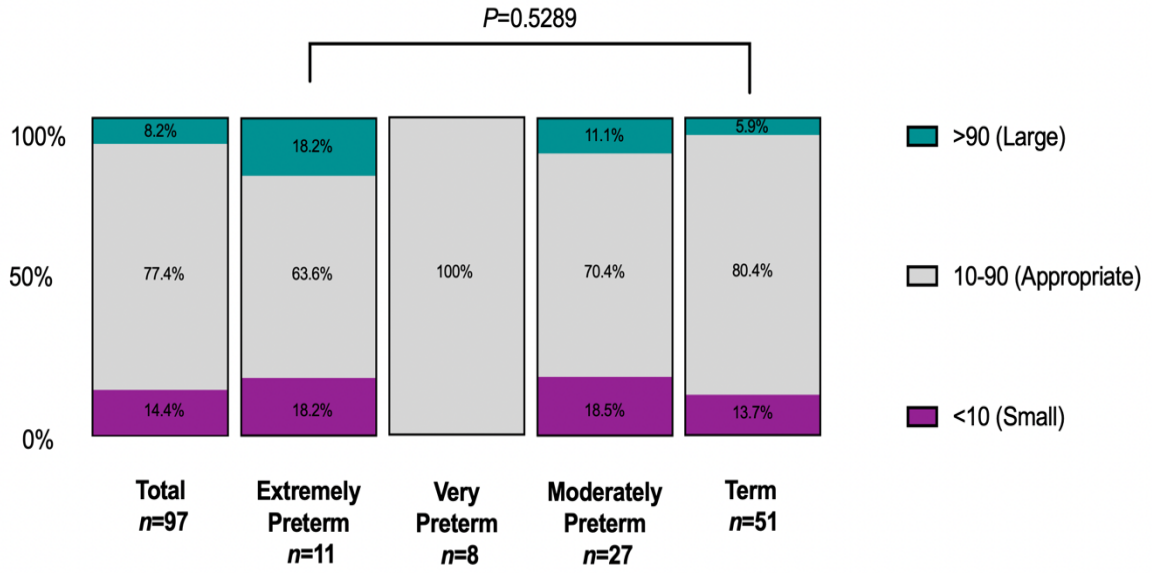

Figure 3. 5 Placental weight centile proportions in the total cohort and by gestational age subcategory.

Placental weight was compared against published data tables to determine a placental weight centile**.** In the whole cohort, and within each gestational age group, most placentas were within the appropriate for gestational age category (10^{th}-90^{th} centile). No significant relationship was shown between preterm categories and small or large for gestational age placentas (<10^{th} or >90^{th} centile, respectively) (*P*=0.5289).

3.3.14 Preterm categories maintain normal fetoplacental weight centiles

Although placental weight may indicate underlying conditions given the association with fetal growth, fetoplacental weight ratios can provide greater depth of information on individual pregnancies and on developmental origins of health and disease (DOHaD). In addition to conditions described previously which may be associated with heavy or light placentas, abnormal fetoplacental weight ratios correlate to fetal growth restriction and, when decreased, cardiovascular disease in adulthood (343). Consequently, effects of fetoplacental weight ratios outside of normal parameters (10^{th} – 90^{th} centile), as presented in Appendix AIII.II and recently updated by Flatley *et al.* (2022) (345), were considered for participants in the cohort where both birthweight and fixed placental mass were available (*n*=91).

The majority (*n*=78; 85.7%) of fetoplacental weight centiles (FPWC) in the total study population were within the 10^{th} - 90^{th} centiles. Nine (20.0%) term placentas were found to be outside the normal range, five (11.1%) >90^{th} centile and four (8.9%) <10^{th} centile. Only four (8.7%) FPWCs outside the normal range were calculated for the entire preterm cohort, two

in the EPT and two in the MPT categories, and each of these was reported to be large for gestational age (>90th centile). Statistical analysis found no significant association between frequencies of large or small for gestational age FPWC between preterm and term cohorts (*P*=0.0924; data not shown), nor was any significant association shown between abnormal FPWC and gestational age category (*P*=0.5347; Figure 3.6), thus demonstrating FPWC is maintained in all PTB categories and at term.

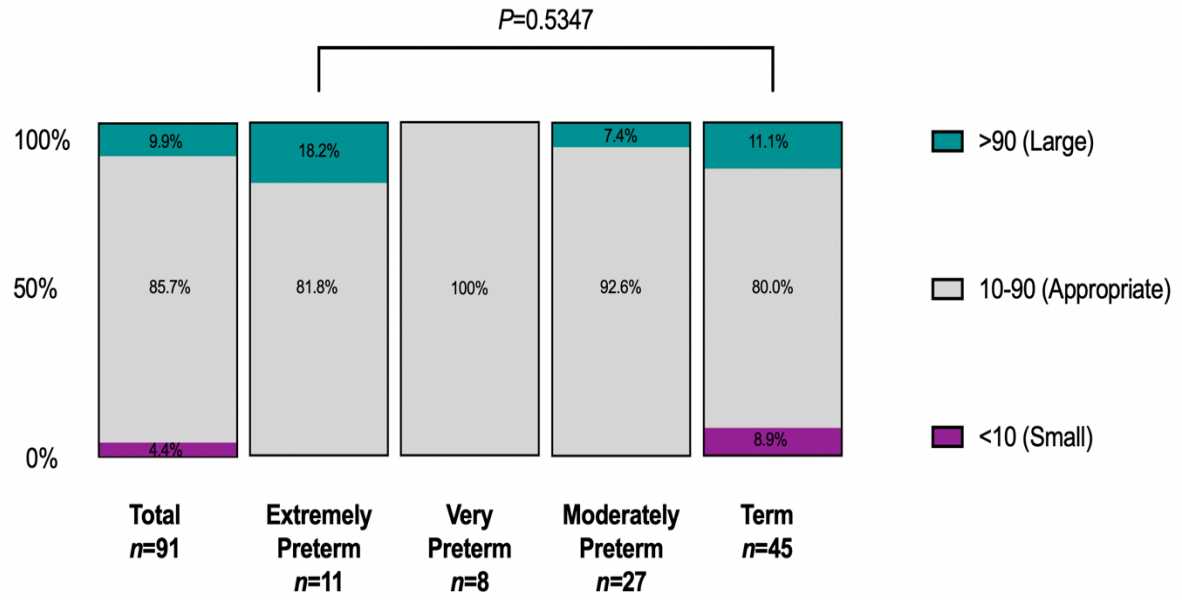

Figure 3. 6 Fetoplacental weight centile proportions in the total cohort and by gestational age subcategory.

Fetoplacental weight centiles (FPWC) were compared against published data tables. Most FPWCs were within the appropriate for gestational age category (10th- 90th centile) and no significant relationship was shown between gestational age categories and appropriate, small or large for gestational age FPWC (*P*=0.5347).

3.3.15 Umbilical cord length and diameter, along with umbilical coiling index were maintained as normal across gestational age categories

Of the ninety-six placentas with an umbilical cord suitable for assessment, the mean (±SD) length was 33.1 ± 11.5 cm, the mean diameter was 1.3 ± 0.5 cm, and, on average there were 8 ± 7 coils. Dividing the number of coils by the length of the umbilical cord (cm) provided an umbilical coiling index (UCI) for each case which was compared against published data (normal range: 0.07 – 0.30) (103); UCIs outside of the normal range were reported as either hypocoiled or hypercoiled. In the total study population, the mean UCI was calculated as 0.25 ± 0.18. Coil depth was predominantly shallow (86 cases; 89.6%), as was a sinistral, or left, coiling direction (68 cases; 70.8%) and a central insertion (58 cases; 60.4%) (Table 3.10).

At term, mean umbilical cord length was recorded as 37.3 ± 11.8 cm, 51.6% (12.7 cm) longer than the EPT mean of 24.6 ± 5.9 cm. Cord length increased by almost 25% between EPT and VPT and MPT and term gestation (22.0% and 23.9%, respectively), but there was minimal change in umbilical cord length in this cohort between VPT and MPT gestations (0.1 cm; 0.3%) (Table 3.10); a significant difference between umbilical cord length and gestational age was expectedly observed when a one-way ANOVA was performed (*P*=0.0011). Umbilical cord diameter appeared to be constant until approximately 32 weeks' gestation, as demonstrated by a mean value of 1.1 cm observed in both the EPT and VPT categories. Beyond this gestation, umbilical cord diameters increased by over a third (36.4%; 0.4 cm) to a term value of 1.5 cm, most likely to accommodate the growing demands of the fetus (Table 3.10). Whilst an overall statistically significant difference was shown between umbilical cord diameter and gestational age by a non-parametric Kruskal-Wallis test (*P*=0.0006), post-hoc Dunn's multiple comparisons test found no statistically significant differences between each progressive gestational age category, i.e. EPT to VPT, etc. (data shown in Appendix III, Figure AIII.VII).

Numbers of coils in each umbilical cord were counted and recorded (Table 3.10), however, this information is of little value alone. Rather, the purpose of noting the number of coils is to calculate the UCI as described above. UCIs ranged from 0.06 to 1.68, however, there was only one report of a hypocoiled cord (<0.07**),** and this was in the MPT group (34.4 weeks' gestation). Hypercoiling was reported in eighteen cases (18.8%), but, with the exception of two extreme UCIs (0.75 and 1.68 reported at 41.8 and 41.3 weeks' gestation, respectively),

these were all calculated as being ≤0.43. Mean UCI values were within the normal range for all gestational age subcategories, although at 0.30 ± 0.10, the VPT UCI was at the upper limit of normal. Discounting this anomalous result, in the remaining subcategories, there was a non-significant increase in mean UCI as gestation progressed; the lowest value was recorded in the EPT group at 0.20 ± 0.09 followed by 0.22 ± 0.11 in the MPT group and 0.27 ± 0.18 at term (*P*=0.4420), overall demonstrating UCI to be appropriate for gestational age (Table 3.10).

3.3.16 Umbilical cord coil depth is significantly shallower in extremely preterm gestations, but coiling direction and cord insertion point are not associated with gestational age

As demonstrated by UCI values, cord lengths and number of coils were appropriate for gestational age and hypo- and hypercoiled cords occurred infrequently and were not shown to be significantly associated with gestational age. Shallow, left helices were predominant findings within all gestational age subcategories; only 12.5% of VPT and 18.4% of term umbilical cords were reported to have deep coils at assessment (Table 3.10) although statistical analysis showed this to be a significant association (*P*=0.0483) and Chi square test for trend post-hoc analysis revealed a significantly greater frequency of deep coil depth at term gestations ($\chi^2_{df=1}$ = 3.995; *P*=0.0456). Nonetheless, there is little clinical significance in coil depth in normocoiled cords (103). More dextral (right) helices were reported at term (38.8%) than at EPT (18.2%), VPT (0%) or MPT (25.0%) gestations, however, sinistral (left) helices were more common in all subgroups and no significant relationship between gestational age category and coil direction was demonstrated (*P*=0.0953). Almost two-thirds of the cohorts' umbilical cords had a central insertion into the placental disc (58/92; 63.0%) and this was replicated in the gestational age groups except in the very preterm group which had a greater number of eccentric insertions (3 cases; 60.0%). Marginal insertions of the umbilical cord were reported in one EPT, three MPT and two term placental examinations. A velamentous insertion was recorded in only two cases, both in placentas delivered at term. Statistical analysis showed no significant relationship between gestational age category and umbilical cord insertion point (*P*=0.4319) (Table 3.10). Figure 3.7 highlights umbilical cord pathology examples as reported within the study cohort; representative examples of normal and pathological umbilical cord insertions defined by the Amsterdam Criteria (103) are shown in Figure 3.8.

Table 3. 10 Mean umbilical cord characteristics and counts of insertion points, coil depth and direction according to gestational age category

	Extremely Preterm (n = 11)	Very Preterm (n = 8)	Moderately Preterm (n = 28)	Term (n = 49)	*P* Value	Total (*n* = 96)
Length (cm) *	24.6 ± 5.9	30.0 ± 10.3	30.1 ± 10.3	37.3 ± 11.8	0.0011	33.1 ± 11.5
Diameter (cm) +	1.1 ± 0.4	1.1 ± 0.3	1.3 ± 0.4	1.5 ± 0.5	0.0006	1.3 ± 0.5
Number of coils	5 ± 3	9 ± 5	7 ± 4	10 ± 9	n/a	8 ± 7
Umbilical coiling index (UCI) *	0.20 ± 0.09	0.30 ± 0.10	0.22 ± 0.11	0.27 ± 0.23	0.4420 ns	0.25 ± 0.18
Coil depth (*n*) ^						
Shallow	11	7	28	40	0.0483	86
Deep	0	1	0	9		10
Coil Direction (*n*) ^						
Left (sinistral)	9	8	21	30	0.0953 ns	68
Right (dextral)	2	0	7	19		28
Insertion (*n*) ^		(*n*=5)	(*n*=27)			(*n*=92)
Central	6	2	15	35	0.4319 ns	58
Eccentric	4	3	9	10		26
Marginal	1	0	3	2		6
Velamentous	0	0	0	2		2

*One way ANOVA, +Kruskal-Wallis test, ^Fisher's exact test (with Freeman Halton extension where necessary). Values are presented as mean ± SD unless stated as (n) which are counts. n/a; statistical test not deemed appropriate, ns not significant. *P*; probability. Two-Tailed *P*<0.05 considered to be statistically significant.

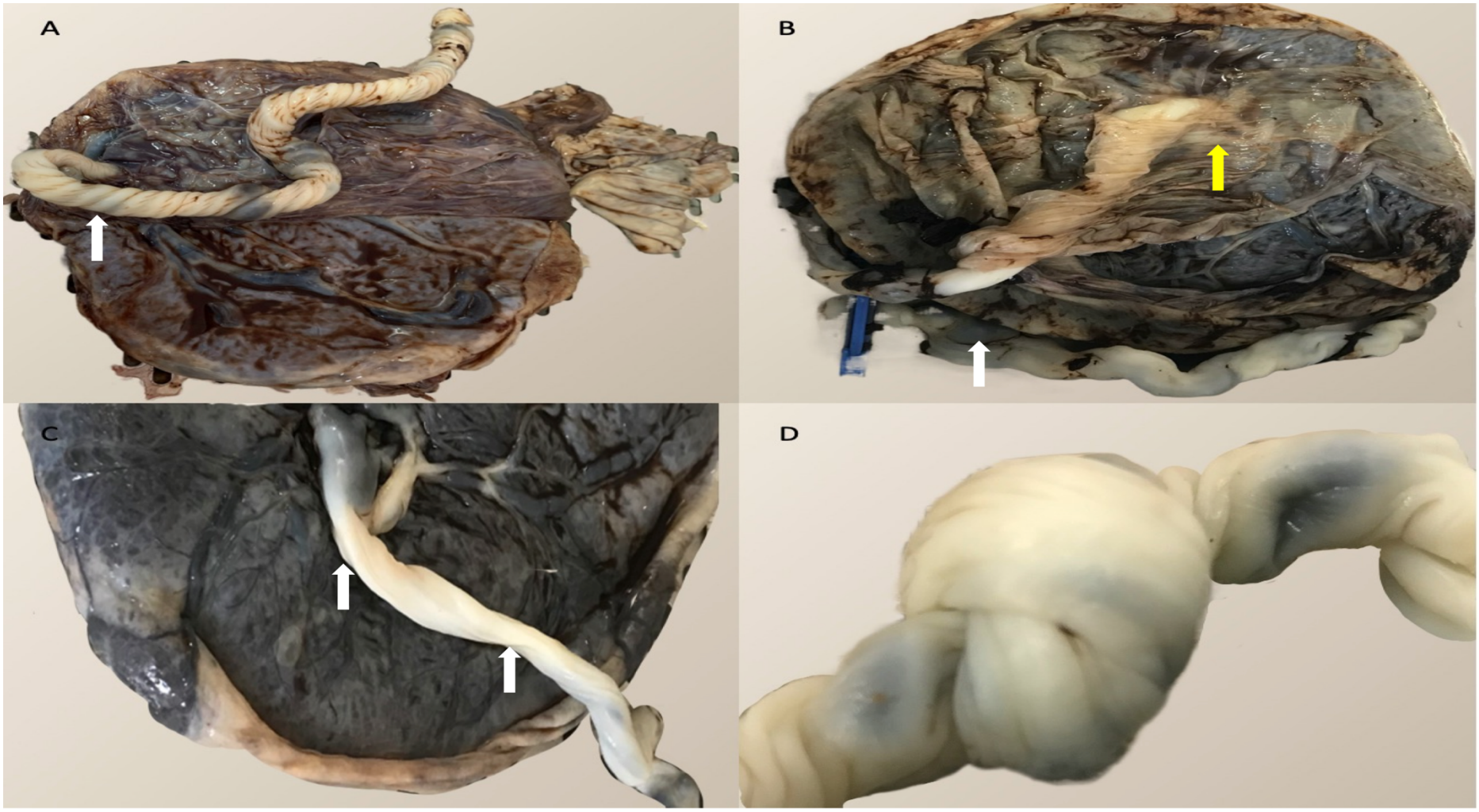

Figure 3. 7 Example pathological findings of umbilical cords as defined by the Amsterdam Criteria.

A term placenta with hypercoiled cord and UCI of 1.68 (white arrow shows regular, tight, shallow coils). **B** thick cord with areas of hypocoiling and engorgement with blood (white arrow shows deep coil). At the insertion point, the cord is tethered to the disc by amniochorion (yellow arrow). **C** hypocoiled term umbilical cord, white arrows point to start and finish points of one deep coil. **D** pseudo umbilical knot cut from preterm placenta at 11 cm from insertion point.

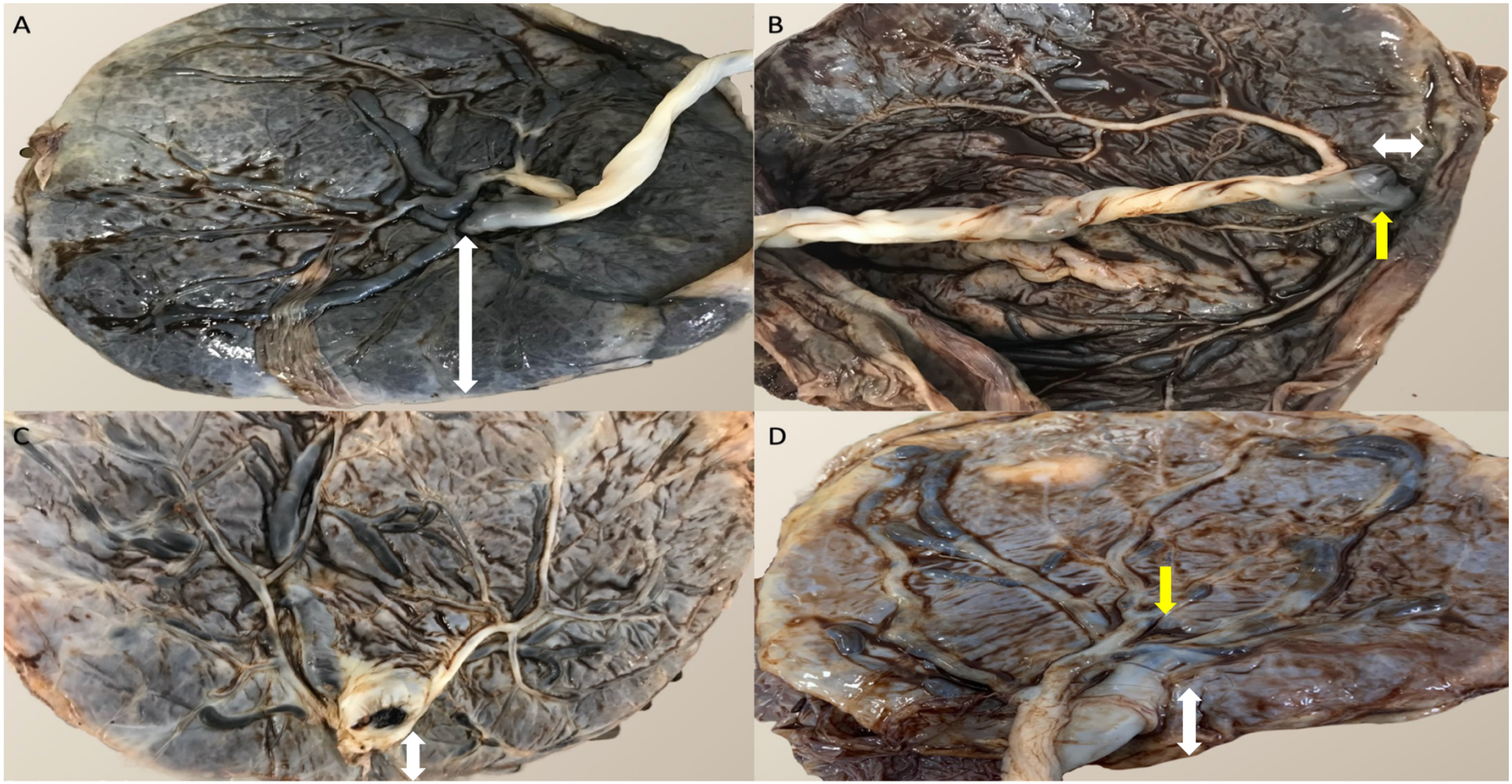

Figure 3. 8 Normal and abnormal umbilical cord insertions as defined by the Amsterdam Criteria.

A term placenta with double-headed arrow to denote distance of normal umbilical insertion into the central aspect of the placental disc. **B** velamentous insertion of umbilical cord into peripheral membranes of preterm placenta. Double-headed white arrow shows vastly reduced insertion distance; yellow arrow shows small area of visible bifurcation of umbilical vessels. **C** eccentric insertion close to the margin of the placental disc (double-headed arrow), but umbilical vessels do not bifurcate until within the disc. **D** marginal insertion of umbilical cord in preterm placenta (double-headed arrow). The yellow arrow highlights distinct bifurcation of the vessels before insertion into the disc exposing the Hyrtl's anastomosis (connection between umbilical arteries).

3.3.17 Normal features were predominantly observed on fetal, maternal and cut surfaces in the total study population

In the whole cohort, fetal (chorionic) surfaces were predominantly translucent in colour (75 cases; 76.5%), representing normal findings; however, sixteen (16.3%) were recorded as being opaque and seven (7.1%) as discoloured (green/brown). Normal chorionic vessels were reported in all but one case (99.0%), a term-delivered succenturiate placenta where the vessels were noted to be limited and narrow (Table 3.11).

Although potentially resulting from referral bias (anecdotal evidence from Prof. Marta Cohen), two-thirds (66 cases; 67.3%) of placental maternal (basal) surfaces were considered to be intact rather than ragged (32 cases; 32.7%). More maternal surfaces were normal coloured (brown when formalin-fixed) rather than pale (55 cases; 56.1% vs. 43 cases; 43.9%). Calcification is not considered to be pathological, unless reported as prominent or premature (103). Although the mechanism of placental mineralisation is not fully defined, it is suggested to be a natural phenomenon resulting from a supersaturated environment in the third trimester (346); in the total study cohort, prominent calcification was reported in less than one-fifth of placentas (19 cases; 19.4%). Blood clots attached to the maternal surface may be associated with retroplacental haemorrhage (103), therefore their presence and measurements were recorded; in the whole study population there were twelve (12.2%) placentas where this was a reported feature. Once cut as described in *Chapter 2: Materials and Methods*, it was noted that most cut surfaces did not have any pale areas (85 cases; 86.7%) but cut surface lesions were present in almost two-thirds of all placentas (61 cases; 62.2%) (Table 3.11).

Table 3. 11 Counts of reported gross features of fetal, maternal and cut surfaces according to gestational age category

		Extremely Preterm (n = 11)	Very Preterm (n = 8)	Moderately Preterm (n = 28)	Term (n = 51)	*P Value	Total (*n* = 98)
Fetal Surface							
Colour (*n*)	Translucent	4	5	22	44		75
	Opaque	5	3	3	5	0.0077	16
	Discoloured (green/brown)	2	0	3	2		7
Normal chorionic vessels (*n*)	Yes	11	8	28	50	>0.9999 ns	96
(*n*=97)	No	0	0	0	1		1
Maternal Surface							
Completeness (*n*)	Intact	4	6	21	35	0.1421 ns	66
	Ragged	7	2	7	16		32
Prominent calcification (*n*)	Yes	0	1	1	17	0.0025	19
	No	11	7	27	34		79
Colour (*n*)	Normal	4	5	21	25	0.0719 ns	55
	Pale	7	3	7	26		43
Attached blood clot (*n*)	Yes	5	1	5	1	0.0005	12
	No	6	7	23	50		86
Cut surface focal lesions (*n*)	Yes	7	5	9	40	0.0006	61
	No	4	3	19	11		37

*Fisher's exact test with Freeman Halton extension. *ns* not significant. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant.

3.3.18 Pathological features of the fetal, maternal and cut surfaces are associated with early preterm gestations

Colour of the fetal surface was significantly associated with gestational age category (*P*= 0.0077) with over 1.7-times the number of EPT placentas (63.6% vs. 36.4%) being reported as either opaque or discoloured than as translucent (5, 2 and 4 cases, respectively). When a Chi square test for trend was performed to assess any linear relationship between fetal surface translucency and opacity or discolouration combined, a statistically significant association was shown ($\chi^2_{df=1}$ = 12.5969; *P*=0.0004) demonstrating a greater frequency of discoloured or opaque fetal surfaces at EPT gestations than at term, likely due to increased rates of chorioamnionitis. With only one report of abnormal chorionic vessels in the total cohort, it was unsurprising that no significant relationship was shown between gestational age group and this fetal surface feature (*P*>0.9999) (Table 3.11).

No statistically significant relationship was shown between gestational age category and maternal surface completeness (*P*=0.1421) or colour (*P*=0.0719) (Table 3.11). However, the presence of prominent calcification, attached blood clots and cut surface focal lesions were all shown to be significantly associated with gestational age subgroup (*P*=0.0025, *P*=0.0005, and *P*=0.0006, respectively; Table 3.11). Nonetheless, no statistically significant trends were revealed by Chi square test for trend analysis for either prominent calcification ($\chi^2_{df=1}$ = 2.1707; *P*=0.4070), attached blood clots ($\chi^2_{df=1}$ = 0.2270; *P*=0.8804) or cut surface focal lesions ($\chi^2_{df=1}$ = 2.7157; *P*=0.0994). These results therefore suggest these placental features of the maternal and cut surfaces occur no more frequently at earlier gestational ages. Example images of normal and pathological features of fetal, maternal and cut surfaces are presented in Figures 3.9 – 3.11.

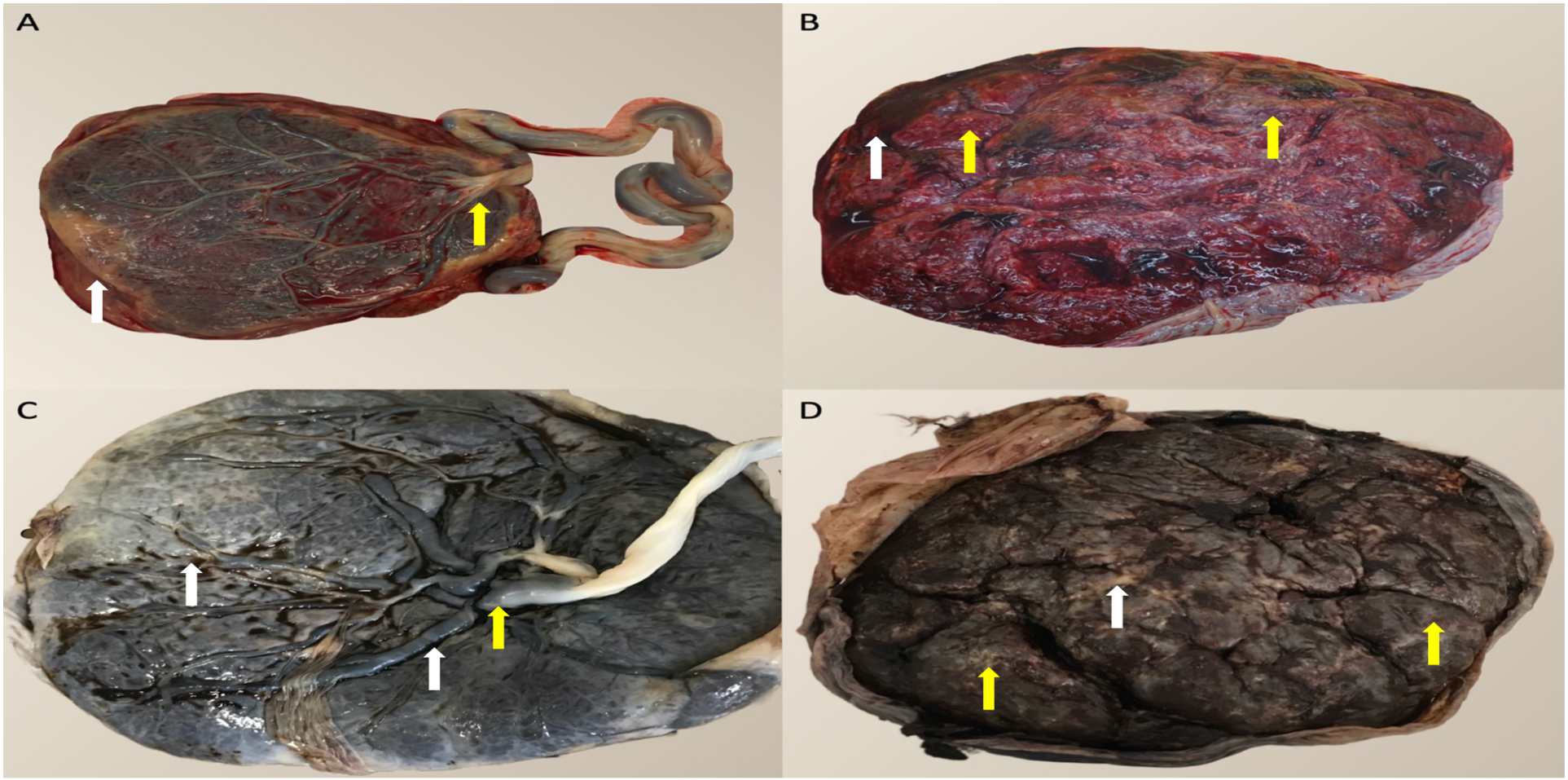

Figure 3. 9 Example images of placental fetal and maternal surfaces as defined by the Amsterdam Criteria.

A fresh term placenta showing fetal surface (chorionic plate), eccentric umbilical cord insertion (yellow arrow), an irregular shaped disc and circummarginate membranes (white arrow). **B** maternal surface (basal plate) of fresh term placenta with membranes reflected back towards the top left of the image (white arrow). Cotyledons are visible although not completely clear (yellow arrows). **C** fixed term placenta. Normal chorionic vessels are spread across the fetal surface (white arrows) and the umbilical cord is inserted para-centrally (yellow arrow). **D** fixed term placenta with membranes removed to expose maternal surface. Cotyledons are apparent (yellow arrows), and the surface is complete and normal in colour. White areas are calcification which is normal at term gestations (white arrow).

Figure 3.10 below shows fresh and formalin-fixed ragged maternal surfaces which are distinctly different from the intact basal plates shown in Figure 3.9.

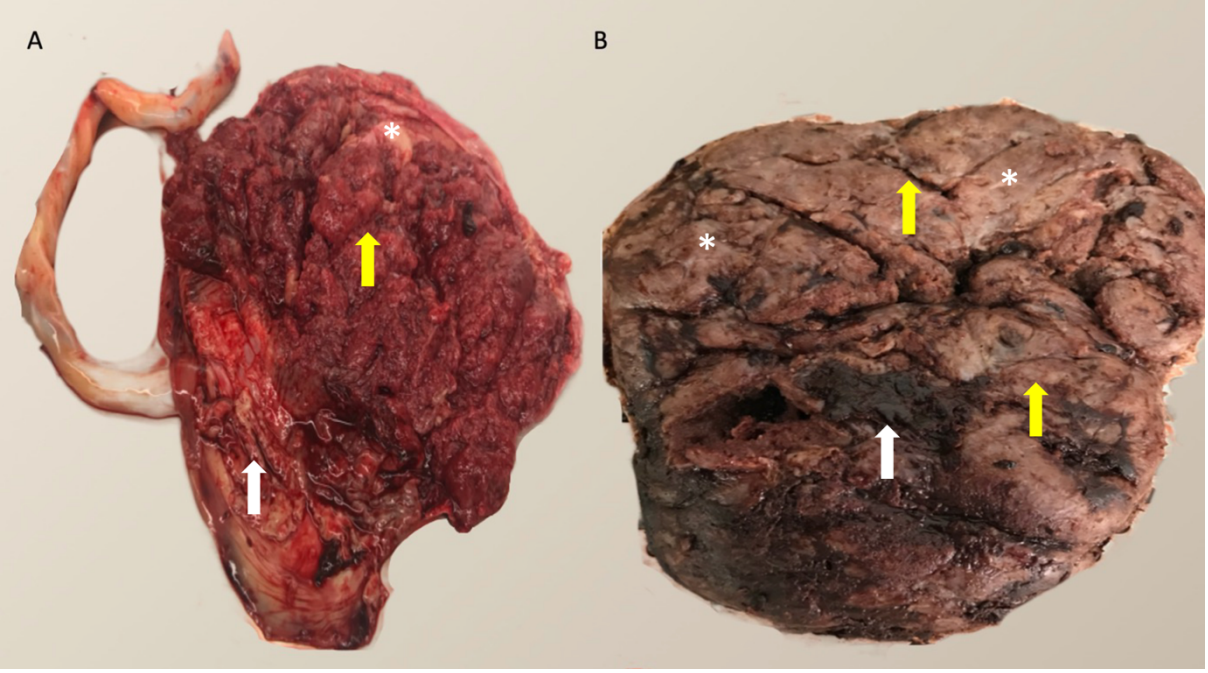

Figure 3. 10 Ragged maternal surfaces as defined by the Amsterdam Criteria.

A fresh ragged maternal surface of preterm placenta where it is difficult to discern the cotyledons (yellow arrow). Damage to the peripheral membranes is also evident (white arrow). **B** fixed term placenta with ragged areas and undefined cotyledons (yellow arrows). There are attached blood clots centrally (white arrow). Pale areas are noted on both the fresh and fixed examples (asterisks).

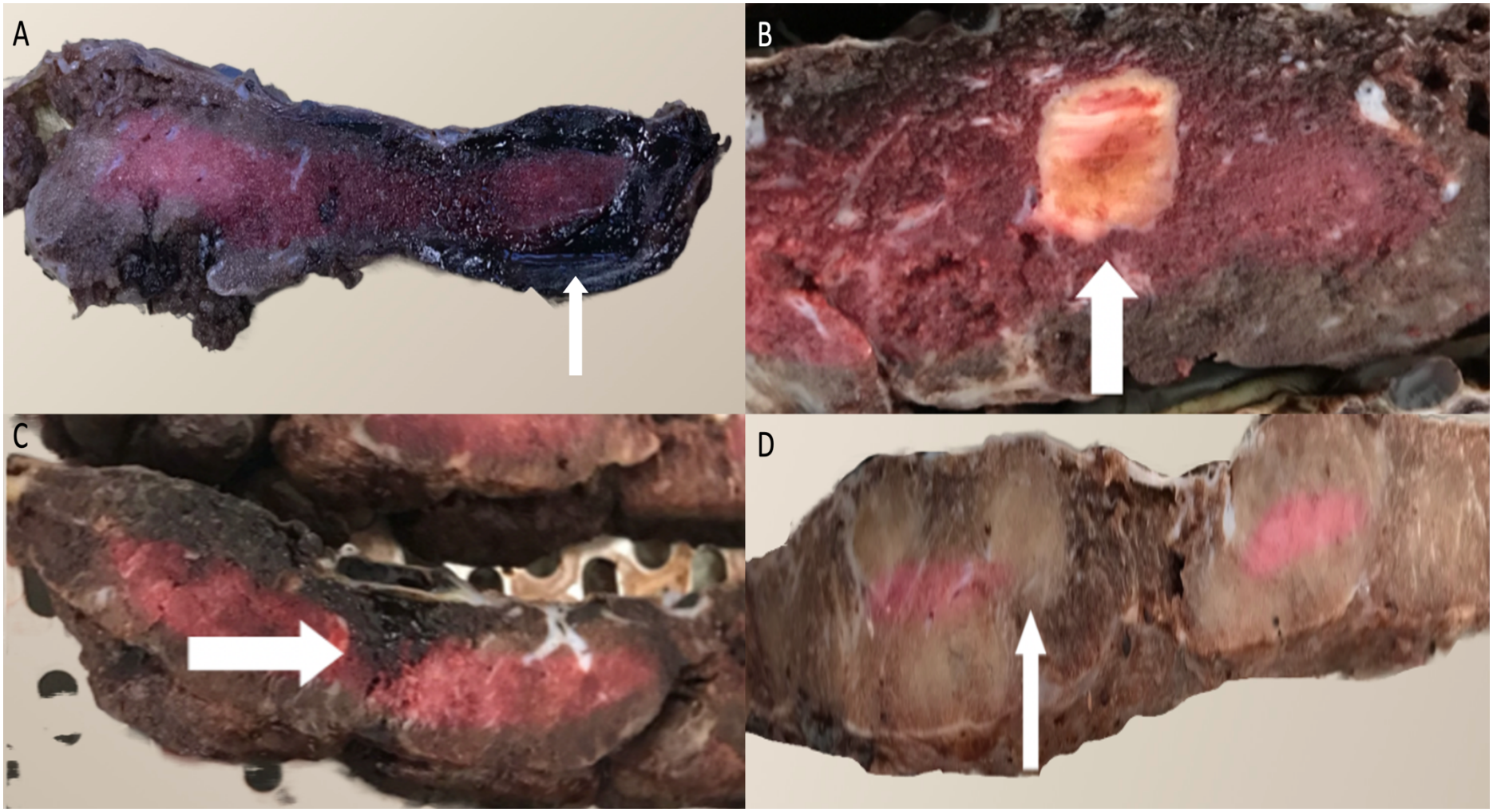

Figure 3. 11 Example images of placental cut surface characteristics and pathological findings as defined by the Amsterdam Criteria.

White arrows denote lesions of interest. **A** preterm placenta with haemorrhage overlying the parenchyma. The remaining tissue to the left is ragged. **B** Firm white lesion seen on the cut surface of a term placenta. Lamination is visible as the lesion has aged. **C** term placenta cut surface with small haemorrhage signifying a fresh lesion. The remainder of the cut surfaces were predominantly normal. **D** term placenta with multiple pale areas.

3.4 Discussion

Histopathological evaluation provides a wealth of clinical information but has limitations in that it can only be performed post-partum, as discussed in *Chapter 1*: *Introduction*. To mitigate the shortage of histopathologists trained in reporting placental evaluations, plus a noticeable decrease in preterm infant mortality rates (347), the RCPath made amendments to their essential referral criterion for preterm births from those occurring <32 weeks (July 2017) to deliveries <30 weeks (October 2019) (318, 348). However, an update to the guidance in September 2022 has seen the RCPath reverse this decision and to refer all preterm births <32 weeks for histopathological examination (136). As positive as the survival to childhood statistics may be, limited data are available on corresponding improvements in adverse functional outcomes, such as cerebral palsy, thus the importance of the placental examination has been reemphasised (347). Thorough macro- and microscopic placental examination can reveal underlying pathophysiologies, determine recurrence risk for a range of obstetric complications and provide guidance on maternal and/or neonatal management or advice in respect of medicolegal issues (349). As such, the histopathological evaluation of placentas must be a priority for future preterm birth management. This chapter describes in detail the gross placental morphological characteristics of singleton neonates liveborn between 23.9- and 42.3-weeks' gestation following spontaneous delivery, each of which were evaluated in accordance with the Amsterdam Criteria and routine clinical practise conducted at SCH (103, 350), and considers their association with PTB. Further, regression models were employed to determine the effect size of maternal and obstetric characteristics on PTB to test the hypotheses that women delivering preterm would have a greater incidence of known clinical and demographic risk factors for PTB than those delivering at term, and that preterm placentas would show an increased prevalence of macroscopic and microscopic pathological characteristics compared to term placentas.

3.4.1 Main findings associated with maternal risk factors of preterm birth

The effect of associations between maternal risk factors of preterm birth and placental morphology, and the underlying biological mechanisms, are currently underreported and many associations are considered to be controversial (351). Despite rising proportions of women aged over 35 years giving birth in high-income countries (HIC), and a global increase in obesity rates, there remains no standard definition of advanced maternal age (AMA) and a

poor understanding of the mechanisms by which obesity leads to PTB (351, 354, 355). No significant difference between maternal age and gestational age was found in the study population (*P*=0.2970), in contrast to evidence that AMA leads to PTB (356). Similarly, teenage pregnancy has previously been linked to PTB, specifically at very preterm gestations (357). Whilst women delivering at very preterm gestations were the youngest group at 25.6 (± 5.4) years, this age falls within what is commonly agreed to be 'normal' for maternal age, i.e. neither adolescent nor AMA (356). Likewise, despite evidence associating obesity with PTB (26, 36-38), potentially through an overweight BMI contributing to the shift to a proinflammatory state and concomitant increase in PTB risk (26, 29), BMI was not significantly associated with gestational age (*P*=0.3955). Both AMA and elevated BMI, especially when combined, are proposed to increase PTB risk through a greater incidence of insulin resistance and GDM; additionally, advanced age during pregnancy contributes to hypertensive disorders and preeclampsia via natural progressive endothelial damage (356, 358). At the opposite end of the spectrum, PTB risk is proposed to be increased in teenage mothers on account of their nutritional status and gynaecological immaturity, however, Khashan, Baker and Kenny argue these theories are contentious and PTB is more likely associated with socioeconomic deprivation and higher rates of smoking and alcohol consumption (357). Within the relatively small study population presented in this chapter, over three-quarters of all women enrolled were aged between 20 and 35 years (76.9%) and more than half (56.3%) had a BMI in the normal range at antenatal booking. Additionally, GDM, hypertension and preeclampsia were reported infrequently, as was being a smoker, and none of these were associated with PTB in any subcategory, nor was gestational age a predictor of GDM in a linear regression model. It is plausible that the placenta is capable of adapting to the proinflammatory state created through an altered inflammatory milieu in environments of AMA and obesity, potentially to a degree sufficient to protect mother and baby (37, 38), yet evidence describes these maternal risk factors as being more commonly associated with iatrogenic PTB, elective Caesarean section delivery and stillbirth (36, 359, 360). As these were outcomes which were excluded from this research, it is suggested AMA, high BMI and GDM may be associated with PTB but not within the selected cohort included in this thesis.

Assessment of the relationship between previous first trimester loss (<13 weeks' gestation) and PTB in the total study population showed first trimester losses to have been reported more frequently by women delivering preterm, specifically where delivery was 32 to <37 weeks' gestation (MPT), irrespective of whether analysis included the term cohort (*P*=0.0116) or not (*P*=0.0045). Mid-trimester losses (≥13 weeks' but <24 weeks' gestation) were an uncommon report in the study population (3 cases; 3.4%) and were only significant when assessing the relationship with PTB in the indicated pregnancy if the term cohort was included. Conversely, a previous PTB did not appear to be associated with an increased prevalence of PTB in this cohort (*P*=0.0562), although there was a significant trend for increased frequency of MPT births where women had experienced a previous PTB (*P*=0.0187). There are two important factors to consider with these findings. Firstly, although independent relationships have been shown by other researchers between miscarriage (loss <24 weeks' gestation) and subsequent increased risk of PTB, it is proposed that this may result from cervical damage caused during surgical management of incomplete miscarriage (41), akin to the increased risk of PTB observed in women with physiological cervical incompetence (361) or following LLETZ (33). Additionally, evidence shows the risk of subsequent spontaneous PTB in women who experience a second stage, full cervical dilation (≥10 cm) Caesarean section (CS) to increase almost 6-fold from 2.3% to 13.5%, likely due to cervical and/or vaginal damage caused during the procedure (362, 363). Data were not collected on whether surgical or medical management of miscarriage was necessary for women enrolled in the study, nor was it possible to record or analyse data on method of delivery and cervical dilation at CS in previous births for all participants, therefore the associations between previous losses or PTBs cannot be reliably attributed to either mechanical factors or to other, unknown, factors. Secondly, management of women who have experienced full dilation CS suggests transabdominal cerclage to be more effective than transvaginal cerclage, but this necessitates subsequent elective CS delivery (364) and would thus exclude women from being enrolled in the study.

In the present study, White British ethnicity was significantly associated with gestational age category, although no significant trend was observed. This was not an unexpected finding since most women self-identified as White British in the total study population (65.5%), however, it is not in accordance with primary research studies and meta-analyses of

observational studies which indicate a positive relationship between PTB and Black ethnicity (44, 365). The small sample size and setting in which the study was conducted is likely to have influenced these data two-fold; 83.7% of the Sheffield population identify as White (366), and, as evidence suggests Black women have greater difficulty in accessing health services than White women (45, 365). This may explain the differences in numbers of Black women delivering in the Jessop Wing, regardless of gestation, despite its position as a tertiary referral centre. Future studies which aim to address associations between PTB and ethnicity, and support efforts to realise health equity, require larger sample sizes and greater geographical coverage than presented in this thesis. Further, analysis of a broader scope of sociodemographic and clinical variables is necessary to corroborate the influence of an individual's social position on disease occurrence and PTB risk.

3.4.2 Associations with indications for placental examination and preterm birth

Results showed an inverse linear relationship between gestational age and antepartum haemorrhage (APH), preeclampsia (PE) and PPROM. Pregnancies complicated by APH or PE had almost three times the likelihood of delivering preterm than those without, whereas PPROM increased the probability 7.5-fold. PPROM preceded 80.9% of PTBs in the study cohort, double the UK estimate of 30% - 40% (367). Multiple linear regression demonstrated individual slopes of PPROM, APH and PE predicted gestational age at delivery and that, individually, PPROM had the greatest effect with a predicted gestational age of 25.2 weeks. Likewise, where PPROM was present in combination with either APH or PE, predicted gestational ages were earlier than an absence of PPROM. The association between PPROM and PTB is not novel, yet each occur due to divergent pathophysiological pathways. It is suggested, though, that inflammation is the fundamental mechanism of both pathways (335). Conversely, PPROM may result from a stepwise process of aberrant collagenolysis resulting in amniochorionic microfractures (368), sometimes even in early pregnancy, which become tears and an eventual rupture. It has been suggested that propagation of inflammatory mediators from damaged membranes results in transition of uterine tissues from a quiescent to an active state (335) and this is supported by evidence that up to 50% of women give birth within 48 hours of PPROM, and 70% - 90% do so within seven days (369), even following administration of prophylactic antibiotics.

Consistent with data reviewed by Varouxaki *et al.* (370), almost 20% (9/47) of preterm deliveries in this thesis were associated with antepartum haemorrhage, however, previous research studies fail to consider a predictive model or a combinatory effect of PPROM and APH. Moreover, research is dated and primarily focuses on APH in the context of placenta previa and resultant iatrogenic PTB rather than spontaneous PTB (371, 372). Data from this cohort showed a negative linear relationship between APH and PTB, but, more striking, a significant slope in the presence of APH and PPROM combined in which the regression model predicted delivery 9.4 weeks earlier than when these conditions were absent. It is, therefore, proposed that a mechanism similar to that underlying PPROM and spontaneous PTB as described above is involved in cases of APH, PPROM and PTB. The results, however, suggest the difference in the cases of these combinatory conditions, is that prior to inflammatory mediators from damaged membranes activating labour processes, their effects are exerted on the decidua basalis causing weakening and haemorrhage. PE-associated PPROM on the other hand is most likely to arise from both these obstetric complications being disorders of deep placentation and failure of physiologic transformation of spiral arteries in the placental bed rather than inflammation (373).

3.4.3 Gross placental morphological features associated with preterm birth

Histopathological evaluation of the placenta is considered the gold standard for identifying pathologies and communicating vital information to parents, carers and clinicians on neonatal and long-term outcomes as well as for risk assessing future pregnancies (103); the first step in this process is gross morphological assessment. Gross morphology data showed placental size, that is its mass, length and width, increased significantly with advancing gestational age. Whilst this was not uniform, placental growth is recognised as not linear beyond 24 weeks' gestation so this finding was considered as normal (375) and was further supported by standard range PWCs and FPWCs (10^{th} -90^{th} centile) for all gestational age categories. Of the ninety-eight assessed placentas, mean thickness was 2.2 cm and this did not differ significantly when preterm gestations were subcategorised (P=0.7003), in line with studies describing a relatively uniform placental thickness throughout the second and third trimesters of uncomplicated pregnancies (376). Divergence from the mean is due to other factors, such as IUGR when placental thickness is decreased, and GDM where an increase is reported (376), but these were not significant complications reported in the present study.

A novel significant, linear association between succenturiate placentas and extremely preterm gestations was revealed (*P*=0.0048), however, with relatively small sample sizes in each of the preterm groups once categorised according to WHO classifications, larger, selected cohorts will be needed to corroborate these findings. Succenturiate, or bilobate, placenta is considered to be a rare and generally incidental finding (377, 378); the limited available literature cites varying prevalence of approximately 3 in 1000 (379) or 5-6% of pregnancies (380). Previously, abnormally shaped placentas, specifically succenturiate, have been associated with infection, postpartum haemorrhage, and retained placenta, conditions which may increase maternal morbidity and mortality (377, 381, 382). Anomalies in placental shape or position in the uterus may be evident as early as during apposition of the fetal membranes and the uterine mucosa, or through the processes of adhesion and invasion generally completed by the end of the third week post-conception (383, 384). Disordered or delayed angiogenesis of placental vasculature has been suggested as a potential explanation of the resultant placental dysfunction associated with abnormal shape (99). It has been further proposed that negative angiogenic effects are greater when placental shape abnormalities occur earlier (385), yet, despite a thorough search of the literature, the association between succenturiate placentas and extremely preterm birth has not previously been identified or reported. The data presented in this thesis are therefore the first to associate placental shape abnormalities and PTB; it is postulated this is linked to vascular malperfusion and placental insufficiency, as discussed further in *Chapter 4*.

Gross morphology results also indicated a relationship between PTB prior to 32 weeks and extrachorial placenta (*P*=0.0390), notably clinically significant circumvallation (324) in 27.3% of placentas delivered extremely preterm, although this was not found to be a linear relationship. The mechanism by which circumvallation develops is unknown, but it is regarded as a disorder of placental shape (386). Previously, circumvallation has been associated with emergency Caesarean section delivery, SGA infants, *abruptio placentae,* intrapartum haemorrhage and preterm birth, primarily due to the annular shape created by an augmented placental disc margin and the preponderance for haematomas to become trapped marginally (324, 386). Circumvallate placentas reported in this research were delivered at preterm and term gestations and were not significantly associated with CS delivery, APH or IUGR (data not shown), although this is likely due to the small number of placentas with a recorded

circumvallation in the cohort. Circummargination does not bring with it the same adverse obstetric outcomes as circumvallation (387). Circummarginate placentas are characterised by an inward insertion of the membranes away from the placental disc margin, but unlike circumvallate placentas, the membranous chorion remains flat (388). In consensus with published data, in this study circummargination did not contribute significantly to placental pathology (382).

Current evaluation of peripheral membrane completeness by perinatal pathologists makes reference to 'incomplete' when membranes appear to be ragged and are not *completely* submitted (103). Limited studies or clinical protocols for placental examination explain the significance of reporting or establishing completeness of the peripheral membranes beyond stating there should be enough membrane to enclose the fetus (103, 144, 319) or describing an association between incomplete membranes and an elevated risk of postpartum haemorrhage (389). Indeed, peripheral membranes are commonly considered to be an adjunct of the placenta and obsolete following delivery (390), yet, in this cohort, PPROM was significantly associated with a report of incomplete peripheral membranes at placental examination (P=0.0069) when assessed by Fisher's exact test. To the best of the author's knowledge, such a relationship has not previously been discussed in published literature. Peripheral membranes are comprised of the amnion and chorion which together make up the innermost intraamniotic cavity layer (amnion) and the fetal tissue providing the connection to the maternal decidua (chorion); collectively, these layers are termed the amniochorion (104, 391). Development of the amniochorion is complete by early- to mid-gestation, although remodelling continues until birth (104); it is therefore suggested that the observed relationship between incomplete membranes and PPROM arose from abnormalities in the structural formation and modification of the amniochorion, likely through excess sloughing of the single cuboidal amnion epithelial cell layer and resultant membrane microfractures (104). Since the chorion has less tensile strength than the amnion (391), the loss of this innermost layer and exposure of the *zona spongiosa* and chorion would permit cells and amniotic fluid to enter the spongy layers, reducing the mechanical integrity of the membranes and increasing the risk of PPROM (104, 392). Alternatively, it is plausible that increasing the latency period following PPROM to allow for administration of corticosteroids and/or prophylactic antibiotics contributes to damage to the amniochorion and resultant incomplete

membranes at placental examination, as demonstrated by the good predictive performance of duration of ROM on completeness of membranes when a simple logistic regression model was applied to the dataset (AuROC = 0.7280; 95%CI: 0.5523-9037, *P*=0.0201). Irrespective of whether impairment of the amniochorion occurs prior, or subsequent, to PPROM, destabilisation of this protective barrier increases the likelihood of MIAC, HCA, initiation of parturition (390) and, as demonstrated in the present study, APH. Future research is proposed to prospectively collect PPROM and membrane completeness data in order to corroborate these findings.

3.4.4 Gross umbilical cord and placental surface morphological features associated with preterm birth

In all preterm categories, and at term, normal umbilical cord anatomy and umbilical coiling indices (UCI) were maintained. Although umbilical cord defects such as a single umbilical artery (SUA), true knots or anomalous insertion (velamentous or marginal) increase the risk of associated congenital or chromosomal abnormalities, PE, IUGR, PTB and fetal death, irregularities in the umbilical cord are generally identifiable on antenatal ultrasound scanning and are, therefore, rare incidental findings on histopathological assessment (103, 393-395). Umbilical cord insertion was found to be predominantly central (normal) across all gestations and, consistent with previous research, extreme cord insertion abnormalities were infrequent findings (396); only two velamentous and six marginal cord insertions were reported in the entire cohort. Left twist coiling was prevalent, in harmony with findings published by the Amsterdam Consensus Workshop Group, although no studies define the significance, if any, of this directionality (103). Shallow indentations also predominated in this cohort, yet this is also considered to be normal unless in cases of hypercoiled cords (103), thus, whilst statistically significant, this finding was not deemed to be of clinical significance.

Since the umbilical cord elongates throughout gestation (108), significant relationships were expected between umbilical cord length and diameter and gestational age subgroups. Discordant with global studies reporting average singleton umbilical cord length, the term cohort was found to be considerably shorter at 37.3 cm than the 57.7 cm, 63.9 cm and 68.0 cm reported from studies conducted on large cohorts in Finland (397), India (108) and Sudan (398). It is likely that these longer umbilical cord lengths arose from inconsistent

methodological processes, notably measurement of cord length when fresh immediately after birth, such as in the Finnish and Sudanese studies, versus after fixation in 10% neutral-buffered formalin as was the case for samples included in this study (103, 397, 398). Nonetheless, ratios calculated between 50th centile umbilical cord lengths at 24- and 40-weeks' gestation presented by Georgiadis *et al*. (397) were similar to those calculated for EPT and term umbilical cord lengths (data not shown), adding credence to the idea that variance in umbilical cord length in this cohort is an artefact of the evaluation protocol. Importantly, this highlights the need for a consistent, replicable methodology for clinical and research assessment of the placenta and the essentiality of large multi-national cohort studies to determine reference ranges and produce centile charts for all placental parameters.

Translucent (normal) fetal surfaces were significantly associated with gestational age subgroup (*P*=0.0077), and an inverse linear trend was reported showing an increased frequency of opaque and discoloured fetal surfaces at extremely preterm gestations (*P*=0.0004). Translucency generally indicates a normal, healthy placenta whereas opacity or discolouration is linked to HCA (399). In further support of this, and in agreement with published literature, fetal surface opacity was positively correlated to HCA (*P*=0.0220) in the study cohort; associations between HCA and PTB are discussed in further detail in *Chapter 4*. Maternal surface colour was not associated with the presence of attached blood clots or where APH had been reported, however, significant positive relationships were shown between gestational age and the presence of prominent calcification of the placental disc (*P*=0.0025), attached blood clots on the maternal surface (*P*=0.0005) and cut surface focal lesions (*P*=0.0006). Calcification of the placenta is reflective of the ageing process so is expected at term; observations of early or excess calcification indicate premature placental ageing or dystrophic, ischemia-related conditions (346, 400). In normal pregnancy, calcitriol (1,25-dihydroxyvitamin D) drives intestinal absorption of serum-ionised calcium and phosphate to ensure adequate supply to the developing fetus; by term, approximately 80% of calcium and phosphate present in the fetal skeleton crossed the placenta in the third trimester (401). This late-onset rapid transfer suggests term placental calcification results from metastatic mineralisation and not a physiological or dystrophic process, as described in 2001 by Poggi *et al.* (346) who demonstrated mRNA expression of critical bone morphogenic proteins to be independent of gestational age, thereby discounting a physiological process,

and a calcium/phosphorous mass ratio from mature placental calcifications similar to stones formed in a metastatic environment when analysed by energy-dispersive x-ray. From this, the authors concluded that the third trimester/term rapidly-formed, supersaturated mineral environment accounts for calcification observed on maternal, fetal and cut surfaces of late preterm and term placentas and that this was a normal phenomenon (346).

3.4.5 Conclusions

On the whole, placentas collected from women enrolled in the study who delivered at term or preterm were reported as having normal gross morphology. However, novel negative associations between succenturiate placentas and extremely preterm births and circumvallate placentas and extremely preterm births were shown which may account for the increased prevalence of clinical features such as APH and emergency Caesarean delivery in the preterm cohorts. Furthermore, to the best of the author's knowledge, this study is the first to model prediction estimates of gestational age for conditions considered as indicators for placental examination and to provide estimated effect sizes for these individually and in combination. This methodology has revealed a significantly increased risk of PPROM and preterm birth in women experiencing antepartum haemorrhage and preeclampsia, potentially improving identification of at-risk women and implementation of more timely management and prevention strategies.

Amongst the limitations of the study were the relatively small number of cases, in particular when assessing women with combinations of confounding clinical conditions such as PPROM, APH and preeclampsia. Another limitation is the inclusion of only women experiencing spontaneous PTB as many of the conditions where pathological placental features were observed, or the regression model predicted negative relationships, would result in expedited elective delivery to reduce the risks of maternal and/or fetal mortality and morbidity. Studies with expanded inclusion criteria and larger cohorts are therefore required to confirm these preliminary findings.

Chapter 4: Histopathological Evaluation of Placentas Elucidates Differential Phenotypes of Preterm Birth and Variances in Placental Inflammatory Responses Which Correlate to Gestational Age

4.1 Introduction

Chapter 3 described morphological characteristics of preterm and term placentas as recorded on placental pathology reports. This chapter details the differences in inflammatory features observed between preterm and term placentas and attempts to identify whether differential phenotypes of PTB exist. It has been established that up to 50% of spontaneous PTBs are complicated by infection or inflammation, either as a result of microbial ascension of the genital tract and subsequent MIAC or uterine activation following increased production of proinflammatory cytokines (402). Even so, there is commonly no clinical indication of infection in most women presenting with threatened or established preterm labour and histopathological evaluation of the remaining 50% of preterm placentas elucidates no features of inflammation (403). Diverse underlying pathophysiologic processes, such as uteroplacental vascular maladaptation, may also result in preterm birth, but, the heterogeneity of causes, and absence of any pathology in around one-fifth of cases, means the precise aetiology of most preterm births remains elusive (403, 404) (Figure 4.1).

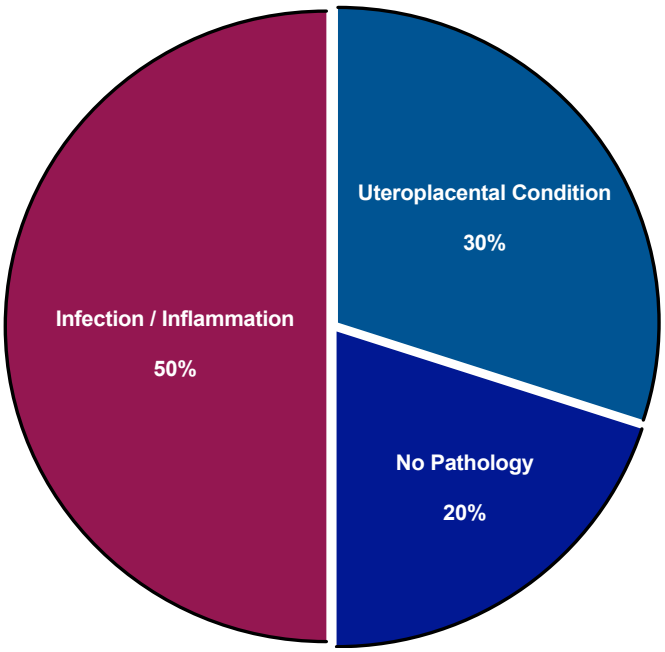

Figure 4. 1 Aetiology of spontaneous preterm birth on histopathological evaluation.

Up to 50% of preterm births can be attributed to infection or a placental inflammatory response and 30% to uteroplacental disease such as vascular arterial malperfusion, following macro- and microscopic histologic evaluation. The remaining 20% reveal no pathology (403, 405).

The 2016 publication of the Amsterdam Placental Workshop Group Consensus Statement introduced agreement to the terminology and microscopic descriptors used in placental pathology reports and provided a framework for sampling and definition of placental lesions (103), as detailed in Section 4.2.1 and Table 4.1. The utility of such a consensus is vital for aiding understanding of the clinical significance of defined placental lesions and ensuring global comparability of placental histopathological study (103). Conversely, there are no agreed standards for placental sampling and storage for research and no clear agreement on what clinical data is relevant for effective interpretation of results. Furthermore, differing access to resources and facilities may restrict the types of sample taken as well as how and for how long they are stored (350). Moreover, data are limited on the type, frequency and severity of histologic placental lesions in term pregnancies with no adverse outcome, whether this leads to the clinical significance of these lesions in preterm placentas being overlooked or misinterpreted is undefined (328).

4.1.1 Placental inflammatory responses

Placental inflammatory responses (PIR), identified by diffuse neutrophilic infiltration, are observed histopathologically in the maternal and/or fetal compartments (103, 406). Maternal inflammatory responses (MIR) are characterised by a chemotactic gradient attracting maternal neutrophils to and then across the chorionic layer, the amnion and into the amniotic space (142, 407). In contrast, fetal inflammatory responses (FIR) are differentiated by fetal neutrophils infiltrating first the umbilical vein, then the umbilical arteries, chorionic vessels and Wharton's jelly (funisitis) (142, 407). Initially defined by Redline *et al*. in 2003 (141), MIR and FIR are classified according to the confluence or intensity of neutrophils, referred to as the *grade*, and the progression of these inflammatory cells within the placental anatomical compartments, recorded as the *stage* (103, 141) (Table 4.1).

4.1.2 Histopathologically-diagnosed acute chorioamnionitis

Relationships between PIR, in particular FIR, PTB and subsequent adverse neonatal outcomes are well established, especially in cases of histopathologically-diagnosed acute chorioamnionitis (HCA). Literature reports increased HCA grade and stage in preterm placentas (408). HCA at advanced stage and grade or in combination with FIR lesions, as seen in cases of necrotising chorioamnionitis or in the presence of acute funisitis, are associated

with delivery at earlier gestation and are proven to contribute to severe adverse neonatal pathologies including neurodevelopmental disorders (409), sepsis (410), necrotising enterocolitis (NEC) (411, 412) respiratory distress syndrome (RDS) (413) or even neonatal death (410).

Estimates of HCA prevalence at term range from 2-34% (142, 408, 414-416). In comparison, HCA is reported to occur in 25 - 80% of PTBs (408, 417, 418). As well as being less widespread in term placentas, HCA is less severe and progressive, predominates in women with spontaneously ruptured membranes (SROM), is unlikely to have occurred prelabour (408, 419) and is non-infectious in nature (420). On the other hand, HCA at preterm gestations commonly results from infectious conditions, is linked to PPROM and MIAC (402, 408), and is reported as being of higher grade and stage (407). There is emerging evidence that the activated inflammatory pathways in term and preterm deliveries are similar, although their origins differ (420), and that uncontrolled activation of the complement system also plays a role in initiating preterm labour (421). Nonetheless, determining the frequencies of HCA which occurred prelabour, at the onset of labour or those which developed during labour remains a substantial challenge (420, 421). Whilst HCA at term is less well-understood than at preterm gestations, as a sterile inflammatory response it is linked to IUGR (422), maternal hypertension (423), low APGAR scores and stillbirth, irrespective of gestational age at delivery (406). Maternal malnutrition has been implicated in the progression of sterile HCA through impaired fetoplacental development, as has chronic inflammation such as that seen in the presence of maternal obesity (424). Since histopathological evaluation of the placenta provides only a temporal characterisation (425), it must be emphasised that HCA at term may exclusively represent a physiological response to labour or an intrinsic aspect of parturition (408) in contrast to evidence that preterm fetuses mount a sufficient inflammatory response to HCA to result in initiation of labour (426).

4.1.3 Preterm birth pathophysiological phenotypes

Recently, there has been a drive to better classify PTB to understand preventative and treatment measures, particularly as PTB phenotype is more likely to be linked to longer term neonatal and infant health than of gestational age alone (427). Proposals have been made as to a phenotypic classification system that incorporates maternal, fetal and placental characteristics with associated aetiology (428). Reporting of antecedent events, and considering these as contributors to PTB, are the primary foci of subcategorising PTBs at present. Presentation at delivery traditionally takes precedent over underlying processes when attempting to phenotype PTB or determine the pathways to parturition (429), however, placental pathologies are known to correlate to the clinical presentation of PTB (430). Despite the global public health significance, immune pathways, molecular stimuli or infectious agents underlying the pathogenesis of preterm birth remain undefined (402). Evidence that disorders of placentation and placental perfusion may also give rise to preterm labour (431) and a lack of understanding of the aetiology of preterm births in the absence of placental abnormalities (404) further highlight the major gaps in knowledge of preterm birth pathophysiology. There are a lack of methods with the ability to predict placental phenotype at preterm gestations, yet addressing this need could provide critical information to support targeted clinical treatment and monitoring of pregnancies with the aim of advancing management or prevention of preterm birth. Analysis of data in this chapter sought to contribute to interpretation of results of placental pathology reports to provide predictive methods of severity of MIR and FIR and a framework for classification of preterm birth phenotype.

4.1.4 Hypothesis and aims

This chapter tested the hypothesis that preterm subcategories are independently associated with placental inflammatory or vascular malperfusion histopathological lesions. The aims of the study were to:

a) describe placental inflammatory and non-inflammatory lesions in the study cohort,
b) categorise placentas based on gestational age and histopathological features, and
c) assess and describe associations between Amsterdam Consensus grading and staging of placental histopathology and clinical outcome in preterm and term deliveries.

4.2 Materials and methods

4.2.1 Participant recruitment, study design and placental histopathological reporting

A full description of ethical approval, participant recruitment, study design, collection, processing and reporting of placental samples, and methods used in this chapter is set out in *Chapter 2: Materials and Methods.* Ninety-eight women enrolled in the study had a full placental histopathology evaluation and had clinical notes available for data analysis.

4.2.2 Histological review and reporting

Following H&E staining, histological review was conducted to identify acute and/or chronic inflammatory lesions and vascular malperfusion lesions. Any additional features observed, whether pathological or indicators of an ageing placenta, for example, increased syncytial knot formation or calcification, were also recorded following guidelines and terminology set out in the Amsterdam Placental Workshop Group consensus statement (103). Definitions and classifications used to report placental inflammatory and vascular malperfusion lesions are presented in Table 4.1 and described below. High quality maternal and demographic clinical data were collected via thorough examination of enrolled patients' records.

Table 4. 1 Definition and classification of inflammatory and vascular malperfusion lesions

Inflammation		**Maternal**	**Fetal**
Stage	1 Early	Acute Subchorionitis Acute Chorionitis	Chorionic vasculitis Acute Phlebitis
	2 Intermediate	Acute Chorioamnionitis	Acute Umbilical Vasculitis (one/both arteries with/ without vein) Acute Panvasculitis (all three vessels)
	3 Advanced	Necrotising Chorioamnionitis	Necrotising Funisitis Concentric Umbilical Perivasculitis Acute Funisitis
Grade	1 Mild – Moderate	No specific Terminology or Not Severe	No specific Terminology or Not Severe
	2 Severe	Acute Chorioamnionitis Subchorionic Abscesses	Intense Chorionic Vasculitis Intense Umbilical Vasculitis
	Other	Chronic Chorioamnionitis Subacute Chorioamnionitis	Associated Fetal Thrombi Peripheral Funisitis Acute Villitis Acute Intervillositis with Intervillous Abscesses Decidual Plasma Cells
Vascular Malperfusion		**Maternal**	**Fetal**
		Increased Perivillous Fibrin Deposition Accelerated Villous Maturation Distal Villous Hypoplasia Increased Syncytial Knots Basal Decidual Vascular Thrombus Retroplacental Haemorrhage Multiple Infarcts Placental Weight <10th Centile Thin Umbilical Cord <10th Centile or <8mm diameter at term Decidual Arteriopathy	Thrombus Segmental Avascular Villi Villous Stromal-Vascular Karyorrhexis Vascular Intramural Fibrin Deposition Stem Vessel Obliteration/Fibromuscular Sclerosis Vascular Ectasia

Adapted from Khong *et al.,* (2016) (103), Parris *et al.,*(2021) (72) and Suresh *et al.,* (2022) (332)

4.2.3 Inflammatory lesions

Stages and grades of MIR and FIR were determined through detailed histological review and description of the constituents and topography of each PIR, as suggested in Khong *et* al., (2016) (103). Terminology used for inflammatory conditions as reported by stage and grade is detailed in Table 4.1.

4.2.3.1 Maternal inflammatory responses

In cases of MIR, presence of neutrophils or, more specifically, polymorphonuclear leukocytes (PMNs), to the subchorion or choriodecidual interface was staged as 1. Extension of PMNs to the fibrous chorion and/or amnion was staged as 2 and amnion necrosis, amnion basement membrane hypereosinophilia and/or PMN karyorrhexis was staged as 3, necrotising chorioamnionitis. Grade 1 MIR was reported where there were no or scant neutrophils or, if present, there was no confluence observed. Grade 2 was considered severe and was characterised by confluent PMNs and/or the presence of subchorionic microabcesses (103, 141, 142, 407).

4.2.3.2 Fetal inflammatory responses

Stage 1 FIRs were characterised by neutrophilic infiltration to the umbilical vein or chorionic vessels; this was increased to stage 2 if PMNs were observed in one or both umbilical arteries *in conjunction* with those reported in the umbilical vein. Involvement of the Wharton's jelly was staged as 3. Grading was similar to that of MIR, specifically at grade 1, in that FIR grade 1 was reported where PMNs were absent or scant and non-confluent. Grade 2, however, was reported upon observation of near-confluent intramural PMNs with associated attenuation of vascular smooth muscle (103, 141, 142). An absence of inflammatory pathology was staged and graded as 0 (zero) for both MIR and FIR for the purposes of this study.

4.2.3.3 Villitis

As an uncommon finding, acute villitis is not routinely staged, but is graded as 2 (severe) where present (103). Histological observations of multifocal neutrophilic infiltration to the chorionic villi or intervillous space were reported as grade 2 (154). Chronic villitis was reported where lymphocytes, macrophages and/or plasma cells were seen to have infiltrated villi or intervillous spaces (171). In the absence of identified acute or viral infections, observations of

maternal cytotoxic $CD8^+$ T cells in the villous tissue were reported as villitis of unknown aetiology (VUE) (171). A separate grading system is in use for VUE, and this was agreed to be applied in any reported cases. As detailed in the Amsterdam Criteria, where inflammation affected less than ten neighbouring villi in any one histologic focus, VUE was recorded as being low grade. High grade VUE was reported if villous inflammation affecting over ten contiguous villi was observed on more than one placental section (103).

4.2.4 Vascular malperfusion lesions

Vascular malperfusion was reported following gross histopathological and microscopic evaluation using terminology and descriptors recommended by the Amsterdam Criteria (103).

4.2.4.1 Gross features of vascular malperfusion

Details were recorded of all preterm placental infarctions and term infarctions involving >5% of non-peripheral tissue. Placental hypoplasia, characterised by placental weight centiles <10 and/or an umbilical cord <10^{th} centile or <8mm diameter at term (103), was also reported as a gross maternal vascular malperfusion (MVM) finding. Where retroplacental haemorrhages were observed, MVM was reported alongside descriptions of any associated indentation of placental parenchyma including length, width and percentage involvement of the basal plate. Attached blood clots submitted with the placenta for pathology were measured in three dimensions, i.e., length, width and depth, and their mass recorded (103). Fetal vascular malperfusion was considered and explored further microscopically when intrinsic or anatomic obstructive pathologies were reported on gross examination of the umbilical cord (UC) or where clinical notes revealed potentially obstructing clinical conditions. Examples where this was the case included hypercoiled UC, long UC and true knots of UC, marginal or velamentous UC insertion, thin (<8mm diameter at term) UC or laterally compressed UC in cases of oligohydramnios (432).

4.2.4.2 Microscopic features of vascular malperfusion

Upon histological examination, MVM was reported where blood compressed the intervillous space and there was villous congestion and/or intervillous haemorrhage indicative of the microscopic features of retroplacental haemorrhage. Observations of increased syncytial knot formation inappropriate for the recorded gestational age, thin and elongated villi and a lack

of total villi resulted in a report of distal villous hypoplasia. The presence of short, hypermature villi for gestational age, often accompanied again by increased syncytial knot formation, warranted a report of accelerated villous maturation. Finally, MVM was reported where decidual arteriopathy was observed as characterised by fibrinoid necrosis, chronic perivasculitis, arterial thrombosis, absences of spiral artery remodelling, acute atherosis or mural hypertrophy (103). Histologically, FVM was reported where arterial or venous thrombosis was observed in fetal compartments, i.e., chorionic plate, stem vessel vasculature or the umbilical cord. The presence of segmental avascular villi, recent or remote intramural fibrin deposition, villous stromal-vascular karyorrhexis, stem vessel obliteration or vascular ectasia was also recorded as FVM as defined by the Amsterdam Criteria (103).

4.2.5 Categorisation of preterm placentas

Data analysis was performed to determine the frequency and type of placental inflammatory and vascular malperfusion lesions in the total study population. Subsequent analyses compared preterm (<37 weeks' gestation) and term (≥37 weeks' gestation) placentas before preterm placentas were sub-categorised according to the WHO definition and as described in Quinn *et al.* (2016) (5). Preterm births were grouped to, and named as previously, that is, extremely preterm (EPT) if they occurred <28 weeks' gestation, very preterm (VPT) if between 28- and 32-weeks' gestation and moderate to late preterm (MPT) if delivered >32 weeks' gestation. Any birth ≥37 weeks was classified as being at term.

4.2.6 Classification of placentas to histological groups

Using data collected from histopathological reports, placentas were classified to one of four histologic groups:

1. Acute placental inflammation (A/PIR). Placentas with a reported presence of neutrophilic infiltration in any compartment of the placenta and at any stage and grade,
2. Vascular malperfusion (VM). Maternal or fetal vasculopathy as described in Table 4.1,
3. Combined vascular malperfusion and acute inflammatory lesions (PIR/VM). Placentas reported to have inflammatory and vascular lesions deemed to be any combination of maternal or fetal responses,
4. No pathology (NP). No inflammatory response or vasculopathy reported.

4.2.7 Statistical analyses

Statistical analyses were conducted as described in *Chapter 2: Materials and Methods* with additional chapter-specific analyses performed as follows. Odds ratios were employed to compare relative odds of the occurrence of PTB in the presence of the variable of interest, for example specific inflammatory or non-inflammatory placental lesions. Application of odds ratios to the data further permitted determination of whether each of these lesions were risk factors for PTB and comparison of the magnitude of the risk. Odds ratios (OR) were calculated on two-by-two frequency tables and the following equation applied:

$$\mathrm{OR} = \frac{\mathrm{a/c}}{\mathrm{b/d}} = \frac{\mathrm{ad}}{\mathrm{bc}}$$

Where:

a = number of preterm placentas with specified lesion

b = number of term placentas with specified lesion

c = number of preterm placentas without specified lesion

d = number of term placentas without specified lesion

In addition, univariable linear regression models were developed to study the relationship between the probability of classification to histologic group and gestational age (in weeks) and to estimate the percentage probability value of that classification for unknown gestational ages.

4.3 Results

Before pathological findings are presented, representative images of normal preterm and term amniochorionic membranes and villous tissue are provided for comparison purposes (Figure 4.2).

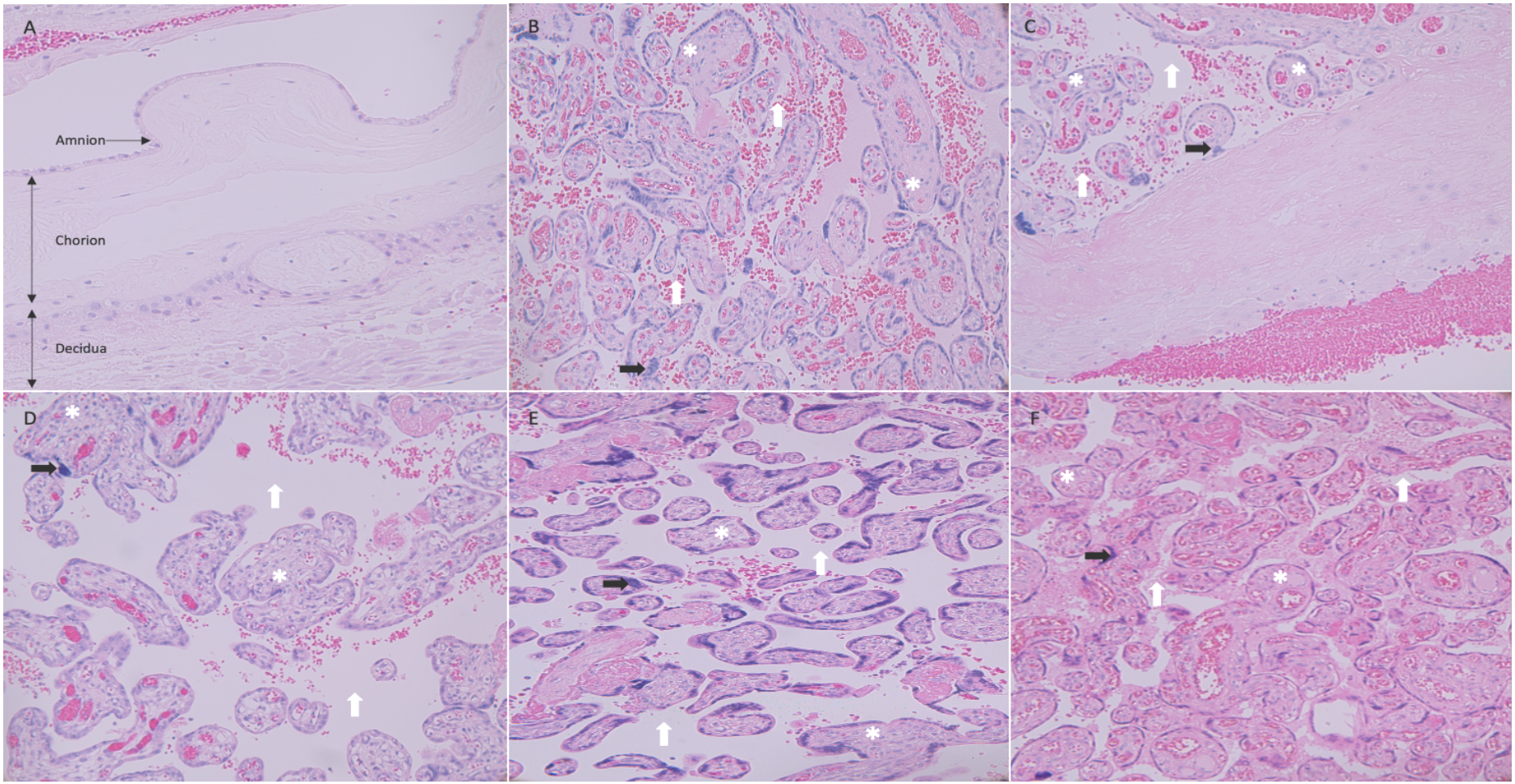

Figure 4. 2 Normal features of placental compartments on histology.

Haematoxylin and eosin staining (H&E), 20X magnification**. A** shows a sample of the fetal membrane roll with the single amnion epithelial layer covering the chorion and decidua layers, **B** is the corresponding villous tissue (VT), and **C** shows the decidua basalis (maternal plate). A, B, and C are images taken from a single term placenta with no pathological features. Asterisks represent villi, white block arrows denote intervillous spaces (IVS), and black block arrows show syncytial knots. **D** preterm placenta VT of 26 weeks and 3 days gestation, **E** preterm placenta VT of 33 weeks and 1 day gestation and **F** term placenta VT of 40 weeks and 5 days gestation. Note larger size of villi and decreased number of syncytial knots along with increased IVS in earlier gestations.

4.3.1 Placental inflammatory histopathological features in the total study population

Seventy-seven incidences of acute histologic inflammatory responses (PIR) were reported from forty-nine (50.0%) placentas in the total study population. A finding of no placental inflammatory response was reported in the remaining 50.0% (49 cases). Eight acute placental inflammatory lesion types were observed and reported. Chronic conditions were exceptionally rare; there was only one report of chronic VUE in the total cohort (Table 4.2).

HCA was the most prevalent inflammatory response in the study cohort and was reported in 55.1% (*n*=27) of the forty-nine placentas with PIR. There were twelve cases of acute subchorionitis (15.6%), ten of necrotising chorioamnionitis (13.0%) and nine of acute funisitis (11.7%) (Table 4.2). Umbilical cord vascular inflammation was the least common inflammatory response. There was one report of report of acute arteritis (1.3%), four (5.1%) of acute phlebitis and six (7.7%) of acute chorionic vasculitis (Table 4.2).

Table 4. 2 Reported incidence of acute inflammatory lesions in the study cohort

Acute inflammatory lesion	Reported Incidence (*n*)	Percentage of overall PIR
Acute Arteritis	1	1.3
Acute Chorioamnionitis (HCA)	27	35.1
Acute Chorionitis	8	10.4
Acute Funisitis	9	11.7
Acute Phlebitis	4	5.2
Acute Subchorionitis	12	15.6
Acute Chorionic Vasculitis	6	7.8
Necrotising Chorioamnionitis	10	13.0
Total	77	n/a

PIR; placental inflammatory response. Percentage calculated from *n*=77 PIR reports and not from number of placentas with PIR (*n*=49) to capture all incidences, including where placentas had report of more than one inflammatory lesion.

4.3.2 Inflammatory features in preterm versus term placentas

Presence of placental inflammatory responses was determined by microscopic visualisation and identification of neutrophils in any compartment of the placenta, as described previously. Twenty-three (46.9%) placentas with a reported PIR were delivered preterm compared to twenty-six at term (53.1%). No significant relationship between the presence or absence of PIR and preterm birth was found in the total study population (*P*=0.6862) (Figure 4.3), highlighting the need to subcategorise preterm gestations as in *Chapter 3.* Representative images of specific MIR and FIR lesions are shown in Figures 4.5 and 4.6.

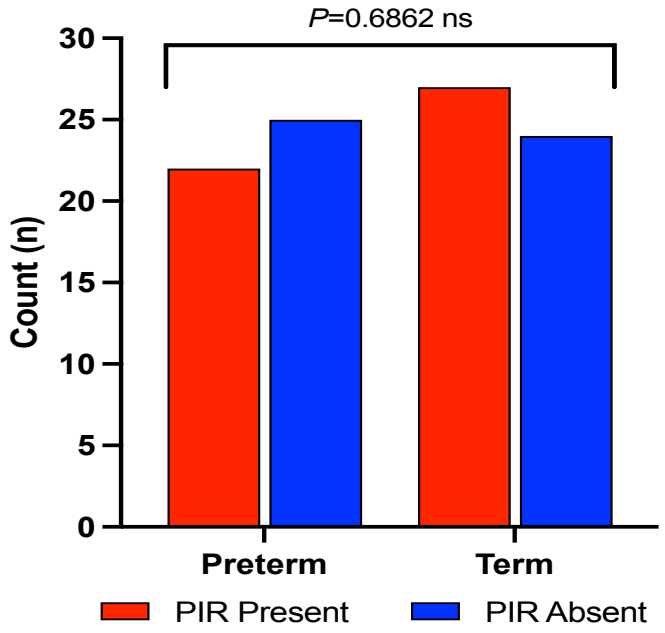

Figure 4. 3 Presence and absence of placental inflammatory responses in preterm and term placentas.

Placental inflammatory responses were reported in both preterm and term placentas and no significant difference was shown in the frequency between the two gestational age categories. *PIR;* placental inflammatory response.

4.3.2.1 Odds of acute subchorionitis are increased in term placentas

Acute subchorionitis was significantly more prevalent at term; calculated odds ratios revealed the odds of a preterm birth decreased by 81.8% where there was a histopathological finding of acute subchorionitis (OR 0.1822 95%CI: 0.03868-0.7484, *P*=0.0292) (Figure 4.4 and Table 4.3). On calculation of the reciprocal odds ratio, that is odds of placentas with acute subchorionitis being delivered at term, acute chorionitis was shown to be 5.5-times more likely in placentas delivered ≥37 weeks' gestation with a 448.8% increase in the odds of placentas with acute subchorionitis being delivered at term compared to delivery at a preterm gestation (OR 5.488; 95%CI: 1.336-25.85, *P*=0.0292) (data not shown).

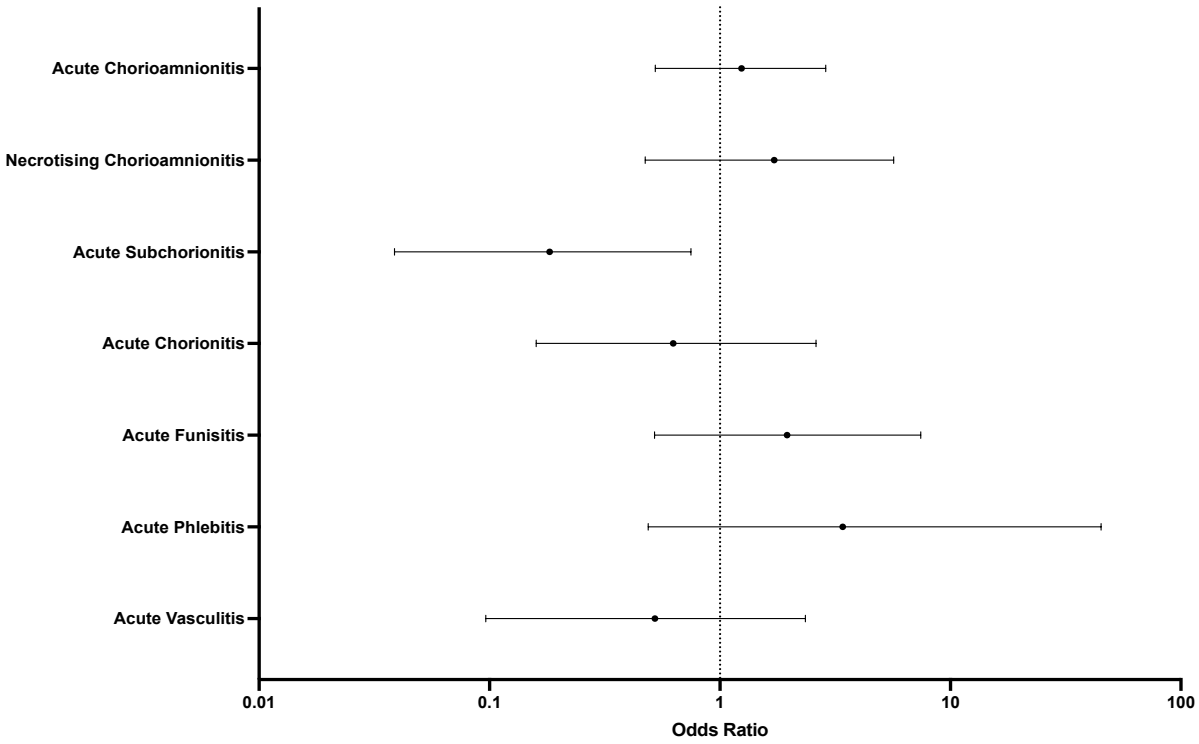

Figure 4. 4 Placental inflammatory response odds ratios.

Odds ratios (black dots) and corresponding 95% confidence intervals (CI) (black lines) calculated for the odds of preterm birth with inflammation. Symmetricity was created by application of a logarithmic (Log^{10}) scale to account for the range of upper limits of the 95% CI.

Table 4. 3 Odds ratios and corresponding 95% confidence intervals for odds of preterm birth with reported

Acute Inflammatory Lesion	Odds Ratio	95% Confidence Interval	*P* Value
Acute Chorioamnionitis	1.240	0.5242 to 2.875	0.6577 ns
Necrotising Chorioamnionitis	1.720	0.4735 to 5.670	0.5134 ns
Acute Subchorionitis	0.1822	0.0387 to 0.7484	0.0292
Acute Chorionitis	0.6273	0.1593 to 2.610	0.7168 ns
Acute Funisitis	1.956	0.5198 to 7.440	0.4902 ns
Acute Phlebitis	3.409	0.4877 to 45.04	0.3475 ns
Acute Chorionic Vasculitis	0.5222	0.0963 to 2.348	0.6792 ns

inflammatory lesions

ns; not significant, *P*; probability value. Two-tailed *P*<0.05 considered to be statistically significant.

Remarkably, no significant relationship was shown between the presence of acute chorioamnionitis (HCA) and PTB, nor were odds of PTB increased in the presence of HCA (OR 1.240; 95%CI: 0.5242-2.875, *P*=0.6577), adding further weight to the decision to subcategorise preterm gestations (Section 4.3.3). Analysis of only preterm vs. term cohorts further showed no association or increased odds of PTB in the presence of necrotising chorioamnionitis (OR 1.720; 95%CI: 0.4735-5.670, *P*=0.5134), acute funisitis (OR 1.956; 95%CI: 0.5198-7.440, *P*=0.3046) or acute phlebitis (OR 3.409; 95%CI: 0.4877-45.04, *P*=0.3475) (Table 4.3 and Figure 4.4). Acute chorionitis, acute arteritis and acute chorionic vasculitis tended to be reported more frequently at term, yet these were not significant relationships and there were no increased odds of preterm birth where placentas were reported with these lesions (Table 4.3).

4.3.3 Inflammatory features in placentas categorised by gestational age category

A statistically significant relationship (*P*=0.0008) (Table 4.4) and trend for increased prevalence at EPT gestations ($\chi^2_{df=1}$ = 12.00; *P*=0.0005) were shown only between acute funisitis and gestational age group, although this inflammatory lesion of the umbilical cord was observed in all groups except MPT. Unexpectedly, due to its frequent report on placental examination, no significant relationships were shown between gestational age category and acute chorioamnionitis either with inclusion of term placentas (*P*=0.0889) or when they were excluded from the analysis (*P*=0.1211). Inflammatory lesions of acute chorionitis (*P*=0.6108), acute subchorionitis (*P*=0.1517), acute phlebitis and acute chorionic vasculitis (*P*=0.0642) were also not significantly associated with gestational age category in this cohort (Table 4.4).

4.3.4 Necrotising chorioamnionitis is most prevalent in extremely preterm placentas

Necrotising chorioamnionitis, as described in Table 4.1 and Section 4.2.2.1, predominated in the EPT group (4 incidences; 36.4%) but was also observed in MPT (2 incidences; 7.1%) and term (4 incidences; 7.8%) placentas. Despite no overall significant association found between necrotising chorioamnionitis and all gestational age categories (*P*=0.0527) (Table 4.4), exclusion of the term cohort to assess the relationship between necrotising chorioamnionitis and decreasing preterm gestational age revealed a significant association between preterm categories and this severe inflammatory lesion (*P*=0.0349). Furthermore, a significant linear relationship ($\chi^2_{df=1}$ = 4.7537; *P*=0.0292) was revealed on application of a Chi square test for

trend, thus demonstrating an increased prevalence at EPT gestations. Representative images of normal morphology and inflammatory lesions of the umbilical cord and peripheral membranes are shown in Figures 4.5 and 4.6 with specific images of necrotising chorioamnionitis depicted as Figure 4.6E and 4.6F.

Table 4. 4 Inflammatory lesions reported on histology for each gestational age subgroup

Inflammatory lesion	**Extremely Preterm (*n*=11)**	**Very Preterm (*n*=8)**	**Moderate to Late Preterm (*n*=28)**	**Term (*n*=51)**	***P* Value**
Acute Arteritis	0	0	0	1	n/a
Acute Chorioamnionitis	4	5	5	13	0.0889 ns
Acute Chorionitis	1	1	1	5	0.6108 ns
Acute Funisitis	5	1	0	3	0.0008
Acute Phlebitis	0	1	2	1	0.2337 ns
Acute Subchorionitis	1	0	1	10	0.1517 ns
Acute Chorionic Vasculitis	0	2	0	4	0.0642 ns
Chronic VUE	0	0	1	0	n/a
Necrotising Chorioamnionitis	4	0	2	4	0.0527 ns
None	**2**	**2**	**20**	**25**	**0.0082**

n corresponds to number of reported incidences of each inflammatory lesion. In some cases, more than one inflammatory lesion was reported, therefore, total numbers do not necessarily correlate with number of placentas within each group. A total of forty-nine placentas had at least one histologically-diagnosed inflammatory lesion and the remaining forty-nine which had none. *Fisher's exact test with Freeman Halton extension. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. *n/a;* statistical analysis not performed due to *n* value.

4.3.5 Placental inflammatory lesions predominate in preterm gestations

Placentas with no inflammatory lesions observed on histology were reported in all study groups, predominantly at MPT (20 cases; 71.4%) and term (25 cases; 49.0%) gestations. Conversely, no inflammatory pathology was uncommon at EPT (2 cases; 18.2%) and VPT (2 cases; 25%) gestations (Table 4.4). Statistical analysis revealed a significant relationship between gestational age and the absence of inflammatory lesions (*P*=0.0082) (Table 4.4), but this was not shown to be a linear association ($\chi^2_{df=1}$ = 2.934; *P*=0.0867). Considering the relationship between PIR absence across only the preterm groups revealed this to be a greater significant association (*P*=0.0025). Furthermore, the presence of at least one placental inflammatory lesion was most prevalent in EPT placentas (9 cases; 81.8%) compared to VPT (6 cases; 75.0%) and MPT (8 cases; 28.6%), a relationship shown to be linearly significant ($\chi^2_{df=1}$ = 10.58; *P*=0.0011). It was therefore considered that inflammatory lesions affect preterm placentas more diffusely than term placentas and that an increased prevalence of PIR is associated with decreased gestational age.

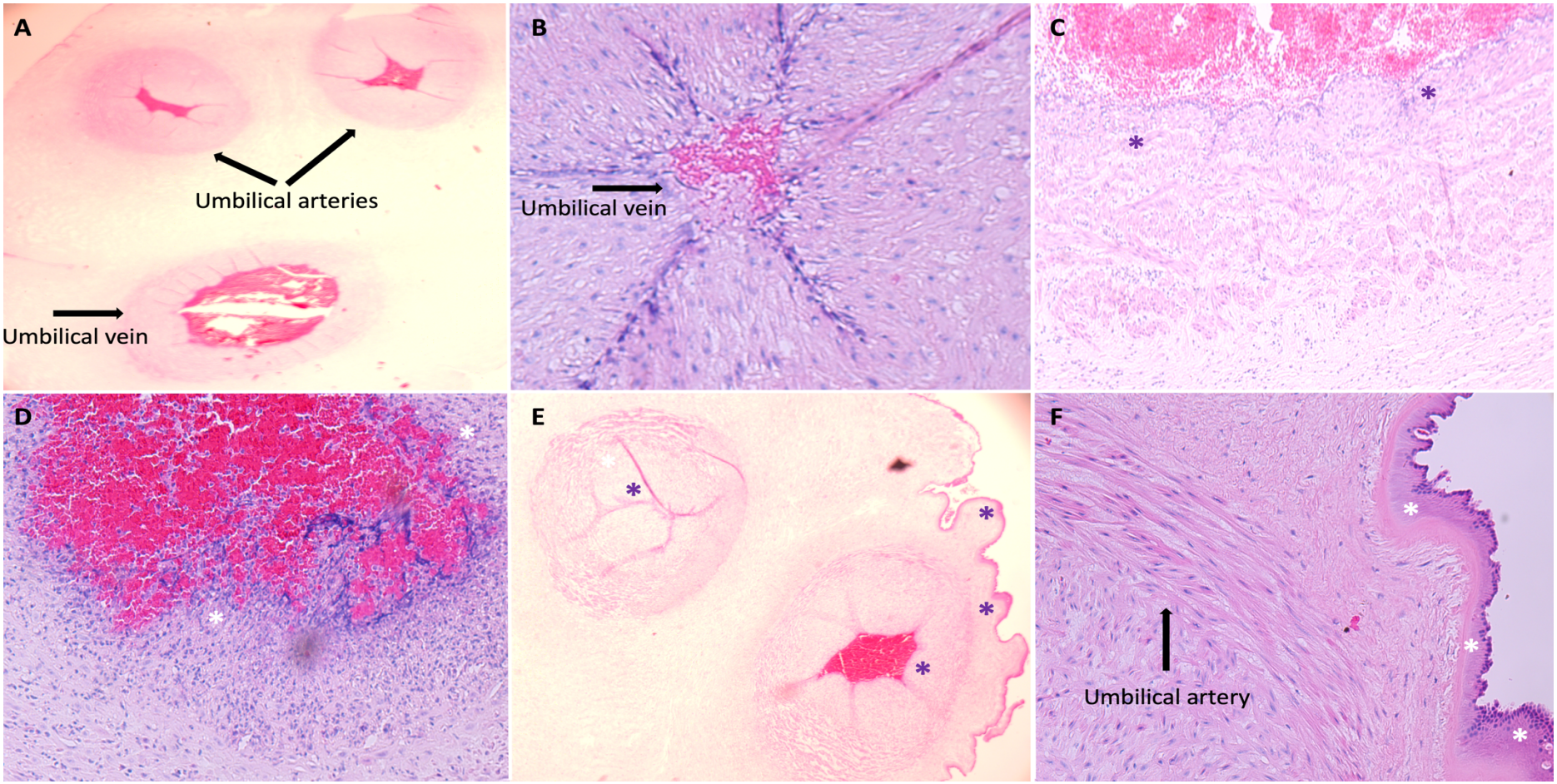

Figure 4. 5 Umbilical cord H&E staining at 20X magnification unless otherwise stated.

Asterisks represent neutrophilic infiltration; differences in colour are for clarity on images only. **A** depicts normal umbilical vessels with the two arteries and one vein noted (2X magnification). **B** shows a normal umbilical vein. **C** neutrophils around the umbilical vessels indicating low-grade vasculitis. **D** advanced grade arteritis with diffuse and confluent neutrophils migrating to an umbilical artery. **E** acute phlebitis, arteritis and funisitis with neutrophilic infiltration around the umbilical vein and artery and into the surrounding Wharton's jelly (4X magnification). **F** Acute funisitis in term placenta; neutrophils evident in the Wharton's jelly. Umbilical artery identified by presence of smooth muscle cells.

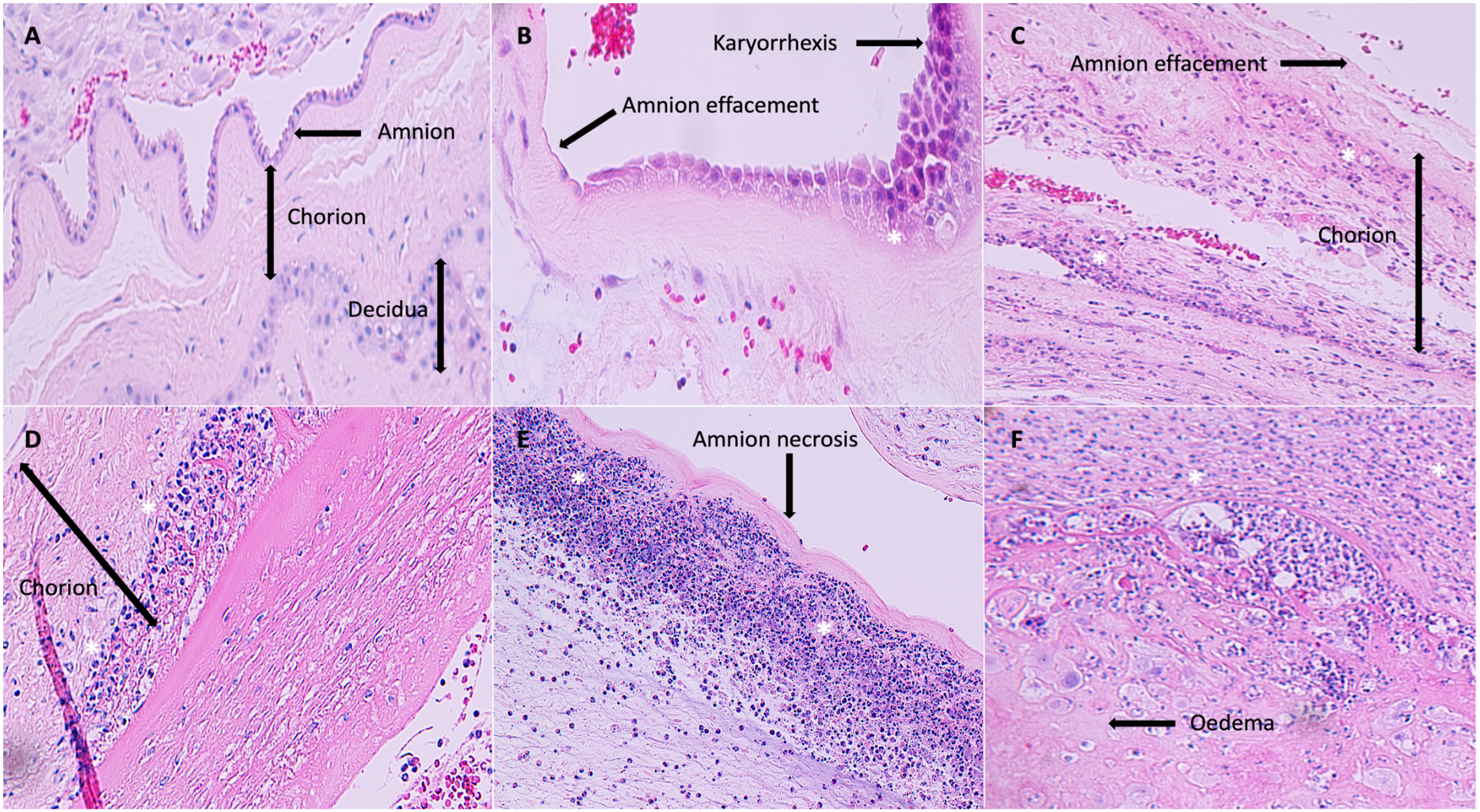

Figure 4. 6 Peripheral membranes H&E staining, original magnification at 20X.

White asterisks represent neutrophilic infiltration. **A** early acute chorioamnionitis (HCA), sparse neutrophils in the amniochorion. **B** HCA with effacement of amnion epithelial layer in places and evidence of karyorrhexis. **C** neutrophils in the chorion indicating chorionitis, effacement of amnion epithelial layer. **D** chorionic and subchorionic neutrophilic infiltration indicative of subchorionitis. **E** term placenta with complete necrosis of the amnion epithelial layer and diffuse, confluent neutrophils indicate necrotising chorioamnionitis. Karyorrhexis is evident. **F** preterm placenta with necrotising chorioamnionitis, karyorrhexis and oedema.

4.3.6 Grades and stages of placental inflammatory responses in the total study population

As per current guidelines, grading and staging of MIR and FIR were completed according to the Amsterdam Placental Workshop Group Consensus Statement as described in *Chapter 2: Materials and Methods* and Khong *et al*., (2016) (103). Grades of MIR and FIR relate to the severity of the inflammatory response and were reported as 1 or 2; stages describe the progression of the placental inflammatory response and were reported on the predefined scale of 1-3 (Table 4.5) (72, 103, 141). For the purposes of this study an absence of PIR was graded and staged as zero (0). Over half of all placentas were reported to have a MIR (53.1%), of these, this was recorded as stage 1 in 40.4% (21 cases), stage 2 in 38.5% (20 cases) and stage 3 in 21.2% (11 cases), an association (*P*=0.0070) and trend shown to be significant ($\chi^2_{df=1}$ = 4.327; *P*=0.0375). Severity was reported predominantly as grade 1 (69.2%; 36 cases) and did not significantly differ within the study population (*P*=0.3649) (Table 4.5).

Although FIRs are most commonly a progression of a maternal response, that is they do not occur in isolation, they are not *guaranteed* to occur following MIR, therefore, numbers of reported FIRs differed from the number of MIRs. In the total study cohort, almost one-third of placentas were reported with FIR (30 cases; 30.6%), all of which had a concomitant MIR. More FIRs were stage 3 (12 cases; 40%) than stage 2 (11 cases; 36.7%) or stage 1 (7 cases; 23.3%), yet no significant association (*P*=0.1093) or linear relationship was shown ($\chi^2_{df=1}$ = 1.875; *P*=0.1709). In placentas with a reported FIR there was an almost equal division between a grade 1 (14 cases; 46.7%) and a grade 2 response (16 cases; 53.3%). Frequencies of each grade and stage were counted for all groups and the total cohort and are detailed in Table 4.5; individual participants' gestational age and MIR and FIR grades and stages are detailed in Appendix IV (Figure AIV.I).

Table 4. 5 MIR and FIR stages and grades reported in preterm and term placentas

	Preterm *n* (%)	Term *n* (%)	*P* Value^	Total *n* (%)
MIR Stage/Grade 0	24 (51.1)	22 (43.1)	0.5437 ns	46 (46.9)
MIR Stage 1	4 (17.4)	17 (58.6)	0.0070	21 (40.4)
MIR Stage 2	13 (56.5)	7 (24.1)		20 (38.5)
MIR Stage 3	6 (26.1)	5 (17.2)		11 (21.2)
MIR Grade 1	14 (60.9)	22 (75.9)	0.3649 ns	36 (69.2)
MIR Grade 2	9 (39.1)	7 (24.1)		16 (30.8)
Total MIR	**23**	**29**		**52**
FIR Stage/Grade 0	28 (59.6)	40 (78.4)	0.0507 ns	68 (69.4)
FIR Stage 1	3 (15.8)	5 (45.5)	0.1093 ns	8 (26.7)
FIR Stage 2	6 (31.6)	4 (36.4)		10 (33.3)
FIR Stage 3	10 (52.6)	2 (18.2)		12 (40.0)
FIR Grade 1	9 (47.4)	5 (45.5)	>0.9999 ns	16 (53.3)
FIR Grade 2	10 (52.6)	6 (54.5)		14 (46.7)
Total FIR	**19**	**11**		**30**

FIR; fetal inflammatory response, *MIR;* maternal inflammatory response. Values are presented as counts (*n*) and percentage of total cases for stage/Grade 0 or of MIR/FIR cases where reported. ^Fisher's exact test. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

4.3.7 Maternal inflammatory response stages are significantly higher in term placentas, but fetal inflammatory response stages are significantly higher in preterm placentas

MIR stage was statistically significantly associated with term gestations (*P*=0.0070) and most commonly reported as stage 1 (58.6%). Conversely, preterm placentas were more likely to be reported as stage 2 and a significant linear increase in MIR stage was demonstrated on application of a post-hoc Chi-square test for trend ($\chi^2_{df=1}$ = 7.706; *P*=0.0055). A linear relationship was also revealed which showed a significant association with a higher FIR stage in preterm placentas ($\chi^2_{df=1}$ = 5.803; *P*=0.0160); no such linear relationship was seen at term ($\chi^2_{df=1}$ = 1.841; *P*=0.1748).

4.3.8 Increased stages but not grades of MIR are associated with preterm birth

MIRs were most prevalent in the EPT and VPT groups (9 cases; 81.8% and 6 cases; 75%, respectively), but also occurred frequently in term placentas (29 cases; 56.9%). A statistically significant association between the presence of MIR and extremely and very preterm birth was shown (*P*=0.0059) (Table 4.6). In addition, there was a significant relationship between MIR stage and gestational age category (*P*=0.0220) (Table 4.6) and linearity tests showed a significant trend for stage 1 MIR in term placentas ($\chi^2_{df=1}$ = 6.548; *P*=0.0105) (data shown in Appendix IV). Grade 1 MIR predominated overall, but no significant relationship was shown between gestational age category and grade of MIR (*P*=0.6451) (Table 4.6).

4.3.9 FIR stage and grade are not significantly associated with preterm birth

FIRs were reported in almost two-thirds of EPT and VPT placentas (7 cases; 63.6% and 5 cases; 62.5%, respectively) and there was a significant association between gestational age category and the presence of FIR (*P*=0.0084) (Table 4.6). However, FIR stages were not significantly associated with gestational age subcategory (*P*=0.2024) (Table 4.6), nor were any linear trends revealed with post-hoc analyses. Likewise, no significant relationships were found between FIR grade and gestational age category (*P*=0.1612) (Table 4.6).

Table 4. 6 Number of reported placental inflammatory responses by gestational age subgroup

	Extremely Preterm *n* (%)	Very Preterm *n* (%)	Moderate to late Preterm *n* (%)	Term *n* (%)	*P* Value^
MIR Stage/Grade 0	2	2	20	22	
MIR Stage 1	1	2	1	17	0.0220
MIR Stage 2	4	4	5	7	
MIR Stage 3	4	0	2	5	
MIR Grade 1	5	4	5	22	0.6451 ns
MIR Grade 2	4	2	3	7	
Total MIR	**9 (81.8)**	**6 (75.0)**	**8 (28.6)**	**29 (56.9)**	**0.0059**
FIR Stage/Grade 0	4	3	21	40	
FIR Stage 1	0	1	2	4	0.2024 ns
FIR Stage 2	1	2	3	5	
FIR Stage 3	6	2	2	2	
FIR Grade 1	1	4	4	5	0.1612 ns
FIR Grade 2	6	1	3	6	
Total FIR	**7 (63.6)**	**5 (62.5)**	**7 (25.0)**	**11 (21.6)**	**0.0084**

FIR; fetal inflammatory response, *MIR;* maternal inflammatory response. Values are presented as counts (*n*). ^Fisher's exact test with Freeman Halton extension where required. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. Total MIR and FIR shown as count (*n*) and percentage (%) of total number of placentas with reported MIR or FIR.

4.3.10 Gestational age is a predictor of MIR stage, FIR stage and grade, but not MIR grade

To complete data analysis to predict placental inflammatory response stages and grades from gestational age in weeks (independent variable), simple linear regressions were performed for the dependent variables MIR stage and grade, and FIR stage and grade. The null hypothesis for these analyses stated that gestational age had no effect on the severity or progression of MIR of FIR. The regression equation, $y = \beta 0X + \beta 1$, was applied, where:

y = the predicted value of the dependent variable (*y*) for any given value of the independent variable (x),

$\beta 0$ = the slope; the change in *y* for each unit change in *x*,

$\beta 1$ = the Y-intercept; the predicted value of *y* when *x* is zero, and

x = the independent variable.

Best fit values of the slope and Y-intercept, along with the standard error of the Y-intercept are reported in Table 4.7 and presented as an equation to define the best-fit line. Estimates of the effect (R^2; regression coefficient) and *P* values representing whether the slope deviates significantly from zero are also shown. Slopes of each independent variable against the dependent variable of gestational age with R^2 and corresponding *P* values are shown in Figure 4.7.

A significant relationship (P=0.0435) between gestational age and MIR stage (*y* = -0.0446) was found (*P*=0.0435) with a 0.04 decrease in MIR stage for every one week increase in gestational age. No such significant relationship was shown (*P*=0.1727) between gestational age and MIR grade (*y* = -0.02112) (Table 4.7 and Figure 4.7). It is therefore concluded that gestational age at delivery has a statistically significant effect on MIR stage, or progression through the placenta, but no statistically significant effect on MIR grade, or severity. When considering FIR, a highly significant association (*P*<0.0001) was shown between gestational age at delivery and FIR stage (*y* = -0.08557) with FIR stage decreasing by 0.09 for each additional week of gestation. Similarly, although not as highly significant (*P*=0.0036), a relationship was shown between gestational age and FIR grade (*y* = -0.04450); for every one week of advancing gestation, the severity, or grade, of FIR decreased by 0.04 (Table 4.7 and Figure 4.7). In summary, both FIR stage and grade are affected by gestational age and FIRs significantly decrease in severity and progression between preterm and term gestations.

Table 4. 7 Simple linear regression of MIR and FIR stages and grades against gestational age in weeks

	Slope (β0)	Y-Intercept (β1)	Standard Error	Regression Coefficient (R^2)	Equation	*P* Value
MIR Stage	-0.04446	2.505	0.7877	0.0418	Y = -0.04446*X + 2.505	0.0435
MIR Grade	-0.02112	1.422	0.5573	0.0193	Y = -0.02112*X + 1.422	0.1727 ns
FIR Stage	-0.08557	3.727	0.7556	0.1493	Y = -0.08557*X + 3.727	<0.0001
FIR Grade	-0.04450	2.057	0.5406	0.0849	Y = -0.04450*X + 2.057	0.0036

FIR; fetal inflammatory response, *MIR;* maternal inflammatory response. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. Simple linear regression values: *R^2; regression* coefficient.

4.3.11 Non-inflammatory placental histopathological features in the total study population

Thirty-three (33.7%) placentas were reported to have vasculopathy in the total study cohort, twenty (20.4%) with maternal vascular malperfusion (MVM), seven (7.1%%) with fetal vascular malperfusion (FVM) and six (6.1%) with features of both maternal and fetal malperfusion (M/FVM). Perivillous fibrin deposition was an infrequent feature (7 cases; 7.1%) found only in MPT and term placentas. One case each of basal plate necrosis and a single umbilical artery was observed in the EPT group and, in the term group, one case each of amnion nodosum, meconium staining in the chorionic plate and pyknosis in umbilical smooth muscle was reported following histologic examination. Placental thrombi were observed in four cases (4.1%); chorioangiosis and excess giant trophoblast cells were also reported but were infrequent at three cases (3.1%) each. Additionally, non-inflammatory features of decreased number of vessels in villi (5 cases; 5.1%), decreased size of villi (4 cases; 4.1%), increased stromal maternal blood cells (4 cases; 4.1%), decreased syncytiotrophoblast layers (4 cases; 4.1%) and large intervillous spaces (5 cases; 5.1%) were described (Table 4.8). Representative examples of non-inflammatory pathologies and variations in placental characteristics are shown in Figure 4.8.

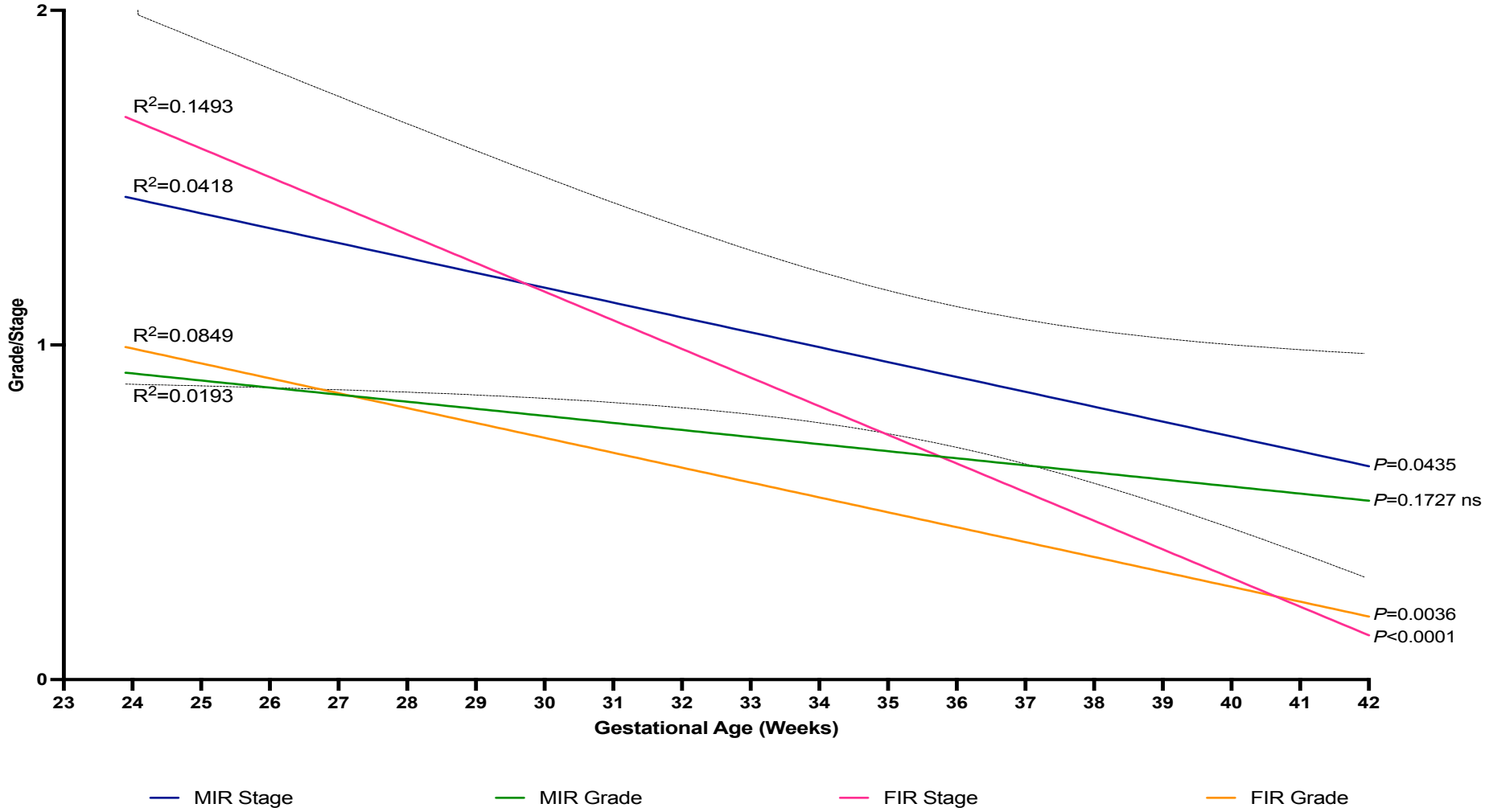

Figure 4. 7 Simple linear regression results demonstrating the predictive value of gestational age on MIR and FIR stages and grades.

Linear regression was performed to determine whether a significant amount of the variance in placental inflammatory response stages and grades could be explained by gestational age. The results demonstrate a predictive value of gestational age against maternal inflammatory response stages but not grades and fetal inflammatory response stages and grades. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. R^2*; regression* coefficient. Black dotted lines represent the 95% Confidence Interval.

4.3.12 Vascular malperfusion lesions occur more frequently in preterm placentas

Vasculopathy predominated in preterm placentas; 90.0% of MVM, 85.3% of FVM and 100% of M/FVM were reported from preterm gestations. MVM and M/FVM were the only non-inflammatory conditions found to have a significant association with delivery preterm versus term (*P*<0.0001 and *P*=0.0012, respectively). Total counts of non-inflammatory characteristics reported in preterm and term placentas and corresponding *P* values for vascular malperfusion lesions are presented in Table 4.8.

Table 4. 8 Total counts of non-inflammatory findings reported following histopathological evaluation in preterm and term placentas

	Preterm (*n*)	**Term (*n*)**
Maternal Vascular Malperfusion	18	2
	P<0.0001	
Fetal Vascular Malperfusion	6	1
	P=0.0525 ns	
Maternal and Fetal Vascular Malperfusion	6	0
	P=0.0012	
Perivillous Fibrin Deposition	1	6
Thrombus	3	1
Chorioangiosis	2	1
Single Umbilical Artery	1	0
Basal Plate Necrosis	1	0
Excess Giant Trophoblast Cells	3	0
Amnion Nodosum	0	1
Pyknosis in Umbilical Smooth Muscle	0	1
Meconium Staining in Chorionic Plate	0	1

P; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. Fisher's exact test applied only to malperfusion lesions as histopathological findings of interest for this thesis. Counts (*n*) show representative findings.

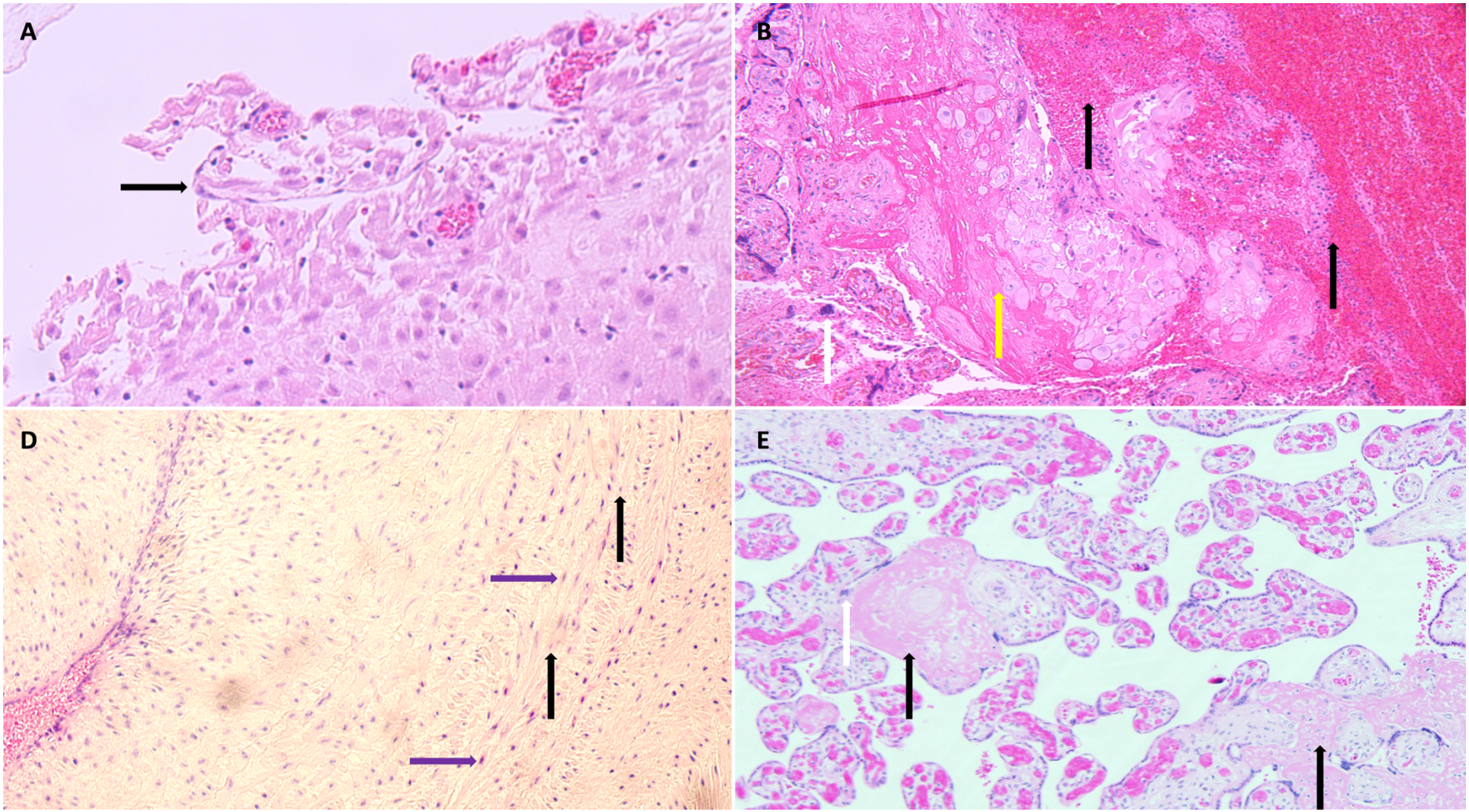

Figure 4. 8 Examples of non-inflammatory placental pathological findings and variations in placental characteristics.

H&E staining at 20X magnification. White arrows in B and E show syncytial knots as normal features. **A** nodules on amnion shown on H&E as amnion nodosum (black arrow), generally secondary to oligohydramnios as was the case in this placenta. **B** term placenta with notable abruption (black arrows) and oedema (yellow arrow). **C** pyknosis in umbilical smooth muscle of term placenta (black arrows), identified by condensation of the cell and compact nucleus. Purple arrows highlight normal sized and shaped cells and nuclei for comparison. **D** preterm placenta with focal excess fibrin deposition (black arrows).

4.3.13 Odds of maternal vascular malperfusion are increased in preterm placentas

Preterm birth was shown to be 15-times more likely in the presence of MVM lesions; MVM increased the odds of preterm birth by 1421% (OR 15.21; 95%CI: 3.678-68.36, *P*<0.0001). FVM was more common in preterm placentas, however, this was not shown to be a significant relationship or to increase the odds of a preterm birth (OR 7.317; 95%CI: 1.091-85.44, *P*=0.0525).

As there were no reports of combined maternal and fetal vascular malperfusion lesions in term placentas it was not possible to calculate odds ratios using the standard Baptista-Pike method, therefore, the Woolf method was used to adjust cell counts and apply the Haldane correction (addition of 0.5 to each cell). A combination of MVM and FVM significantly increased the likelihood of preterm birth by 16-times, thus, maternal and fetal vasculopathy combined increased the odds of preterm birth by 1513% (OR 16.13; 95%CI: 0.8829-294.8, *P*=0.0102) (Figure 4.9 and Table 4.9).

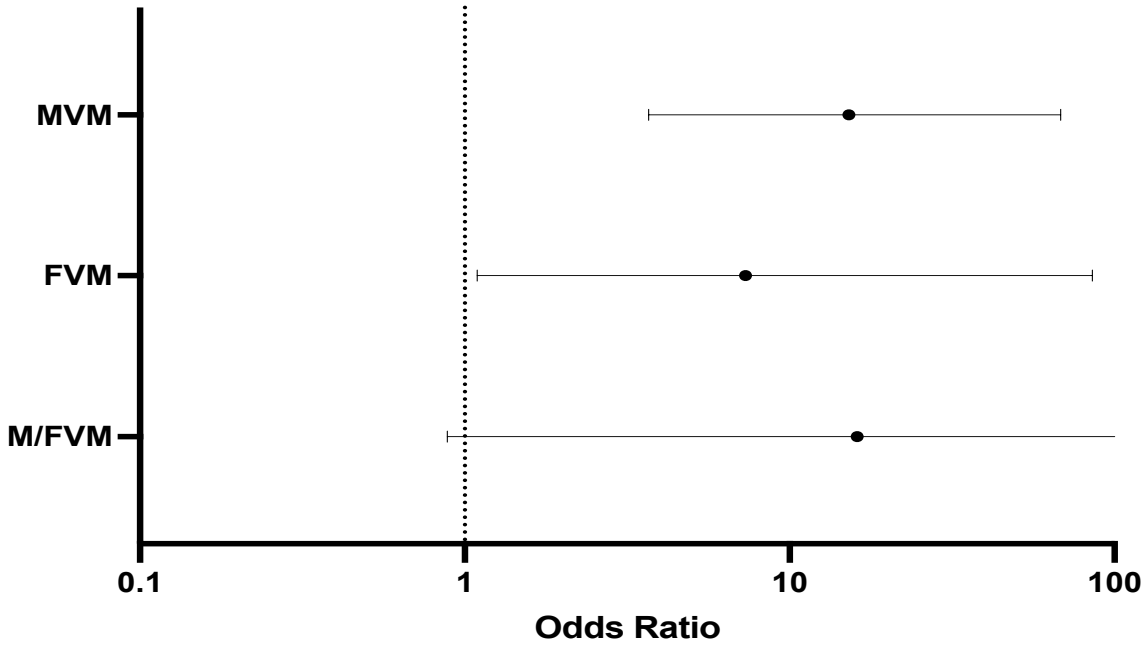

Figure 4. 9 Placental vascular malperfusion odds ratios.

Odds ratios (black dots) and corresponding 95% confidence intervals (CI) (black lines) calculated for the odds of preterm birth with placental vasculopathy. Symmetricity was created by application of a logarithmic (Log^{10}) scale to account for the range of upper limits of the 95% CI. *MVM;* maternal vascular malperfusion, *FVM;* fetal vascular malperfusion, *M/FVM*; maternal and fetal vascular malperfusion.

Table 4. 9 Odds ratios and corresponding 95% confidence intervals for odds of preterm birth with reported placental non-inflammatory responses

Vasculopathy	Odds Ratio	95% Confidence Interval	*P* Value
MVM	15.21	3.678-68.36	<0.0001
FVM	7.317	1.091-85.44	0.0525 ns
M/FVM	16.13	0.8829-294.8	0.0102

FVM; fetal vascular malperfusion*, MVM;* maternal vascular malperfusion, *M/FVM*; maternal and fetal vascular malperfusion*. P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

4.3.14 Maternal and fetal vascular malperfusion predominate in preterm gestations

MVM was most common in the MPT group (12 cases; 60%) followed by the EPT group (4 cases; 20%). Only two cases were reported in the VPT and term groups, each accounting for 10% of MVM placentas. The relationship between the four study groups and presence of MVM was found to be both highly significant (*P*<0.0001) and linear when assessing proportionality within the groups ($\chi^2_{df=1}$ = 9.434; *P*=0.0021). FVM was also most common in the MPT group (4 cases; 57.1%) with only one case (14.3%) reported in each of the other three groups. Unlike MVM, FVM was no more prevalent across preterm gestations (*P*=0.0854) and no linear relationship was shown ($\chi^2_{df=1}$ = 1.875; *P*=0.1709). Six placentas had both MVM and FVM; all of these were in the preterm groups, but most frequently at MPT gestations (3 cases; 50%). One-third of the M/FVM placentas were delivered at EPT gestations (2 cases; 33.3%) with the remaining case observed in the VPT group (16.7%). The association between M/FVM and gestational age was found to be significant (*P*=0.0138) and linear ($\chi^2_{df=1}$ = 6.979; *P*=0.0082).

4.3.15 Distinct histologic groups exist between preterm and term placentas

To determine whether it is possible to identify subgroups of spontaneous preterm birth due to acute inflammatory or non-inflammatory vascular lesions, placentas were categorised according to whether they had acute placental inflammatory features only (A/PIR; Group 1), vascular malperfusion lesions only (VM; Group 2) or a combination of acute PIR and vascular malperfusion lesions (PIR/VM; Group 3), as these were the most common findings across the total cohort. Placentas with no PIR or vasculopathy were classified as no pathology (NP; Group

4). Acute inflammatory lesions (A/PIR) were the most common histopathological finding in the whole cohort (35.7%) and occurred more frequently in term placentas. VM and PIR/VM predominated in preterm placentas (94.7% preterm vs. 5.3% term and 85.7% preterm vs. 14.3% term, respectively). An absence of pathology (NP) was more commonly reported in term (80.0%) placentas than preterm (20.0%) (Table 4.10).

Table 4. 10 Histologic grouping of preterm and term placentas

Group	Histologic Features	Preterm (*n*)	Term (*n*)	Total (*n*)
A/PIR	Acute Placental Inflammatory Response Only	11	24	35
VM	Vascular Malperfusion Only	18	1	19
PIR/VM	Combined Acute PIR and Vascular Malperfusion	12	2	14
NP	No Pathology	6	24	30

n corresponds to number of reported placentas within each group. *PIR;* placental inflammatory response.

A/PIR was a significantly more common placental feature than VM (*P*=0.0160) and PIR/VM (*P*=0.0009). On the other hand, there was no statistically significant difference between the frequency of A/PIR and NP (*P*=0.8781). Placentas were reported to have VM lesions more frequently than PIR/VM lesions (*P*=0.0009), but significantly less often than no pathology (*P*=0.0300). Likewise, placentas with PIR/VM lesions were reported significantly less frequently than placentas with no pathology (*P*=0.0018) (Figure 4.10).

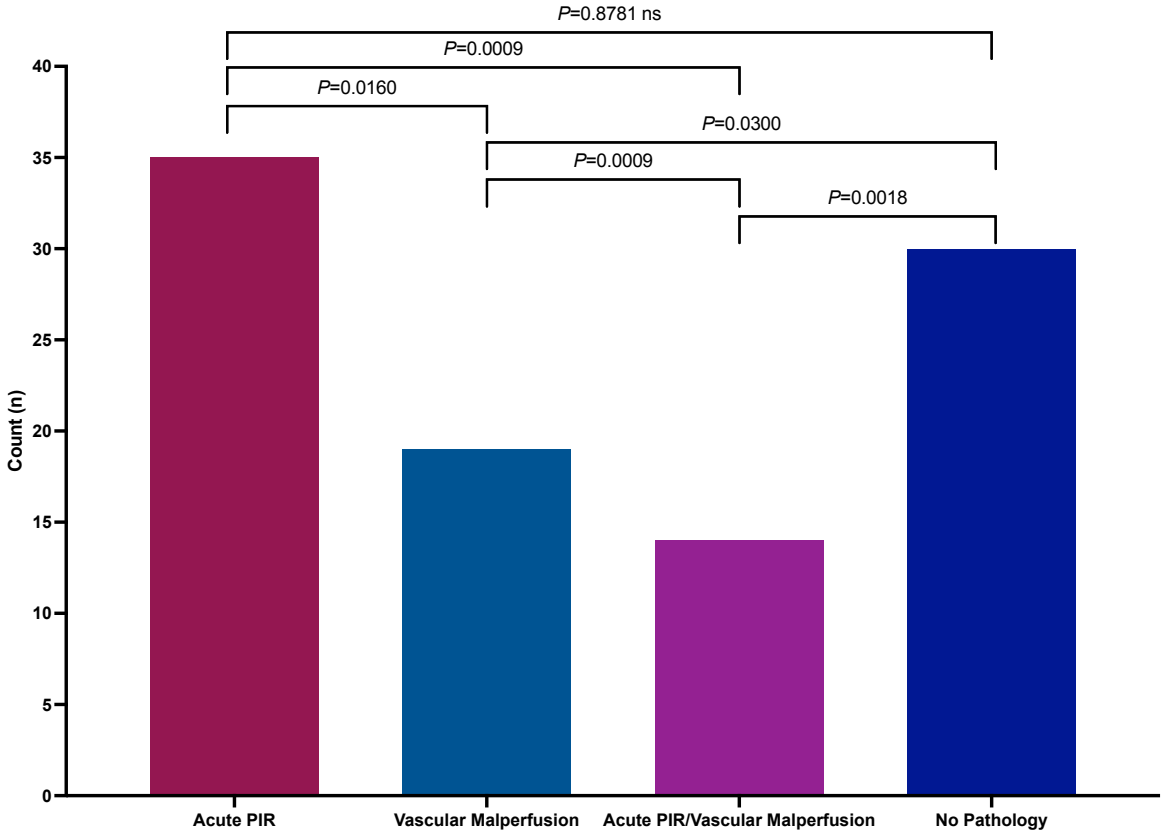

Figure 4. 10 Categorisation of placentas to histologic group.

Acute placental inflammatory responses and no pathology were significantly more common in the total study population except when compared to each other. Vascular malperfusion lesions occurred more frequently than acute PIR and vascular malperfusion combined but less frequently than no pathology. A combination of acute PIR and vascular malperfusion was the least common pathological finding reported in the total study population. *PIR; placental inflammatory response. P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant

4.3.16 Odds of vascular malperfusion and combined inflammation and vascular malperfusion are significantly increased in preterm placentas

To establish whether preterm delivery is a predictor of histologic group, odds ratios were calculated which revealed vascular malperfusion (VM) lesions were 31-times more likely in preterm than term placentas. This exceptional increase of 3003% in the odds of having pathological vascular malperfusion lesions in preterm placentas compared to term placentas was shown to be highly significant (OR 31.03; 95%CI: 4.658-330.3, *P*<0.0001) (Figure 4.11 and Table 4.11).

Whilst not as high as vascular malperfusion lesions alone, the odds of having a reported combination of acute PIR and vascular malperfusion (PIR/VM) lesions were significant and

found to be 8-times greater in preterm compared to term placentas (OR 8.400; 95%CI: 1.835-38.85, *P*=0.0030). This signified a 740% increase in the odds of these combined inflammatory and non-inflammatory lesions in preterm delivered placentas (Figure 4.11 and Table 4.11).

Conversely, placentas delivered at preterm gestations were found to be less likely to have acute PIR with no additional pathology (A/PIR) than those from term births. Preterm birth decreased the odds of a histopathological finding of A/PIR by 65.6% (OR 0.3438; 95%CI: 0.1451-0.7961, *P*=0.0201) (Figure 4.11 and Table 4.11). The calculated reciprocal of the odds ratio, i.e., the odds of placentas with acute PIR being delivered at term, showed this outcome to be almost 3-times more likely. Therefore, there was a 190.9% increase in the odds of placentas with A/PIR being delivered at term compared to preterm (OR 2.909; 95%CI: 1.256-6.892, *P*=0.0201, data not shown).

Placentas with no pathological (NP) lesions were 83.5% less likely to result in a preterm birth than a term birth (OR 0.1646; 95%CI: 0.0599-0.4656, *P*=0.0004) (Figure 4.11 and Table 4.11). The calculated reciprocal odds of no pathology being reported in term placentas were shown to be 6-times higher than in preterm placentas, demonstrating a 507.4% increase in the odds of term placentas having no pathological findings on histopathological evaluation (OR 6.07; 95%CI: 2.148-16.68, *P*=0.0004, data not shown).

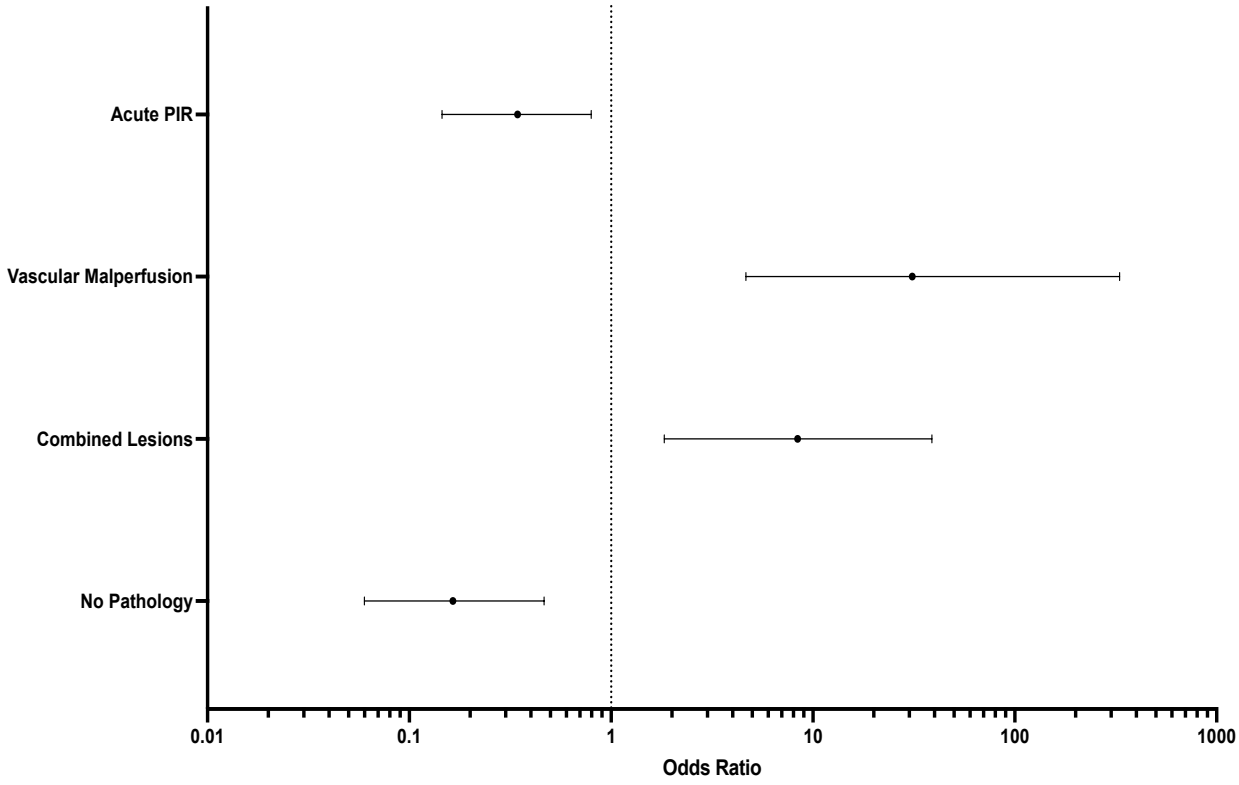

Figure 4. 11 Odds ratios of histologic categorisation.

Odds ratios (black dots) and corresponding 95% confidence intervals (CI) (black lines) calculated for the odds of preterm birth significant placental non-inflammatory responses. Symmetricity was created by application of a logarithmic (Log^{10}) scale to account for the range of upper limits of the 95% CI.

Table 4. 11 Odds ratios and corresponding 95% confidence intervals of preterm birth by histologic group

Histologic Group	Odds Ratio	95% Confidence Interval	*P* Value
Acute Placental Inflammatory Response (A/PIR)	0.3438	0.1451-0.7961	0.0201
Vascular Malperfusion (VM)	31.03	4.658-330.3,	<0.0001
Acute PIR and Vascular Malperfusion (PIR/VM)	8.400	1.835-38.85	0.0030
No Pathology (NP)	0.1646	0.0599-0.4656	0.0004

FVM; fetal vascular malperfusion*, MVM;* maternal vascular malperfusion, *M/FVM*; maternal and fetal vascular malperfusion*. P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant

4.3.17 Vascular malperfusion or combined acute PIR and vascular malperfusion lesions are more common at preterm gestations

The mean (± SD) gestational age for each of the histologic groups and total counts of placentas categorised to gestational age subcategories are presented in Table 4.12. In the whole study population, the mean gestational age at delivery was 35.92 ± 4.9 weeks. Placentas categorised as A/PIR were delivered at a significantly later gestation than those in the VM histologic group (*P*=0.0019) and the PIR/VM group (*P*=0.0005). Similarly, gestational age was significantly greater in placentas with no pathology (NP) compared to the VM (*P*=0.0053) and PIR/VM (*P*=0.0013) histologic groups. There was no such difference between the gestational age of VM and PIR/VM groups (33.84 ± 3.7 vs. 31.26 ± 5.1 weeks; *P*>0.9999) or between placentas with A/PIR and those with no pathology where the mean gestational ages were observed to be early term (*P*>0.9999) (Table 4.12).

4.3.18 Acute placental inflammatory responses and no pathological abnormality are significantly associated with term birth

Eleven (31.4%) preterm and twenty-four (68.6%) term placentas were reported as having only A/PIR; within the preterm subgroups these were relatively evenly distributed with four cases each in the EPT and MPT and three in the VPT group (Figure 4.12). Statistical analysis showed A/PIR to be statistically significantly more prevalent in the term group (*P*=0.0259) although this was not a linear relationship ($\chi^2_{df=1}$ = 1.338; *P*=0.2475).

Of the thirty placentas with no reported pathology, 80.0% (24 cases) were from term births, 16.7% (5 cases) from MPT births and 3.3% (1 case) from VPT births. No placentas categorised to the EPT group had a report of no pathology. Fisher's exact test and post-hoc Chi-square test for trend revealed term placentas to be significantly more likely to have no pathology (*P*=0.0014) (Figure 4.12) and that as gestation progressed the chance of having no pathology increased linearly ($\chi^2_{df=1}$ = 13.13; *P*=0.0003).

VM as the only placental pathology was reported in nineteen placentas, most frequently in the MPT group (15 cases; 78.9%). VM was an uncommon finding in EPT (2 cases; 10.5%), VPT (5.3%; 1 case) and term (5.3%; 1 case). As a result, there was a highly significant (*P*<0.0001) (Figure 4.12) and linear ($\chi^2_{df=1}$ = 4.232; *P*=0.0397) association between VM histologic findings and gestational age, with a preponderance for an increased number of cases towards late preterm. Fourteen placentas were categorised to the PIR/VM histologic group with 35.7% (5 cases) delivered EPT, 21.4% (3 cases) VPT, 28.6% (4 cases) MPT, and 14.3% (2 cases) at term. This relationship was found to be significant (*P*=0.0007) (Figure 4.12), as was the likelihood of PIR/VM lesions decreasing in a linear manner with advancing gestational age ($\chi^2_{df=1}$ = 16.25; *P*<0.0001).

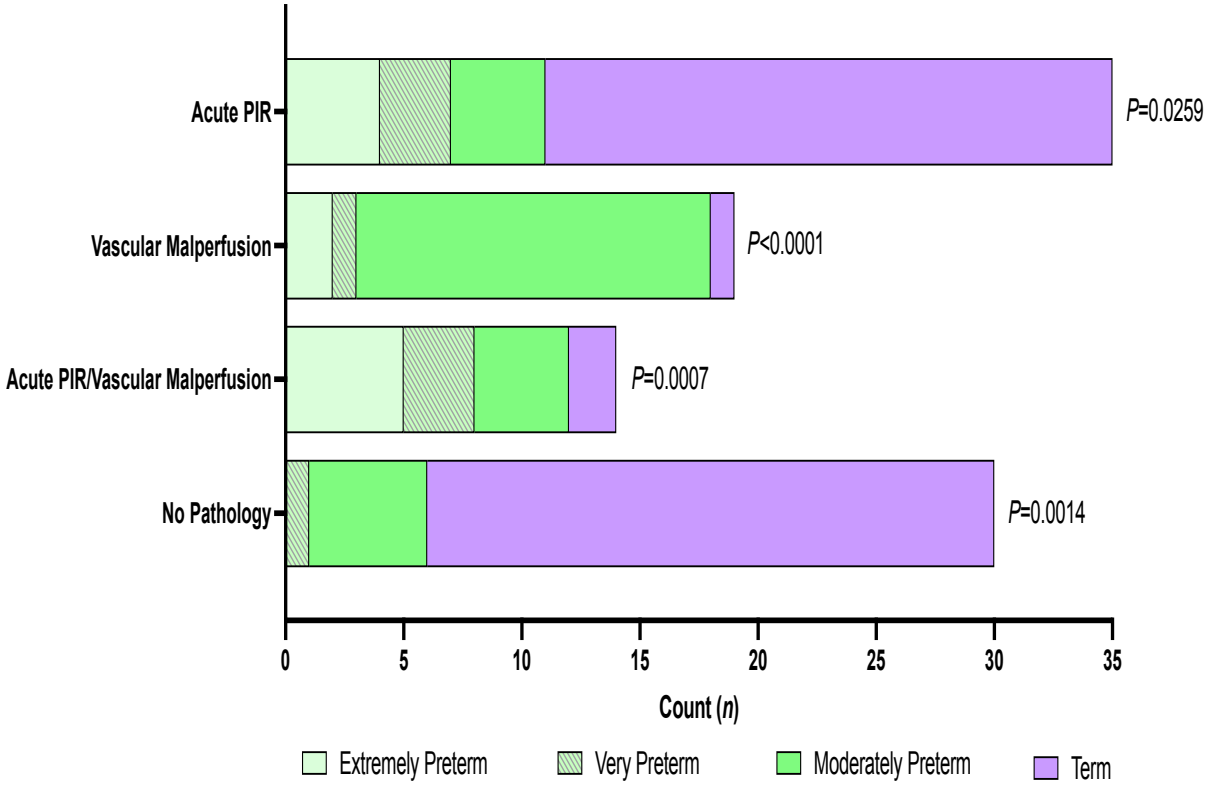

Figure 4. 12 Number of placentas per histologic group by gestational age subgroup.

Acute placental inflammatory responses and no pathology were significantly more common findings in term placentas. Vascular malperfusion predominated in the MPT group, whereas a combination of acute inflammatory and vascular malperfusion lesions occurred most frequently in the EPT group. *EPT;* extremely preterm, *VPT;* very preterm, *MPT;* moderate to late preterm, *T*; term. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant

4.3.19 Placentas can be classified to discrete histologic groups according to gestational age subgroup

Statistical analyses were performed between gestational age subgroups and histologic group to determine differences between these variables. As detailed below, it was possible to classify placentas from each of the gestational age subgroups to a discrete histologic group.

4.3.19.1 Extremely preterm placentas

Combined PIR/VM predominated in extremely preterm placentas and was significantly more frequent than NP (P=0.0351) but not A/PIR (P>0.9999) or VM (P=0.3615). No significant associations were found between A/PIR and VM (P=0.6351), A/PIR and NP (P=0.0902) or VM and NP (P=0.4672) (Table 4.12).

4.3.19.2 Very preterm placentas

Placentas in the very preterm subgroup were most commonly reported to have A/PIR or PIR/VM lesions, however, no significant relationships were found between histologic groups in the very preterm cohort (Table 4.12).

4.3.19.3 Moderate to late preterm placentas

VM was observed significantly more frequently than A/PIR (P=0.0041), PIR/VM (P=0.0041) and NP (P=0.0011) in the moderate to late preterm group. There were no further significant differences between histologic groups in moderate to late preterm placentas, that is, A/PIR was no more common than PIR/VM (P>0.9999) or NP (P>0.9999) and there was no relationship between PIR/VM and NP (P>0.9999) (Table 4.12).

4.3.19.4 Term placentas

Reports of A/PIR or NP were equally as common in term placentas and no significant relationship was found between these two histologic groups (P>0.9999); however, both were significantly more common than VM (P<0.0001) and PIR/VM (P<0.0001). VM and PIR/VM were reported infrequently in term placentas and no significant association was shown between them (P>0.9999) (Table 4.12).

Table 4. 12 Total counts of placentas categorised by gestational age subgroup and histopathological category

Gestational Age	Acute PIR (A/PIR) (*n*=35)	Vascular Malperfusion (VM) (*n*=19)	Combined Lesions (PIR/VM) (*n*=14)	No Pathology (NP) (*n*=30)	*P* Value					
					A/PIR vs. VM	A/PIR vs. PIR/VM	A/PIR vs. NP	VM vs. PIR/VM	VM vs. NP	PIR/VM vs. NP
EPT^ <28 weeks	4 (11.4%)	2 (10.5%)	5 (35.7%)	0 (0%)	0.6351 ns	>0.9999 ns	0.0902 ns	0.3615 ns	0.4672 ns	0.0351
VPT^ ≥28 – <32 weeks	3 (8.6%)	1 (5.3%)	3 (21.4%)	1 (3.3%)	0.5692 ns	>0.9999 ns	0.5692 ns	0.5692 ns	>0.9999 ns	0.5692 ns
MPT^ ≥32 - <37 weeks	4 (11.4%)	15 (78.9%)	4 (28.6%)	5 (16.7%)	0.0041	>0.9999 ns	>0.9999 ns	0.0041	0.0011	>0.9999 ns
Term^ ≥37 weeks	24 (68.6%)	1 (5.3%)	2 (14.3%)	24 (80%)	<0.0001	<0.0001	>0.9999 ns	>0.9999 ns	<0.0001	<0.0001
Mean ± SD†	37.16 ± 5.379	33.84 ± 3.651	31.26 ± 5.140	37.97 ± 2.570	0.0019	0.0005	>0.9999 ns	>0.9999 ns	0.0053	0.0013

PIR; placental inflammatory response. †Kruskal-Wallis with Dunn's multiple comparison, ^Fisher's exact test with Freeman Halton extension. *Mean ± Standard Deviation (SD). *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

4.3.20 Gestational age is a predictor of histologic group

Data were analysed to assess whether it was possible to predict the categorisation to a histologic group (dependent variable) from gestational age in weeks as the independent variable based on the hypothesis that placentas could be categorised to a specific histologic group dependent on gestational age at delivery. Simple linear regressions were performed and, as described in Section 4.3.10, the regression equation, $y = \beta 0X + \beta 1$ was applied. Best fit values of the slope and Y-intercept, along with the standard error of the Y-intercept are reported in Table 4.13 along with estimates of the effect (R^2) and *P* values representing significance of the slope's deviation from zero. Figure 4.13 is a graphical representation of the slope of each dependent variable against gestational age and shows the predictive value (scaled 0.1-0.6 representing % probability value), R^2 value and corresponding *P* values.

Analysis revealed gestational age to account for a 20.8% likelihood of categorisation to the A/PIR histologic group at the upper EPT limit of this cohort compared to 15.5% at term gestations (27.9 weeks vs. 39.9 weeks; Appendix IV, Figure AIV.IV); however, this was not a significant finding (*P*=0.0611). Conversely, a significant (*P*=0.0384) regression equation was demonstrated for the variable of categorisation to the VM histologic group. When considering the upper limit of each gestational age category, i.e., EPT, VPT, MPT and term, specifically for this dataset, a 33.3% predictive value of VM presence at 27.9 weeks' gestation was calculated compared to 25.9% at 31.5 weeks, 17.6% at 36.4 weeks and only 13.1% at 39.9 weeks' gestation, thereby demonstrating a greater risk of these non-inflammatory lesions at earlier gestations (Figure 4.13 and Appendix IV, Figure AIV.IV).

The largest difference in predictive risk was observed between EPT and term gestations and the presence of combined PIR/VM lesions where the predictive value was shown to be 37.9% at 27.9 week's gestation and 4.9% at 39.9 weeks' gestation (Figure 4.13 and Appendix IV, Figure AIV.IV). Overall, a highly significant regression equation was shown for prediction of categorisation to the PIR/VM histologic group from gestational age (*P*<0.0001) (Table 4.13 and Figure 4.13). The regression model also showed the predictive value of categorising placentas with an absence of pathologic lesions based on gestational age to be significant (*P*=0.0054) although this was a positive relationship in contrast to the predictive value of categorisation to the VM and PIR/VM groups (Table 4.13 and Figure 4.13). At 27.9 week's

gestation, the predictive value of classification to the NP group was calculated as 10.0%, rising to 16.4% at 31.5 weeks, 29.7% at 36.4 weeks and 42.2% by term at 39.9 weeks' gestation. Line data for histological group categorisation is presented in Appendix IV (Figure AIV.IV).

Table 4. 13 Simple linear regression of histological category against gestational age in weeks

	Slope (β0)	Y-Intercept (β1)	Standard Error	Regression Coefficient (R^2)	Equation	*P* Value
A/PIR	0.01868	-0.3140	0.3574	0.03605	Y = -0.01868*X – 0.3140	0.0611 ns
VM	-0.01701	0.8049	0.3574	0.04390	Y = -0.01701*X + 0.8049	0.0384
PIR/VM	-0.02806	1.151	0.2448	0.1525	Y = -0.02806*X + 1.151	<0.0001
NP	0.02639	-0.6419	0.3363	0.07775	Y = -0.02639*X - 0.6419	0.0054

A/PIR; acute placental inflammatory response, *NP;* no pathology, *PIR/VM;* combined placental inflammatory response and vascular malperfusion, *VM;* vascular malperfusion. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. Simple linear regression values: *F;* ratio of the mean regression sum of squares divided by the mean error sum of squares, (*DFn,DFd);* degrees of freedom in the numerator, degrees of freedom in the denominator, R^2*;* coefficient of determination.

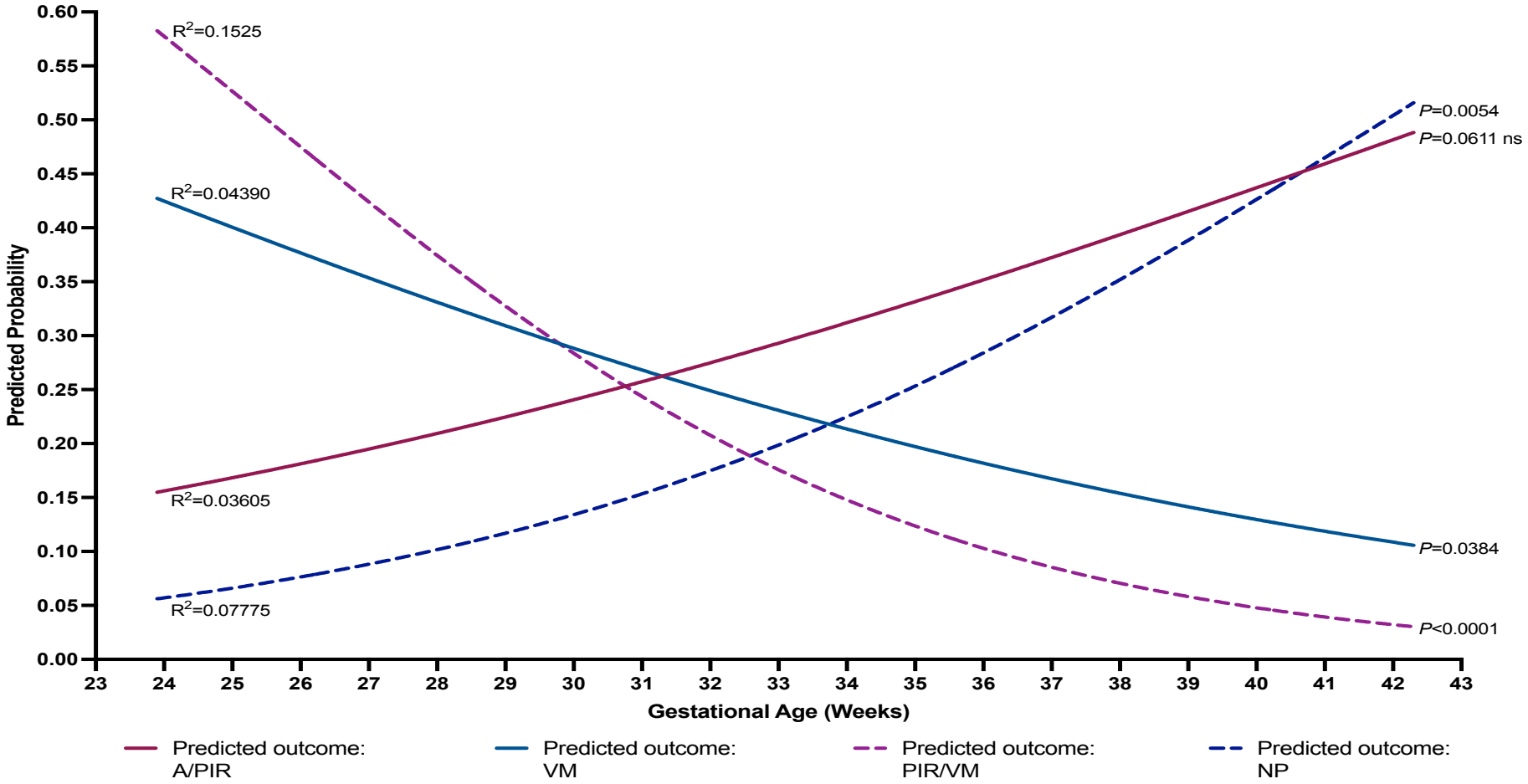

Figure 4. 13 Simple linear regression results demonstrating the predictive value of gestational age on histologic group.

Linear regression was performed to determine the probability of placentas being categorised to histologic group based on gestational age. The results demonstrate a predictive value of gestational age against vascular malperfusion lesions, combined acute inflammatory and vascular malperfusion lesions and no pathological findings, but not of acute inflammatory responses only. *A/PIR;* acute placental inflammatory response, *NP;* no pathology, *PIR/VM;* combined placental inflammatory response and vascular malperfusion, *VM;* vascular malperfusion. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. R^2*;* coefficient of determination.

4.4 Discussion

The principal aims of this chapter were to analyse placental histology in preterm and term births, determine if the prevalence and type of inflammatory lesion differ between preterm and term placentas and to evaluate whether staging and grading of placental inflammatory responses varies with gestational age. In attempting to answer these research questions, the data emphasised the need to consider whether histologic features and PIR grades and stages were influenced by subgroups of preterm birth as well as assessing the contribution of non-inflammatory histologic lesions. Consequently, secondary aims of the chapter were to categorise cases according to histopathological assessment, investigate whether gestational age was a useful predictor of placental pathology and, in the case of acute placental inflammatory lesions, the stage and grade of the response. Three key aspects were highlighted in the results from the study: 1) acute placental inflammatory responses are equally prevalent at preterm and term gestations, notably where the response was reported as acute chorioamnionitis, 2) placental inflammatory responses are of higher grade and stage at earlier gestations, and 3) preterm placentas have a distinct histopathological phenotype. These three key points are discussed in the subsections below.

4.4.1 Acute inflammatory responses are equally prevalent in preterm and term placentas

There are conflicting data on the prevalence of acute PIR in term placentas, potentially deriving from the absence of assessment of placentas considered to be normal, or studies which consider all PIR together rather than focussing only on histologically-diagnosed acute chorioamnionitis. One study where acute PIR was appraised as a single condition without accounting for gestational age found there to be a 20.23% (95% CI 18.42–22.16%) prevalence in 1770 placentas where a histopathological examination had been conducted. However, when all 5910 placentas included in the study were considered, the prevalence was much lower at only 6.06% (95% CI 5.48–6.69%) (164). Histopathological evaluation of placentas collected for this thesis demonstrated acute inflammatory lesions in 50% (49/98) of the total cohort. At least one acute PIR lesion was reported in almost half (46.9%; 23 cases) of preterm placentas, consistent with previously published findings (403, 405, 433), but term placentas contributed 51.0% (26 cases) to the reported acute PIR cases, an unexpected observation and one which refutes the status quo (408).

Only after preterm placentas were categorised to distinct gestational age groups were discrete differences seen in the presence of inflammatory lesions. Acute PIR lesions were present more often than not in both the extremely (81.8%) and very (75.0%) preterm cohorts, but not in the MPT category (28.6%). Exclusion of the term cohort revealed acute PIR lesions to be linearly significant (*P*=0.0011) with a minimal number of placentas in the ≤32 weeks' gestation group being spared of an inflammatory insult and resulting acute PIR. It is plausible that the increase in placentas with acute PIR at preterm gestations is due to PPROM (80.6% of all preterm) or a combination of PPROM and extended periods of rupture of membranes (305.9 ± 578.2 hours), generally indicated to prolong pregnancy and/or complete a full course of corticosteroids. This protracted interlude in physical protection from the membranes may be permissive of microbial invasion of the amniotic cavity (MIAC) and resultant acute PIR, especially in earlier preterm gestations (434).

Alternatively, as MIAC has been shown to be an independent risk factor for PPROM with placental infection and inflammation established before labour initiation (142, 434), the increased number of women in the preterm cohort experiencing PPROM may be accounted for by an entrenched infection. Comparatively, membrane rupture duration was 15.96 (± 43.25) hours in term births, a period potentially less likely to culminate in MIAC and resultant acute PIR. However, previous studies have suggested that MIAC is present in up to 75% of women with spontaneous labour and that this occurred during latency (68, 254). As the total cohort in this study was selected from women who spontaneously laboured, it is suggested that a significant proportion of the acute PIR observed at term and preterm gestations is an observable response to MIAC during labour. Nonetheless, as data on the time between observable histologic PIR and MIAC origination is scant (435), it is challenging to determine whether infection went unnoticed in the cases of negative histopathological evaluations or if inflammatory lesions were reported which were non-infectious in origin and simply a function of physiological labour. This warrants further investigation to compare the prevalence of acute PIR in placentas at term from elective Caesarean sections and those where labour was spontaneous to determine the contribution of non-infectious inflammatory labour processes. Additional study of the effects of duration of membrane rupture may also elucidate the mechanisms underlying histopathological variation in preterm and term placentas.

4.4.2 Acute chorioamnionitis is a common finding of placental histopathological evaluation

Acute chorioamnionitis was the most prevalent histologic inflammatory lesion irrespective of gestational age at delivery. Of the total study population, 27.6% of placentas met the criteria for diagnosis of HCA, a finding comparable with research conducted by Queiros da Mota *et al.,* (2019) (414) and Bhola *et al.,* (2008) (415) in which HCA prevalence was reported at 26.9% and 26.0%, respectively. However, in contrast to these studies where participants were only included if there was confirmed or suspected maternal or neonatal infection (414) or from high-risk deliveries (415), the present study included all livebirths from spontaneous deliveries where mothers were over the age of 16 years, regardless of known maternal, fetal or neonatal infection. As term placentas from low-risk pregnancies are rarely histopathologically evaluated, the reported frequency of HCA at term is ambiguous (328). Data collected as part of this chapter show an almost equal distribution of HCA in preterm and term placentas with 14 cases delivered preterm and 13 cases at term, a relationship found not to be significant (P=0.6557). Overall, 29.8% of preterm placentas were reported to have HCA compared to 25.5% of those delivered at term, results in discord with those presented by Kim and colleagues in their expert review which revealed a HCA prevalence of 54% in preterm placentas compared to 3.9% at term (142). Similarly, the term HCA incidence has been reported by others to be low at only 2-5% (142, 416), but, in some cases with no apparent abnormality, HCA at term has been shown to be a feature in 20% of deliveries (408). As with acute PIR overall, a potential explanation for this observation is that HCA is a histopathologically-observable maternal response to non-inflammatory processes at term with a discernible absence of microorganisms in the placenta (142, 420).This suggestion of sterile-inflammation at term is supported by published data that show an overexpression of neutrophil-specific chemokines, such as CXC motif ligand 1 (CXCL1), CXCL2 and interleukin-8 (IL-8), in the amniotic membranes of women who have spontaneously laboured (142) and a finding of 96% of all term HCA cases occurring without infection (408). Alternatively, at both term and preterm gestations, the risk of maternal pyrexia and HCA have been shown to increase in women receiving epidural analgesia during labour (420, 436) and in extended durations of labour (142), neither of which were findings replicated in participants in this study. As noted previously, preterm placentas had a lower-than-expected prevalence of HCA (29.8%). Categorising preterm placentas to EPT, VPT and MPT also did not reveal a significant relationship between gestational age group and increased prevalence of HCA in this cohort

(*P*=0.0889), although the likelihood trended towards an increase at earlier gestations. Placentas from VPT births were the most likely to have HCA (62.5%), in contrast to published data which found the prevalence at gestations of 28 – 32 weeks to be 35.4% (142), followed by those from EPT births (36.4%). This is in line with Kim and colleagues who found the prevalence of HCA in this gestational age group to be 39.6% (142). Only five placentas delivered in the MPT group had HCA (17.6%), but this prevalence was still higher than published evidence (10.7%) (142). These findings not only highlight the heterogenous nature of HCA, its risk factors and presentation (408), but may also emphasise the variability in pathologists' reporting of HCA which is based primarily on the abundance and confluence of neutrophils in placental compartments and is, therefore, a potentially subjective finding (350).

4.4.3 Advanced maternal and fetal inflammatory responses are associated with extreme prematurity

Necrotising chorioamnionitis and acute funisitis represent more severe and advanced maternal and fetal inflammatory responses, respectively. Despite more cases of both lesions in preterm placentas, neither presented increased odds of preterm delivery when assessed in the total preterm cohort. Similarly, histopathological evaluation of term placentas in this study showed an increased incidence of acute chorioamnionitis, acute arteritis and acute chorionic vasculitis when compared to preterm placentas but no reduction in the odds of a preterm birth. On the other hand, acute subchorionitis prevailed in term placentas and was 5.5-times more likely to result in a term birth than a preterm birth. Isolated acute subchorionitis is not generally associated with adverse neonatal outcome (437), instead, it represents an early infection likely to have developed within the preceding 12 hours (153, 438). Whilst it may appear reasonable to disregard increased odds of acute subchorionitis at term, clinically significant early HCA may be missed when neutrophilic infiltration is limited to the trophoblastic layer of the chorion laeve or the subchorionic fibrin (439). Conversely, the increased prevalence of acute subchorionitis in the term cohort may be representative of a physiological response to labour in the way described above, or an early consequence of insults to the chorioamniotic membranes, most likely due to spontaneous rupture of membranes (SROM) (408). Of the women whose clinical notes recorded whether membrane rupture was spontaneous or not, 71.4% of those with acute subchorionitis had experienced

SROM at term or PPROM. It is therefore most likely that the reported acute subchorionitis in these placentas originated from SROM and a maternal response to this membrane insult.

Categorising preterm placentas revealed a contradictory picture to that of comparing all preterm with term births. Acute funisitis, a fetal inflammatory response involving the umbilical vein and one or both arteries in conjunction with neutrophils traversing through to the Wharton's jelly, was reported in almost half of placentas delivered EPT (45.5%; 5 cases). In contrast, it was a feature in only one (12.5%) VPT case, no MPT cases and three at term (5.9%). Acute funisitis prevalence is inversely correlated to gestational age (440), as demonstrated in the placentas analysed as part of this study. Furthermore, the clinicopathological significance of acute funisitis in term placentas differs from that in preterm placentas (441). There was a negative relationship with gestational age and necrotising chorioamnionitis, but only across the preterm cohort, and a predominance for necrotising chorioamnionitis at EPT gestations (36.4%; 4 cases). The adverse effects on neonates where acute funisitis and necrotising chorioamnionitis are present have been well-documented, and the increased risks of neurological disease (409), respiratory distress syndrome (413), necrotising enterocolitis (411) and neonatal sepsis or death (410) are widely recognised. As the presence of these lesions can only be established after delivery and subsequent histopathological evaluation, additional research to establish and document the preterm phenotype and provide targeted investigations or interventions should be considered to reduce the burden of neonatal morbidity and mortality.

4.4.4 High stages and grades of placental inflammatory responses predominate in preterm gestations

Staging and grading placental inflammatory responses allows more than one lesion type to be categorised to early, intermediate or advanced, and mild-moderate or severe responses (Table 4.1) (72), as well as providing a semi-quantitative evaluation of placental inflammatory lesions. Since many placentas will be reported as having a collection of lesions, staging and grading permits a more detailed assessment of placental status (103). However, unlike reporting of specific MIR or FIR lesions there is potential for a more subjective approach based on perinatal pathologists' expertise, for example, HCA is generally reported as stage 2, but, when considered in relation to collective placental inflammatory lesions and neutrophilic

confluence, it may be reported as stage 1 or 3 and as grade 1 or 2 (103). Additionally, a stage and/or grade may be assigned to a placental inflammatory response without a lesion type being specified, as was the case in three term placentas included in this study (samples SU0024, SU0042 and SU0054). Each of these samples were reported as MIR stage 1/grade 1 with a note made in the placental pathology report that early HCA had been observed but no recognised HCA was ascribed, and that the MIR was most likely an artefact of labour. This variance in staging and grading compared to classification of inflammatory lesions also accounts for the discrepancy in numbers of reported acute PIR lesions (26 term cases) versus reported MIR staged placentas (29 term cases); no such issue was reported in preterm placentas.

There is an abundance of research emphasising the detrimental effects of inflammation on the neonate, predominantly negatively affecting the neurodevelopment, and the cumulative adverse effects seen at earlier gestations or in very low birthweight infants (442, 443). There is a relationship between poor neonatal outcome and both MIR and FIR (103, 153, 444), but the association is most frequently described with FIR (72). Previously published data and reviews support the findings of this study in that not only was the frequency of reported MIR in the total cohort greater than FIR (53.1% vs. 30.6%), but MIR stage 1 (40.4%) and grade 1 (69.2%) predominated in contrast to FIR where stage 3 and grade 2 were most prevalent (40.0% and 53.3%, respectively). Whether MIR is present or absent was significantly associated with predefined gestational age groups (P=0.0059) with MIR observed to dominate in EPT placentas (81.8%). As gestation progressed, the frequency of MIR decreased to 75.0% in VPT and 28.6% MPT placentas, yet, at term, rose to over half (56.9%) of placentas having a reported MIR. Crucially, however, the stage, and therefore advancement of the MIR, was significantly lower at term (stage 1: 58.6%) than all preterm placentas with MIR combined (stage 1: 17.4%; P=0.0070) and when assessed across EPT, VPT and MPT groups (stage 1: 11.2%, 33.3% and 12.5%, respectively; P=0.0220). The inverse correlation between advanced inflammatory responses and earlier gestations was more evident in FIR prevalence, or, more accurately, combined MIR and FIR since no placentas were reported to have MIR without a corresponding FIR. Most of these placentas had been delivered preterm (82.6%), supporting the suggestion that the risk of adverse obstetric outcome increases the earlier a fetal immune response is deployed (254). The frequency of FIR decreased in conjunction with increasing

gestation; extremely and very preterm placentas were more likely than not to have a reported FIR (63.6% and 62.5%, respectively) in contrast to placentas from moderate to late and term births where FIR was less apparent (25.0% and 21.6%, respectively).

4.4.5 Gestational age is an independent predictor of MIR and FIR stage and FIR grade

The results presented in this chapter reveal gestational age to be an independent predictor of MIR stage, FIR stage and FIR grade, but not MIR grade. Data from this study show the predicted MIR stage of 1.44 at a preterm gestation of 23.9 weeks to be 1.97-times higher than the predicted 0.73 at a term gestation of 39.9 weeks. Predictions of FIR stage and grade showed an increase of 5.42- and 3.54-times, respectively, at the same gestations, decreasing from a FIR stage of 1.68 to 0.31 and grade of 0.99 to 0.28 as gestation progressed (data presented in Appendix IV, Figure AIV.III). Before 20-21 weeks' gestation, the fetus does not have the capacity to respond to bacterial colonisation, but once in the mid-trimester the fetus mounts an immune response identifiable on histopathological evaluation as the FIR (72, 142, 161). It has been hypothesised that in the presence of HCA and/or acute funisitis, the response from preterm fetuses is more intense than from term fetuses and that this results in upregulation of inflammatory genes and subsequent initiation of preterm labour (441, 445). When including term births, there was no significant association between stage or grade of FIR and gestational age category (P=0.2024 and P=0.1612, respectively), however, the results of this study demonstrate stage 3, the most advanced FIR, to be significantly more prevalent in EPT placentas (P=0.0471, data not shown). These data support the suggestion that preterm fetuses respond to bacterial colonisation and inflammatory insults in a more robust manner than term fetuses and that this is connected to weaker immune competence at earlier stages of gestation (407). Alternative hypotheses have suggested that it is the duration of the insult and extension beyond the chorio-decidua which leads to an increase in the FIR (153, 446), yet no relationship was revealed between the presence of MIR or FIR, or their stage and grade, and the duration of labour or duration of rupture of membranes, factors shown to increase the chance of MIAC and PIR (442). Therefore, these data suggest that the gestational age of the fetus determines and drives the FIR in earlier gestations, and that inflammatory stimuli at term are more likely to be maternal in origin (407).

Clinically, it is of critical value to identify neonates at risk of inflammation-related injury and the associated respiratory (413), neurodevelopmental (442) and gastrointestinal (410, 411) sequelae previously discussed. In the absence of clearly defined non-invasive antenatal tests for maternal and fetal inflammatory responses, prediction of the advancement and severity of both MIR and FIR may provide opportunity for more appropriate and targeted treatment or management of women presenting with PPROM or PTL. Large cohort studies are required to confirm gestational age as an independent predictor of MIR and FIR stages and grades and to develop histopathologic standards for assessment and routine clinical practise.

4.4.6 Histologic characterisation of preterm birth placental phenotypes

In addition to the documented effects of inflammation on timing of delivery, placentation and placental perfusion disorders are purported to contribute to preterm labour (431); the results of this chapter support this hypothesis. Established fetal risks of malperfusion in either the maternal or fetal circulation include IUGR (447), neuroimpairment (448) and fetal death (449). Additionally, placental malperfusion has been linked to recurrent pregnancy loss (448) and may be predictive of hypertension and cardiovascular disease in both the neonate's and mother's later lives (448, 450-452). Consequently, this chapter sought to establish whether preterm placentas could be classified according to histopathological findings and to determine if at-risk fetuses could be identified based on gestational age. Although identification of vascular malperfusion in preterm placentas is not a novel phenomenon and has been reported by others over the last three decades (403, 453), previous studies have generally discussed inflammatory and vascular malperfusion disorders as two distinct subtypes of preterm birth pathophysiology (3, 403, 430). The results presented in this chapter reveal distinct differences in PTB and confirm that it is possible to classify PTB to discrete histopathological groups, each of which has defining clinical characteristics. Although less prevalent than inflammatory lesions, vascular malperfusion was a feature in over one third (33.7%) of placentas in the total study population and predominated in preterm births (63.8% vs. 5.9% at term). Calculated odds ratios showed PTB to be over 15-times more probable where maternal vascular malperfusion lesions were present but more than 16-times more likely if histopathological evaluation revealed both maternal and fetal vasculopathy. Assessing vascular malperfusion lesions across the four gestational age study groups, maternal and combined maternal and fetal vascular malperfusion were significantly more prevalent in the

MPT group (60.0% and 50.0%, respectively), an interesting finding since inflammatory lesions had been observed least frequently in this category.

As discussed in detail in this thesis, inflammation is a pervasive feature in preterm placentas, and, as in the case of results presented in this chapter, a common finding in term placentas. It was, therefore, not unexpected when histopathologically-classifying placentas to discover most met the criteria for acute placental inflammatory responses (A/PIR) as the only finding (35.7%). Term births dominated this histopathological group (68.6%), a finding which was expected following analysis of inflammatory lesions from the total study population and presented in this chapter. Only thirty placentas were reported as having no pathology (NP), predictably, the majority of these were delivered at term (80.0%). On the other hand, further categorising histopathological findings to take into account vasculopathy revealed 75.0% of the twenty-four preterm placentas with no inflammation to be affected by pathological lesions. Studies determining the proportion of preterm placentas with no pathology report values of approximately 20.0% (403, 405) which is higher than the prevalence shown in this study cohort (12.8%). When odds ratios were calculated these data revealed the probability of preterm placentas having vascular malperfusion lesions to be 31-times more likely than term placentas when in isolation (VM) and over eight times more frequent when combined with acute PIR (PIR/VM), further highlighting the significance of vasculopathy in PTB. In data not shown, vascular malperfusion disorders were frequently reported in the cases of IUGR (71.2%) and fetal distress (50.0%), all of which were from PTBs. Preterm placentas were unlikely to be reported as having no pathological lesions or acute PIR lesions only, demonstrated by the increased likelihood of classification to these groups at term (6.1- and 2.9-times greater odds, respectively).

Categorisation of preterm placentas to WHO defined groups uncovered further variance in the distribution of histopathological lesions and allowed for discrete classification of gestational age groups to the predefined histopathological groups. The presence of pathological lesions increased significantly as gestational age decreased with 100% of EPT placentas being reported as having inflammatory and/or vascular malperfusion lesions. VPT and MPT placentas had similar rates of pathology (87.5% and 82.1%) before a sharp decline was noted at term (52.9%). EPT placentas, those delivered before 28 weeks' gestation, most

frequently had combined inflammatory and vascular malperfusion lesions, to date, an unrecognised distinct preterm phenotype. VPT placentas were equally as likely to be assigned to the PIR/VM or A/PIR groups, however, in this gestational age group these findings did not reach significance, likely due to the small sample size (*n*=8). The greatest proportion of placentas with vascular malperfusion lesions in isolation were delivered in the MPT group (15/19; 78.9%) with these being the reported pathology in over half of all placentas delivered between ≥32 and <37 weeks' gestation (53.6%). Term placentas were equally as likely to have acute inflammatory lesions or no pathology (47.1% per histopathological group) and contributed minimally to the reported vasculopathies (3/33; 9.1%). These data support the earlier discussions which suggest that divergent inflammatory processes are seen in preterm and term placentas. The observations of increasingly frequent acute inflammatory responses at term, coupled with the decreased stage and grade of these lesions, corroborates others' findings that inflammatory processes at term are non-infectious and primarily a consequence of labour (142, 420). Notably, there was an increased prevalence of vascular malperfusion in preterm placentas, a concerning finding given the increased risks of adverse neonatal and maternal outcomes in the presence of these lesions (447-450, 453). The mechanisms underlying vascular malperfusion are not fully understood, however, as per the placental origins of preeclampsia hypothesis, it has been suggested that uteroplacental hypoxia results from inadequate spiral artery remodelling and poor maternal-fetal nutrition in early pregnancy (454, 455). Furthermore, vascular occlusion or high-velocity blood flow through insufficiently remodelled spiral arteries may lead to oxidative damage and inflammation (103, 455, 456), yet it is unclear whether the interactive and multiplicative effect of combined inflammatory and vascular malperfusion lesions results from early placental dysfunction and oxidative damage which cannot be remedied in early gestation, or through later exposure of an insufficient placenta to infectious pathogens. Irrespective of the cause, the results of this study suggest that the effect is an exaggerated fetal response which progresses in the manner discussed previously (441, 445), particularly in extremely and very preterm fetuses.

4.4.7 Prediction of placental histopathological group in preterm birth

As for stage and grade of PIR, an important goal was to determine the possibility of predicting placental histopathological lesions based on gestational age at delivery. Simple linear regression models showed gestational age was not an independent predictor of acute inflammatory lesions alone (*P*=0.0611), but it was possible to predict the probability of histopathological classification to the vascular malperfusion, combined inflammation and vasculopathy and no pathology groups based on gestational age. The importance of identifying fetuses at risk of hypoxic-ischemic and/or inflammation-related injury cannot be overstated, nor can the need for a robust prenatal standard of assessment. Results from this study show predicted probabilities of combined inflammatory and maternal vascular malperfusion lesions (PIR/VM) to decrease 12-fold from 57.8% at 24 weeks' gestation to 4.8% at term (40 weeks' gestational age); the probability of vascular malperfusion lesions alone (VM) decreased 3.3-fold from 42.5% to 13.0% in the same gestational period. Prediction of no pathological lesions was also possible, but inverse to vascular malperfusion and combined lesions and, whilst the probability of no pathology was minimal (5.7%) at 24 weeks' gestation, this increased by 5.7-fold to 42.6% at 40 weeks (Figure AIV.II). The capability to predict discrete histopathological findings by gestational age is a crucial step towards early identification of at-risk fetuses and mothers and advancements in monitoring and intervention. The results of this study highlight the need to consider strategies beyond expectant management of PTL, a practice which may actually increase the prevalence of vascular malperfusion and inflammation, potentially intensifying adverse short- and long-term health outcomes of the neonate and mother, as described in a recent study conducted by Feenstra *et al.* (457). Furthermore, provision of a predictive assessment tool such as the data presented in this chapter could lead to a targeted approach of second and third trimester ultrasound to assess placental size and mass and identify placental lesions (458), or uterine artery Dopplers to evaluate flow impedance in uterine arteries (459), both of which may be additional viable strategies to identify at-risk pregnancies prior to PTB occurring.

4.4.8 Conclusions

Understanding the clinical significance of histologic lesions and their contribution to adverse outcomes in pregnancy and long-term health is a fundamental rationale for histopathological placental evaluation (103, 328). At present, the consensus is that PTB is a syndrome rather than a discrete pathophysiological event and that an association exists between pregnancy complications and the onset of preterm labour (11, 460). Suggestions as to the physiological mechanisms leading to preterm labour and the contribution of the placenta have been made previously (11, 460). Nevertheless, unique to this study are the categorisation of preterm placentas to predefined groups which elucidated differential phenotypes correlated to gestational age and the presentation of a method to antenatally predict stage and grade of placental inflammation and placental pathology. Having the capacity to determine the probability of histopathological findings and predict the severity of placental inflammatory responses is crucial in identifying susceptible fetuses, providing surveillance in high-risk pregnancies and mitigating the effects of placentally-derived adverse outcomes.

Chapter 5: Immunohistochemical Staining for Targeted Viruses Associated with Placental Inflammation and Preterm Birth

5.1 Introduction

A variety of viral pathogens have been linked to PTB, many of which are common in the general population, including cytomegalovirus (CMV), herpes simplex viruses 1 and 2 (HSV-1/2) and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (174-177). Having the potential to infect the placenta causing dysfunction and resultant spontaneous PTB means these viruses are key targets for investigation (35, 177, 178, 180). Previous chapters have described the inflammatory and non-inflammatory characteristics associated with preterm birth, herein, this chapter details investigations into CMV, HSV-1/2 and SARS-CoV-2 and their contribution to placental inflammation and PTB. Initially, it was intended to examine and quantify CMV and HSV-1/2 by polymerase chain reaction (PCR), a technique previously shown to be reliable, specific and sensitive for both viruses (461-463). However, introduction of Covid-19 pandemic laboratory restrictions and reduced availability of reagents from March 2020 led to modifications to the evaluation of CMV and HSV-1/2 in placental tissue. Immunohistochemistry was selected as a method to detect the presence of CMV and HSV-1/2 due to enhanced safety features of working on formalin-fixed paraffin-embedded (FFPE) tissue. Not only did this prevent transmission of the target viruses, in this study it also conferred protection from SARS-CoV-2 in any samples and allowed for the project to continue. IHC was preferred over H&E only as, although CMV often presents with abnormally large cells and characteristic ‘owl’s eye’ intranuclear inclusions surrounded by a clear halo, and HSV-1/2 may be characterised by Cowdry Type B bodies which present as nuclear inclusions, margination of chromatin and ‘ground-glass’ areas in nuclei (187), both are often patchy infections with minimal evidence of these cytopathic changes (463). Additional benefits of IHC over PCR analysis were the ability to localise CMV and HSV-1/2 if their presence was detected and report these with observed histopathological characteristics. A combination of two monoclonal antibodies was used to detect CMV, specifically clones DDG9 and CCH2 which bind to CMV major immediate entry protein-72 (p72) and delayed early DNA binding protein-52 (p52), respectively. As a marker of acute infection, p72 is able to detect CMV in samples 16 - 24 hours after infection, whereas p52 is a marker expressed later in infection and is associated with the nuclear membrane (464). Using the combination of these antibodies ensured active infections were identified irrespective of whether they occurred in early or late pregnancy. An unconjugated mouse monoclonal antibody was selected to active HSV-1/2 with an immunogen directed against all major glycoproteins in the viral envelope

and at least one core protein required for viral entry (465, 466). PCR on the other hand would amplify all CMV and HSV-1/2 DNA irrespective of whether the virus was active or latent. Further, PCR requires the placental tissue to be homogenised prior to DNA extraction procedures, it is therefore possible that contamination with CMV or HSV-1/2 DNA following delivery or from the intrauterine environment would have been amplified providing false positive results. Consequently, it was decided to perform IHC analysis on a subset of samples to detect the presence of CMV and HSV-1/2.

5.1.1 *Herpesviridae* family

The *Herpesviridae* family, commonly known as herpesviruses, consists of large, double stranded (ds) DNA genome enveloped viruses classified into 3 subfamilies (*alpha-, beta-,* and *gammaherpesvirinae*), 13 genera and 107 species, nine of which infect humans (467-469), as outlined in Table 5.1. Human herpesviruses (HHV) share hallmark characteristics; they are ubiquitous, rarely cause overt disease in immunocompetent individuals and are one of the few known viruses to exhibit true latency, that is displaying both persistence and reversibility through tethering of the viral genome in a circular episome to the host cell chromosome (470, 471). Classification of herpesviruses is based on the cells in which the virus convalesces; specifically, alphaherpesviruses establish latency in neurons and gammaherpesviruses within lymphocytes. Conversely, betaherpesviruses demonstrate broad cell tropism, including macrophages and B cells of the immune system (469, 470).

Table 5. 1 Classification of Human Herpesviridae Family

Species	Genus	Acronym	Common Name	Common Acronym
Subfamily: *Alphaherpesvirinae*				
Human α–herpesvirus 1	Simplexvirus	HHV-1	Herpes Simplex Virus-1	HSV-1
Human α–herpesvirus 2	Simplexvirus	HHV-2	Herpes Simplex Virus-2	HSV-2
Human α–herpesvirus 3	Varicellovirus	HHV-3	Varicella Zoster virus	VZV
Subfamily: *Betaherpesvirinae*				
Human β–herpesvirus 5	Cytomegalovirus	HHV-5	Human Cytomegalovirus	HCMV
Human β–herpesvirus 6A	Roseolovirus	HHV-6A	Roseolovirus	
Human β–herpesvirus 6B	Roseolovirus	HHV-6B	Roseolovirus	
Human β–herpesvirus 7	Roseolovirus	HHV-7	Roseolovirus	
Subfamily: *Gammaherpesvirinae*				
Human γ–herpesvirus 4	Lymphocryptovirus	HHV-4	Epstein-Barr Virus	EBV
Human γ–herpesvirus 8	Rhadinovirus	HHV-8	Kaposi Sarcoma Virus	KSHV

Classification of human herpesviruses and common names adapted from (467, 468).

The large genome size of members of the family *Herpesviridae* permits immune evasion and molecular mimicry as well as supporting latency (472). The evolutionary conserved glycoproteins found on the surface of herpesviruses allow entry via binding to multiple receptors on the host cell surface. Tegument proteins support the viruses' survival once in the host cell through formation of tegument complexes required for modulating focal adhesion complex activity and maintenance of attachment of infected cells to their surroundings (473). A prototypical structure of a herpesvirus is shown in Figure 5.1.

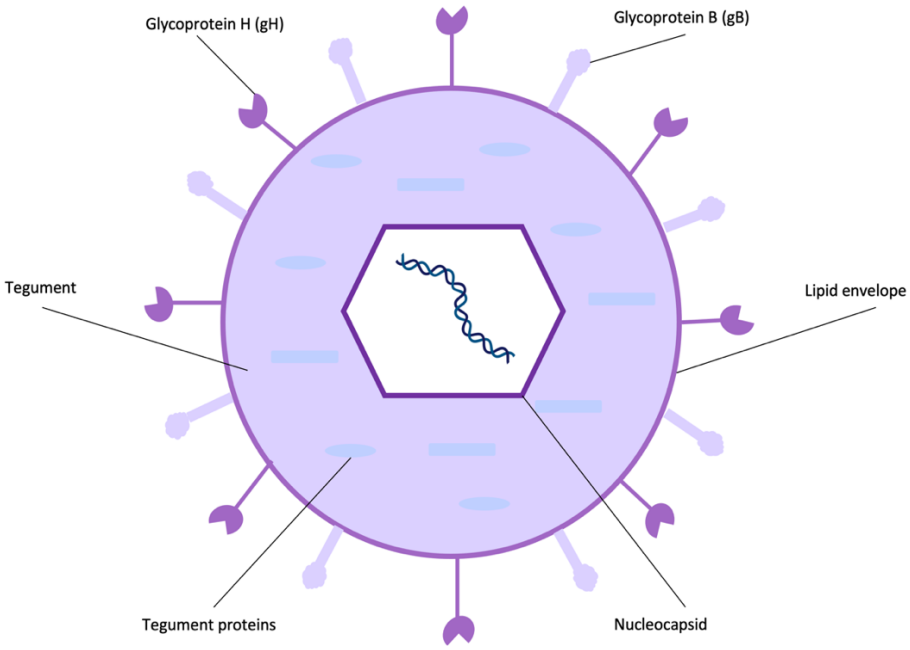

Figure 5. 1 Prototypical structure of herpesviruses.

In this example of a cytomegalovirus virion, glycoprotein B (gB) and glycoprotein H (gH) are two of the multiple glycoproteins found on the lipid bilayer; both are essential for viral entry into host cells. The tegument surrounds the nucleocapsid and contains proteins essential for the virus' survival in the host cell (472).

5.1.2 Cytomegalovirus

Before considering the role of CMV in adverse pregnancy outcomes and, specifically, preterm birth, it is necessary to discuss the biology and pathogenesis of the virus. Human cytomegalovirus, the largest of the herpesvirus family and one of the nine herpesviruses with the capability of infecting humans, is a 200-230nm diameter enveloped viral particle containing a linear double-stranded DNA (dsDNA) genome of ~235 kilobase pairs (kbp). Initial estimates of the number of protein coding open reading frames (ORF) originally ranged from 164 to 220, however, advances in RNA-sequencing technologies have identified 751 translationally active ORFs (474), four long non-coding RNAs (lncRNA) (475) and 14 CMV-encoded micro RNAs (miRNA) with biological relevance and the ability to alter cellular protein expression in host cells (476). As for other *betaherpesvirinae*, CMV displays broad cell tropism, including endothelial, epithelial and smooth muscle cells, macrophages and fibroblasts (472, 477, 478); it is this wide-ranging infective capability that influences the pathogenesis of acute CMV infection (479).

Globally, seroprevalence rates of CMV range from 56% to 94% and are inversely proportional to the socioeconomic status of an individual's country of residence (478, 480). Screening of newborns in developed countries such as the US and UK found CMV infection in 0.5-1.0% of live births compared to rates of 0.6-6.1% in low- to middle-income countries (LMICs), unsurprising considering the prevalence of CMV in LMICs is almost 100% by early childhood (481). Rates of infected individuals increase with age in developed countries with the risk of infection greatest in those who are in regular contact with children under 3 years of age such as mothers with young children, day-care providers and teachers. Studies have shown infection rates 5-10-fold higher in those who work in day-care settings compared to the general population, most likely due to CMV's asymptomatic mode of infection and infants shedding CMV in body fluids such as blood, saliva, tears and urine for months or years post-infection (478, 481, 482). CMV also efficiently spreads through sexual activity (478, 483). Primary or reactivated maternal CMV infection leading to congenital CMV infection through placental transmission or ascent via the vagina is vastly underappreciated despite being the leading cause of prenatal viral infection. Fetuses exposed *in utero* to CMV are at risk of serious neurodevelopmental sequelae, sensorineural hearing loss, altered language development and retinopathy which is seen in 15% of neonates (481, 484, 485). Following maternal primary infection, the rate of transmission to the fetus is estimated to be 35%, as noted in a review by Pass and Boger published in 2018 (481). This article highlighted the challenges of estimating the transmission rate since gestational age at infection affects the rate of transplacental transmission, as does prior maternal immunity (481). However, the reported rates were in accordance with the 30 - 50% maternal-fetal transmission rates previously discussed by Adler and Nigro (2013) (486). Conversely, other studies have shown increased frequency of maternal-fetal transmission (487). Enders *et al.* (2011) determined the risk of maternal-fetal transmission in a cohort of 248 pregnant women and showed that rates of transmission in the first trimester were lowest at 30.1% (25/83) before increasing slightly in the second trimester to 38.2% (29/76). The transmission rate of CMV in the third trimester was found to be 72.2% (26/36).

Whether CMV is associated with spontaneous preterm birth (sPTB) is disputed. A recent study of 86 women in western Kenya who experienced sPTB and 86 matched controls reported cervical CMV shedding may increase the risk of sPTB through elevated levels of the

inflammatory cytokine interleukin-6 (IL-6) (488), a factor previously linked to non-CMV-associated sPTB (489). However, upon further analysis, the same study found no association between cervical CMV shedding or CMV DNA levels and sPTB directly. Furthermore, cervical CMV reactivation was observed in over one-quarter (*n*=45; 28.2%) of women in the study and did not significantly differ between cases and controls (*P*=0.5000) (488). An earlier study supporting a relationship between CMV and preterm birth demonstrated a prevalence ratio of 2.7 (95% CI: 1.4-5.1) for preterm births on examination of 3972 newborn dried blood spots collected as part of a routine screening programme and tested for CMV DNA (490). Although the study included a large population, only seven preterm infants were positive for CMV DNA and all but one had a clinical or demographic characteristic associated with sPTB which had not been controlled for in the study (490). Pitlick *et al.,* (2015) suggested CMV infection as a risk factor for sPTB in a study conducted in Iowa, USA, yet revealed a prevalence of congenital CMV in a large cohort of preterm infants lower than that found in the general Iowa population (0.3%) (491). Conversely, several studies do not consider CMV as a risk factor for sPTB. Screening programmes, early diagnosis, prevention of maternal-fetal transmission or mitigating adverse obstetric outcomes related to placental dysfunction are instead the foci of recent and current research (485, 491-493). Whilst there may be limited data on the relationship between sPTB and CMV, understanding the mechanisms by which CMV leads to abnormalities in placentation and trophoblast invasion and migration and altered maternal cytokine profiles, work which is currently underway (494), will undoubtedly be of benefit in reducing the number of iatrogenic PTBs. This study, therefore, sought to determine whether placental CMV infection was higher in preterm placentas compared to term placentas.

5.1.3 Herpes simplex virus

Herpes simplex virus-1 and Herpes simplex virus-2 (HSV-1 and HSV-2, respectively) are structurally similar to other herpesviruses with a proteinaceous tegument surrounding an icosahedral capsule enveloped in a membrane containing glycoproteins (495). Smaller than CMV, HSV-1 and HSV-2 are approximately 160nm in diameter and contain a linear dsDNA genome of ~157 kbp (496, 497). Homologous sequences account for approximately 50% of the HSV-1 and HSV-2 genomes, and both code for around ninety transcription units, over 90% of which encode proteins (498). HSV infection follows the interaction of ubiquitous glycoproteins, primarily glycoprotein D (gD), glycoprotein H (gH), glycoprotein L (gL) and

glycoprotein B (gB). Initially, gD on the virion's surface binds to the host cell surface which activates a gH-gL heterodimer, initiates a conformational change in gB and permits the viral capsid to enter the cell (495, 499) as shown in Figure 5.2. After the initial infection, HSV establishes latency in neurons (495). It is expected that every individual will be infected by at least one herpesvirus in their lifetime. Recent estimates of prevalence rates of HSV-1 and HSV-2 amongst 15 - 49-year-olds demonstrated widespread infections and rates of 70.3% oral HSV-1, 5.1% genital HSV-1 and 13.2% HSV-2 (180, 469). In the same age group, infection with genital HSV-1 or HSV-2, or both together, was shown to affect 16.0 - 17.6% of the global population. Prevalence of HSV-1 and HSV-2 increased with age, as did the number of new infections. HSV-2 infections in females were over 1.75-times greater than those in men and greatest in the WHO African Region, whereas men were marginally more likely to be infected with genital HSV-1 (5.3% versus 5.1%). Prevalence for both sexes was highest in the WHO Region of the Americas and the WHO Europe Region (500). A meta-analysis investigating the influence of Herpes simplex viruses type 1 and type 2 on pregnancy outcome found maternal HSV-1 and HSV-2 are associated with adverse pregnancy outcomes, including preterm birth (pooled odds ratio (OR) 3.83 (95% CI:1.17-12.54; I^2=84.7%), low birth weight (pooled OR of 3.84 (95% CI: 0.45–32.90; I^2 = 81.8%) and stillbirth (OR of 1.78 (95% CI: 1.08–2.95; I^2 = 10.2%) (180). HSV-2 has been linked to cervical collagen remodelling and sPTB (501), and an increased risk of sPTB where infections are acquired in the first two trimesters and subsequently remain untreated (502). The biological mechanism of viral infection, including of herpesviruses, is poorly defined and understanding commonly comes only from animal models. One theory of this complex process is that viral-associated PTB is in fact a result of a polymicrobial infection whereby the placentitis occurs from the initial viral infection which leads to type I interferon downregulation and hyperresponsiveness to bacterial endotoxins of a secondary infection (503, 504).

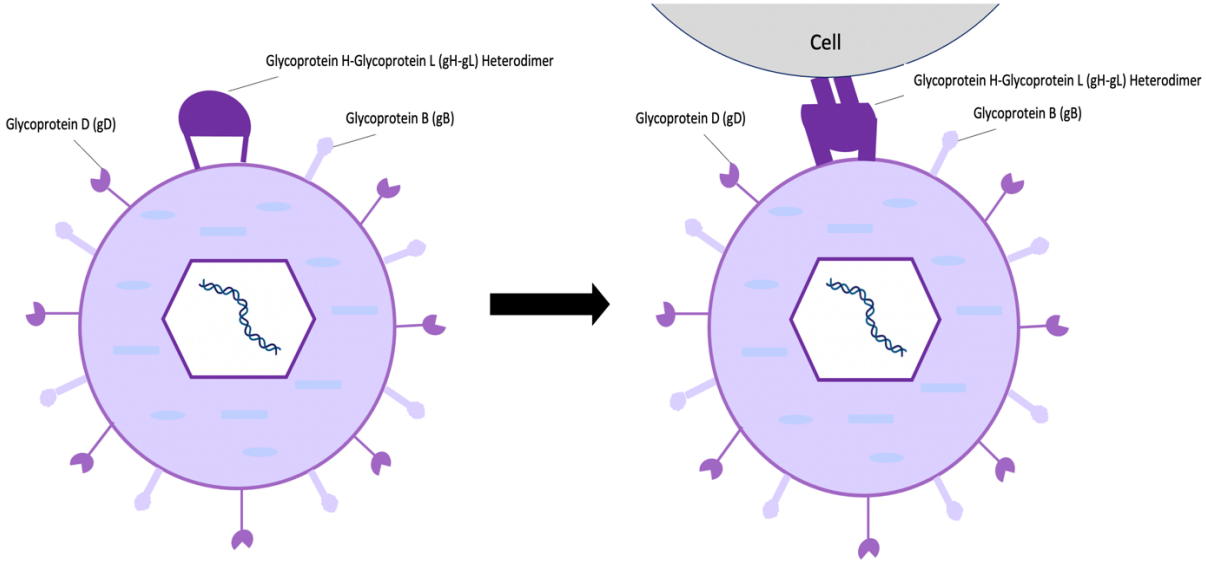

Figure 5. 2 Herpesvirus-1 and herpesvirus-2 entry mechanism.

Initial fusion with the host cell occurs between glycoprotein D (gD) and the host cell plasma or endosomal membrane. gD binding to host cell membrane receptors signals to the glycoprotein H-glycoprotein-L (gH-gL) heterodimer to initiate a transformational change in glycoprotein B (gB). This in turn triggers gB to introduce hydrophobic fusion loops into the membrane and permit HSV to enter the cell (499).

In summary, it is unclear exactly how HSV-1, HSV-2 or CMV affect gestation, despite studies showing an active maternal infection (mRNA-positive) increases the incidence of maternal-fetal transmission and adverse pregnancy outcomes when compared to latent infections (180, 505). However, whether HSV infections during pregnancy increase the risk of spontaneous preterm birth directly remains unclear.

5.1.4 Severe Acute Respiratory Syndrome Coronavirus-2

In December 2019 a novel zoonotic disease, coronavirus disease 2019 (Covid-19), emerged in Wuhan, Hubei Province, China, following reports of a cluster of severe pneumonia of unknown aetiology cases. Next-generation meta-transcriptomic sequencing revealed a complete viral genome of a human coronavirus belonging to the *Coronaviridae* family. Its close relationship to the genome of the virus responsible for the 2002-2003 severe acute respiratory syndrome-coronavirus (SARS-CoV) and the 2012 Middle East respiratory syndrome-coronavirus (MERS-CoV) outbreaks saw it named 'severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (506-509). On 11 March 2020 the WHO declared the Covid-19 outbreak a global pandemic after increasing numbers of cases were reported outside of China (506). Despite mass testing and vaccination programmes being rolled out,

Covid-19 remains a formidable global health challenge (510, 511). Unlike other viral respiratory infections, particularly those caused by viruses of the *betacoronavirus* genus, SARS-CoV-2 affects a plethora of organs in addition to the lungs and increases the risk of multi-organ failure in Covid-19 patients (507, 509, 510). Pregnant women are a vulnerable population for Covid-19 and an increase in obstetric complications, including PTB, PPROM, stillbirth, IUGR and maternal death, has been reported following confirmed or suspected maternal SARS-CoV-2 infection (507, 512-514). Importantly, however, illness is generally mild for most pregnant women infected with SARS-CoV-2 and does not compromise the health of the neonate (515, 516)**.**

Histopathological and morphological assessment revealed distinct changes and features in the placentas of women infected with SARS-CoV-2, commonly MVM and FVM, intervillositis and massive perivillous fibrin deposition (189, 510, 517-519). Expression analysis of the SARS-CoV-2 entry factors, angiotensin converting enzyme-2 (ACE2) and transmembrane protease serine 2 (TMPRSS2), showed a negligible to low level of co-expression in trophoblasts at term (516, 520) and a significantly lower expression level of *ACE2* mRNA in the placentas of women with mild or asymptomatic Covid-19 when compared to those with severe disease (520). It is hypothesised that it is this which provides trophoblasts with a resistance to SARS-CoV-2 infection and prevents progression to viral particles entering cells in the chorionic villi which subsequently leads to a fetal inflammatory or hypoxic response (516). Vertical transmission of SARS-CoV-2 has been reported in only a minimal number of cases with scant evidence of transplacental transmission. However, most recent studies of histopathological features associated with SARS-CoV-2 infection report small numbers of cases, did not consider control groups and placental examination was not blinded to the clinical condition (518).

It is important to understand placental pathology in cases of maternal Covid-19, regardless of whether these were asymptomatic, mild or severe, in order to characterise disease trajectories in pregnant women and mitigate or prevent risks posed to the fetus and mother. A range of viral infections have been identified which posses the ability to cross the placental barrier and reach the fetus, often with devastating effects, including Ebola virus, human immunodeficiency virus (HIV) and, more recently Zika virus (ZIKV) (504, 521). Given that similar placental cytopathology and destructive lesions have been observed in cases of PTB

where maternal viremia has been confirmed for CMV, HSV-1/2 and SARS-CoV-2, it is crucial to determine the placental cell types and compartments affected and the mechanism of tissue injury in order to establish any similar potential risks, such as PTB and compromised fetal development associated with HIV infection (155) to the more severe neuronal necrosis and microcephaly seen in ZIKV infection (504).

5.1.5 Hypothesis and aims

The hypothesis for this chapter was that CMV and HSV-1/HSV-2 are more prevalent in preterm births and that active viruses would be detected to a greater extent in preterm placentas. Latterly, an additional hypothesis was that Covid-19-related placental dysfunction would be observed in placentas delivered preterm but no evidence of active viral SARS-CoV-2 nucleocapsid (N) protein would be present. The aims of this chapter were to:

a) identify CMV and HSV-1/HSV-2 viruses using immunohistochemistry in term and preterm placentas,
b) compare prevalence of herpesviruses between term and preterm births,
c) identify and localise SARS-CoV-2 using immunohistochemistry in term and preterm placentas, and
d) compare prevalence of SARS-CoV-2 in placentas from term and preterm births following negative maternal nasopharyngeal swab test.

5.2 Materials and methods

5.2.1 Participant recruitment and study design

A full description of ethical approval, participant recruitment, study design, collection, processing and reporting of placental samples and methods used in this chapter is set out in *Chapter 2: Materials and Methods.* Following the outbreak of the novel coronavirus SARS-CoV-2 and its associated disease, Covid-19, an additional study arm was included by way of amendment to the original STH approval to investigate the presence of SARS-CoV-2. During the period of 29 October 2020 and 16 April 2021, defined as the UK's second Covid-19 wave (522), forty-two placentas were collected from women delivering in the JW, Sheffield, sixteen of whom delivered preterm and twenty-six at term. From these cohorts, six term and six preterm were retrospectively randomly selected to be immunostained for the presence of viral SARS-CoV-2 N protein. An additional inclusion criterion was any woman being recruited to the research studies described in this thesis from October 2020 must have received a negative result from the SARS-CoV-2 RNA RT-PCR test collected routinely by nasopharyngeal swab on admission to wards within the JW maternity unit.

5.2.2 Cytomegalovirus (CMV) and Herpes Simplex Virus-1/2 (HSV-1/2) staining

A total of thirty-four placentas were selected for CMV detection analysis, of which fourteen were delivered preterm and twenty at term. Thirty-five placentas were selected for HSV-1/2 detection analysis, eighteen from the preterm cohort and seventeen from the term cohort. All placentas included in this chapter were collected as part of the PRIME study between April 2019 and March 2020 (pre-Covid19 pandemic recruitment restrictions). Histopathological evaluation was performed as previously described in *Chapters 2, 3 and 4* and by Khong *et al.*, (2016) (103). Full thickness placental tissue samples were processed for histopathological and IHC staining as described in *Chapter 2: Materials and Methods*. Briefly, IHC stains were performed with mouse monoclonal anti-CMV antibodies to DDG9 and CCH2 clones (Abcam 17073, 1:40 dilution) and rabbit polyclonal anti-HSV-1/2 antibodies (Dako Agilent, California, USA, IR521, ready-to-use) on an Autostainer Link 48 (Dako, California, US) in the Department of Histopathology, Sheffield Children's Hospital (SCH). Horseradish peroxidase (HRP) anti-mouse and HRP anti-rabbit were used for primary antibody localisation. Complex visualisation was achieved by the addition of 3,3'-diaminobenzidine (DAB) as the substrate chromogen with haematoxylin and eosin as a counterstain. Full thickness control samples of placentas

shown to be positive by IHC and PCR for CMV and HSV-1/2 were kindly provided by SCH and included for each staining run.

5.2.3 SARS-CoV-2 staining

Histopathological evaluation was performed as previously described in *Chapters 2, 3 and 4* and by Khong *et al.*, (2016) (103). Full thickness placental tissue samples were processed for histopathological and IHC staining as described in *Chapter 2: Materials and Methods*. Briefly, IHC stains were performed with mouse monoclonal anti-SARS-CoV-2 nucleocapsid (N) protein antibody clone B46F (Cat. No. MA1-7404, Thermo Fisher Scientific, Waltham, Massachusetts, USA, 1:100 dilution) on an Autostainer Link 48 (Dako, California, US) in the Department of Histopathology, SCH. Full thickness placental positive control samples for SARS-CoV-2 were kindly provided by SCH; staining of all samples was performed as a single run.

5.2.4 Immunohistochemical analysis

As described in *Chapter 2: Materials and Methods*, samples stained for CMV, HSV-1/2 and SARS-CoV-2 were visualised and imaged using an Eclipse 80i Y-THS light microscope (Nikon, Minato City, Tokyo, Japan) located at SCH Department of Histopathology. All placenta samples from IHC experiments were evaluated by the author (KMP) and reviewed by Professor Marta Cohen (MCC) for CMV and SARS-CoV-2 samples and Dr Sophie Stenton (SRS), Consultant Paediatric and Perinatal Pathologist, SCH, for HSV-1/2 samples.

5.3 Results

5.3.1 Maternal age, BMI and parity do not differ between study populations, nor does neonatal sex

An overview of clinical characteristics for the CMV, HSV-1/2 and SARS-CoV-2 cohorts are shown in Table 5.2. Of the women randomly selected for inclusion in the CMV, HSV-1/2 and SARS-CoV-2 studies, no significant differences were seen in maternal age between those delivering preterm and those who delivered at term. Likewise, there were no significant differences in BMI or parity between women experiencing preterm birth and those whose who gave birth at term and significant associations between neonatal sex and PTB (Table 5.2).

5.3.2 Active CMV and HSV-1/2 were not detected in preterm or term placental samples

No characteristic morphological features suggestive of CMV or HSV-1/2 infection were observed in either the term or preterm cohorts on histopathological evaluation. Although CMV affects the placenta in a patchy manner, slides were fully screened for each sample including the chorionic plate, villous tissue and decidua. No DDG9 or CCH2 clones were detected to signify the presence of active CMV, nor were any HSV-1/2 antibodies detected in any placental samples. Positive control samples were positive and negative control samples were all negative (Figure 5.3 and 5.4). Individual results for CMV clones and HSV-1/2 antibody staining are shown in Table 5.3.

Table 5. 2 Mean maternal age, BMI, gestational age and birthweight, median parity and counts of neonatal sex of study cohorts

	CMV			HSV-1/2			SARS-CoV-2		
Characteristic	**Preterm (*n* = 14)**	**Term (*n* = 20)**	***P V*alue***	**Preterm (*n* = 18)**	**Term (*n* = 17)**	***P V*alue***	**Preterm (*n* = 6)**	**Term (*n* = 6)**	***P V*alue***
Maternal age (years)	29.12 ± 6.78	28.32 ± 5.84	0.7149 ns	28.66 ± 7.39	28.32 ± 5.94	0.8832 ns	26.49 ± 3.31	31.20 ± 6.12	0.1282 ns
BMI (kg/m^2) †	28.80 ± 10.63	25.69 ± 5.85	0.6127 ns	27.96 ± 9.82	25.36 ± 5.61	0.5723 ns	26.67 ± 6.08	24.65 ± 7.82	0.4286 ns
Gestational age (weeks)	32.76 ± 4.01	39.29 ± 1.38	n/a	32.69 ± 3.75	39.41 ± 1.42	n/a	32.23 ± 3.83	40.30 ± 1.66	n/a
Parity (Median: Range)	1: 0 – 2	0: 0 – 2	0.2638 ns	0.5: 0 – 6	0: 0 – 2	0.2081 ns	0.5: 0 – 1	0.5: 0 – 2	0.6867 ns
Birthweight (g)	1936 ± 702.5	3356 ± 479.6	n/a	1893 ± 646.9	2880 ± 877.2	n/a	1554 ± 787.3	3357 ± 413.9	n/a
Sex (*n*) § Male	7	12	0.7282 ns	9	11	0.4998 ns	2	3	>0.9999 ns
Female	7	8		9	6		4	3	

BMI; body mass index. Values are presented as mean ± SD unless stated otherwise. *Student's *t*-test, §Fisher's exact test, ^Fisher's exact test with Freeman Halton extension, †Mann-Whitney *U* test. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

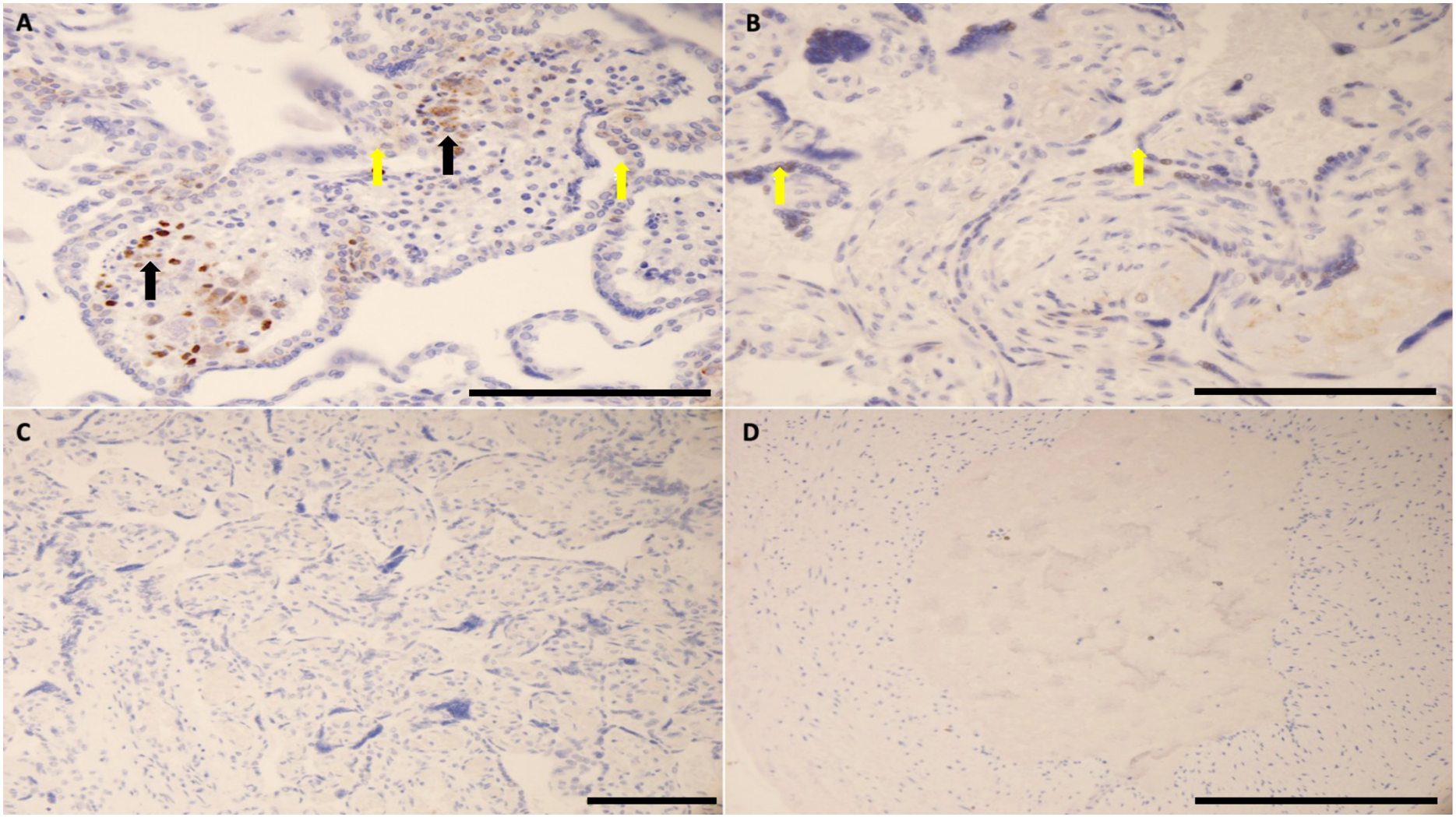

Figure 5. 3 Immunostained CMV positive and negative controls and example images.

A, B and **D** original magnification 40X, **C** original magnification 20X. Black arrows refer to areas of positive immunostaining for active CMV DDG9 and CCH2 clones and yellow arrows represent background staining. Scale bars represent 10μm.

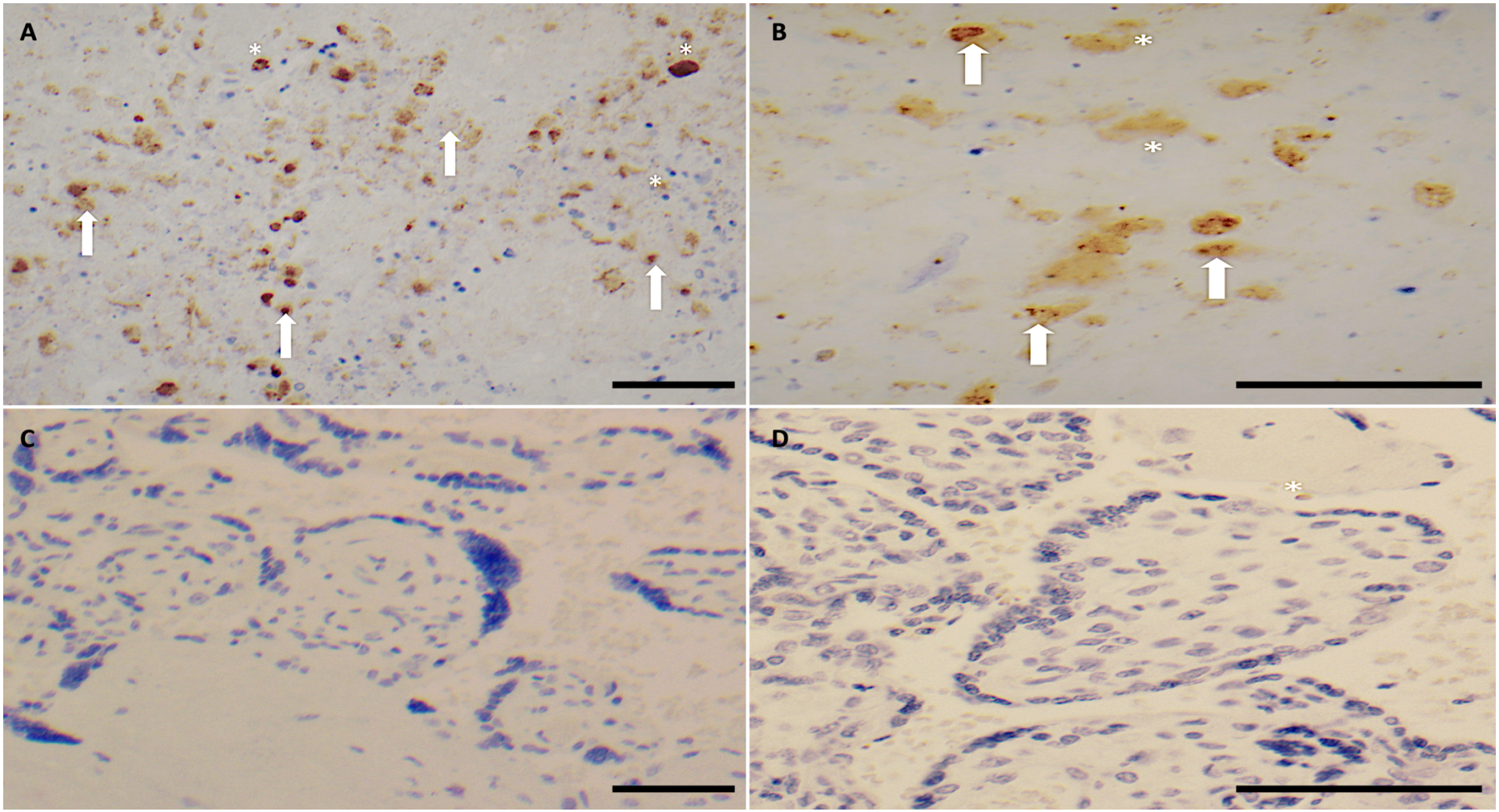

Figure 5. 4 Immunostained HSV-1/2 positive and negative controls and example images.

A and **C** original magnification 10X, **B** and **D** original magnification 40X. Arrows refer to areas of positive immunostaining for HSV-1/2 antibodies and asterisks represent background staining. Scale bar represents 10μm. As noted in **A, B** and **D**, background staining presents as dark brown dots whereas it is possible to see that the antibody has been taken up into the cell in immunostained positive control cells (**A** and **B**). Despite an automated process being used, contrast on the HSV-1/2 slides was suboptimal, however, it was possible to determine negative immunostaining in all cases.

Table 5. 3 Delivery outcome and viral presence of all placental samples immunostained for CMV and HSV-1/2

Unique ID	Outcome	CMV (DDG9/CCH2)	HSV-1/2	Unique ID	Outcome	CMV (DDG9/CCH2)	HSV-1/2
SU0002	Term	Negative		SU0045	Preterm	Negative	Negative
SU0006	Preterm	Negative	Negative	SU0046	Preterm	Negative	Negative
SU0014	Preterm	Negative	Negative	SU0047	Preterm	Negative	Negative
SU0016	Preterm		Negative	SU0049	Preterm		Negative
SU0020	Term	Negative		SU0050	Term	Negative	Negative
SU0021	Preterm	Negative	Negative	SU0051	Term	Negative	Negative
SU0025	Preterm	Negative	Negative	SU0052	Term	Negative	Negative
SU0026	Preterm	Negative	Negative	SU0053	Preterm	Negative	Negative
SU0029	Preterm	Negative	Negative	SU0054	Term	Negative	Negative
SU0030	Preterm		Negative	SU0058	Term	Negative	Negative
SU0032	Term	Negative	Negative	SU0059	Term	Negative	Negative
SU0034	Preterm	Negative	Negative	SU0060	Term	Negative	Negative
SU0035	Term	Negative	Negative	SU0063	Term	Negative	Negative
SU0036	Preterm		Negative	SU0064	Term	Negative	Negative
SU0040	Preterm	Negative		SU0065	Term	Negative	Negative
SU0041	Preterm	Negative	Negative	SU0068	Term	Negative	Negative
SU0042	Term	Negative	Negative	SU0069	Term	Negative	Negative
SU0043	Term	Negative	Negative	SU0070	Term	Negative	Negative
SU0044	Term	Negative	Negative	SU0072	Preterm	Negative	Negative

CMV; Cytomegalovirus, *HSV-1/2;* Herpes simplex virus 1/2, grey shaded box=not tested.

5.3.3 SARS-CoV-2 was a rare finding in a cohort of women with negative RNA RT-PCR nasopharyngeal swab results

Summary clinical and demographic data are provided for women randomly selected for enrolment to the SARS-CoV-2 arm of the study (Table 5.4), individual brief clinical and demographic details are presented in Table 5.4. Additionally, of the twelve women included, 50% identified as White British, 25% were Asian/British Asian (Other) and the remaining 25% self-identified as Asian/British Asian (Pakistani), Black (Other) and Mixed White-Black (8.3%; 1 case each).

Table 5. 4 Brief overview of clinical characteristics of cases in SARS-CoV-2 study

Case Number	Maternal Age (Years + Months)	Ethnicity	Gestational Age (Weeks + Days)	Birthweight (g)	Neonatal Sex	APGAR Score (1min/5min)
1	20+8	White British	37+3	3400	Male	9/9
2	27+11	White British	40+1	2880	Male	8/9
3	35+11	Asian/ British Asian (Other)	41+1	3030	Male	5/9
4	35+6	Mixed-White/Black	42+2	4020	Male	8/9
5	36+2	Asian/ British Asian (Other)	39+6	3210	Female	9/9
6	31+0	Black - Other	41+0	3600	Female	6/9
7	24+2	Asian/ British Asian (Pakistani)	29+5	1170	Male	NR
8	28+2	Asian/ British Asian (Other)	35+3	1900	Female	6/8
9	21+3	White British	35+4	1600	Male	NR
10	28+0	White British	36+1	2920	Female	7/9
11	30+4	White British	27+2	770	Female	NR
12	26+9	White British	29+3	963	Female	NR

NR; not recorded. APGAR values represented are scores at 1 min (1-9) and 5 min (1-9)

5.3.4 Placental features associated with SARS-CoV-2 were not observed in the study cohort

Upon histopathological evaluation of samples retrospectively selected for analysis of the presence of SARS-CoV-2 N protein, three placental masses were found to be outside the normal range (10^{th} – 90^{th} percentile), one delivered at term and two preterm. Two fetoplacental weight ratios were below the normal range and two were above (normal range: 10-90 percentile). No other placental features associated with SARS-CoV-2 were noted in any participant in the study (Table 5.5).

Table 5. 5 Placental histopathological characteristics of cases in SARS-CoV-2 study

Case ID	Unique ID	Placental Mass (g)	Placental Weight Percentile	Fetoplacental Weight Ratio	Fetoplacental Weight Ratio Percentile	Histopathological features
1	SU0075	332	5-10	10.3	>97	Normal term placenta, evidence of avascular villi but of no clinical significance
2	SU0080	433	10-25	2	<3	Normal term placenta, no evidence of PIR
3	SU0081	598	75-90	5.1	<3	Term placenta with acute HCA and acute subchorionitis. Acute vasculitis in UC. Normal maturation and development. Small fresh intervillous thrombi of no clinical significance
4	SU0085	641	75-90	6.3	10-25	Term placenta with subchorionitis and arteritis
5	SU0088	428	25-50	7.5	75-90	Normal term placenta, no evidence of PIR
6	SU0089	541	50-75	6.6	25-50	Normal term placenta, no evidence of PIR
7	SU0092	203	10-25	5.7	75	Preterm placenta with acute HCA
8	SU0093	282	5-10	6.7	50-75	Preterm placenta, acute HCA, small for gestational age (<10th centile) and accelerated villous maturation.
9	SU0144	417	50-75	6.2	25-50	Preterm placenta with normal weight and normal fetoplacental ratio. Hypercoiled UC and accelerated villous maturation
10	SU0145	550	75-90	5.3	10	Normal preterm placenta with some microscopic features compatible with maternal arterial malperfusion abnormality; patchy accelerated villous maturation
11	SU0146	141	<5	5.46	90-95	Normal-sized preterm placenta with high fetoplacental weight ratio (90-95th centile) showing features of maternal arterial malperfusion
12	SU0147	234	25-50	4.1	10-25	Normal-sized preterm placenta with adequate fetoplacental weight ratio. Hypocoiled UC and features of maternal arterial malperfusion including advanced decidual arteriopathic changes. Peripheral membranes show decidual arteriopathy (mural hypertrophy). Accelerated chorionic villous maturation and multifocal remote villous infarct in chorionic villi. Multifocal recent infarction haematoma in IVS and basal plate shows decidual arteriopathy. Abnormal UCI positively correlates to conditions noted.

HCA; histologically-diagnosed acute chorioamnionitis, *IVS;* intervillous space, *PIR;* placental inflammatory response, *UC;* umbilical cord, *UCI;* umbilical coiling index.

5.3.5 SARS-CoV-2 Immunopositive case

Immunostaining for SARS-CoV-2 nucleocapsid (N) protein showed positivity in placental syncytiotrophoblasts of case #6, SU0089 (Figure 5.5). To minimise potential false-positive and false-negative results, positive and negative control samples were included with cohort sample runs. Positive control samples to assess antibody specificity were immunopositive (Figure 5.5A and 5.5C). Negative control samples where no SARS-CoV-2 antibody was included in the IHC stain were shown to be immunonegative with no background staining (Figure 5.5E). No inflammatory infiltrate or cytotoxic damage such as trophoblastic necrosis was evident even at high magnification (Figure 5.5D, 40X magnification). SARS-CoV-2 infection of the placenta has been shown to be patchy, and, according to Hanna *et al*., (2021) (523), IHC provides a more sensitive analysis than RT-PCR, therefore RT-PCR was not performed on the immunopositive case of SARS-CoV-2. Further detail and rationale are provided in the discussion.

5.3.5.1 Background to the patient

Clinical and demographic history of the asymptomatic positive index case (Case #6), including pregnancy, delivery and any administered treatments are, briefly, 31-year-old woman with a previous term livebirth attended the Jessop Wing at 40+3 weeks gestational age having previously reported reduced fetal movements in the 23rd week of pregnancy. On examination, auscultation and ultrasound scanning, no fetal abnormalities or growth restriction were identified at that time. The patient reported no symptoms of Covid-19 in her household during the initial telephone contact with JW staff and a negative RT-PCR SARS-CoV-2 RNA test was reported prior to her face-to-face visit. A further report of obstetric complications was made to the patient's GP at 28 weeks' gestation, again the patient reported no household Covid-19 symptoms, and a negative RT-PCR SARS-CoV-2 RNA test was recorded. At the antenatal booking appointment, the patient had reported being a non-smoker who did not consume alcohol or use illicit drugs. BMI was within normal range and no significant medical conditions were recorded; however, the patient had experienced a third-degree (3b) tear during her previous delivery; an elective Caesarean section delivery was offered but declined. Four days prior to delivery, the mother was admitted to the JW experiencing mild uterine contractions and having again reported reduced fetal movements; a routine negative RT-PCR SARS-CoV-2 RNA test was returned within 6 hours of admission to the ward. Labour was

induced one day post-admission using a Propess 10mg vaginal delivery system (Ferring Pharmaceuticals, Saint-Prex, Switzerland) and intravenous syntocinon at 6ml/hr. Established labour was recorded after 3 days on the antenatal ward following which membranes were artificially ruptured. Entonox and epidural were used for pain relief during labour and delivery of a female neonate weighing 3600g at 41 weeks and 0 days' gestation. Active management of the third stage, involving administration of a prophylactic uterotonic, resulted in delivery of the placenta 15 minutes after the neonate. A third-degree tear (3b) and 1.9 L postpartum haemorrhage occurred, both of which were managed according to standard clinical Trust procedures.

5.3.5.2 Placental histopathological findings were unremarkable for SARS-CoV-2 positivity

The placenta showing immunopositivity for SARS-CoV-2 N protein in case #6 had a trimmed mass of 541g and, although slightly above the 496g mean for 41 weeks' gestation, was determined to be within the 50^{th}-75^{th} percentile. From the neonatal birthweight of 3600g, a fetoplacental weight ratio (FPWR) of 6.6 was calculated (25^{th}-50^{th} percentile). As for placental mass, this was within the normal range for the gestational age. No significant abnormalities were detected in placental shape or size, membrane completeness, colour and insertion or umbilical cord coiling index and insertion. It was reported that the fetal surface was blue-tinged, and the maternal surface was ragged, had pale areas and a 7 cm x 3 cm attached blood clot. Since there had been a reported postpartum haemorrhage, maternal surface features were not considered to be abnormal. The colour of cut surfaces was normal and firm white or haemorrhagic lesions identified accounted for <1% of the total volume. On microscopic examination, maturation was seen to be normal and there was no evidence of the reported key morphological changes of SARS-CoV-2 infection such as CHIV and massive PVFD (507, 519).

5.3.6 Positive IHC staining was shown in term villous syncytiotrophoblasts

Immunostaining for SARS-CoV-2 N-protein showed positivity in placental syncytiotrophoblasts, control samples were positive and negative, respectively (Figure 5.5A, C & E). No inflammatory infiltrate or cytotoxic damage such as trophoblastic necrosis was evident even at high magnification (Figure 5.5D, 40X magnification).

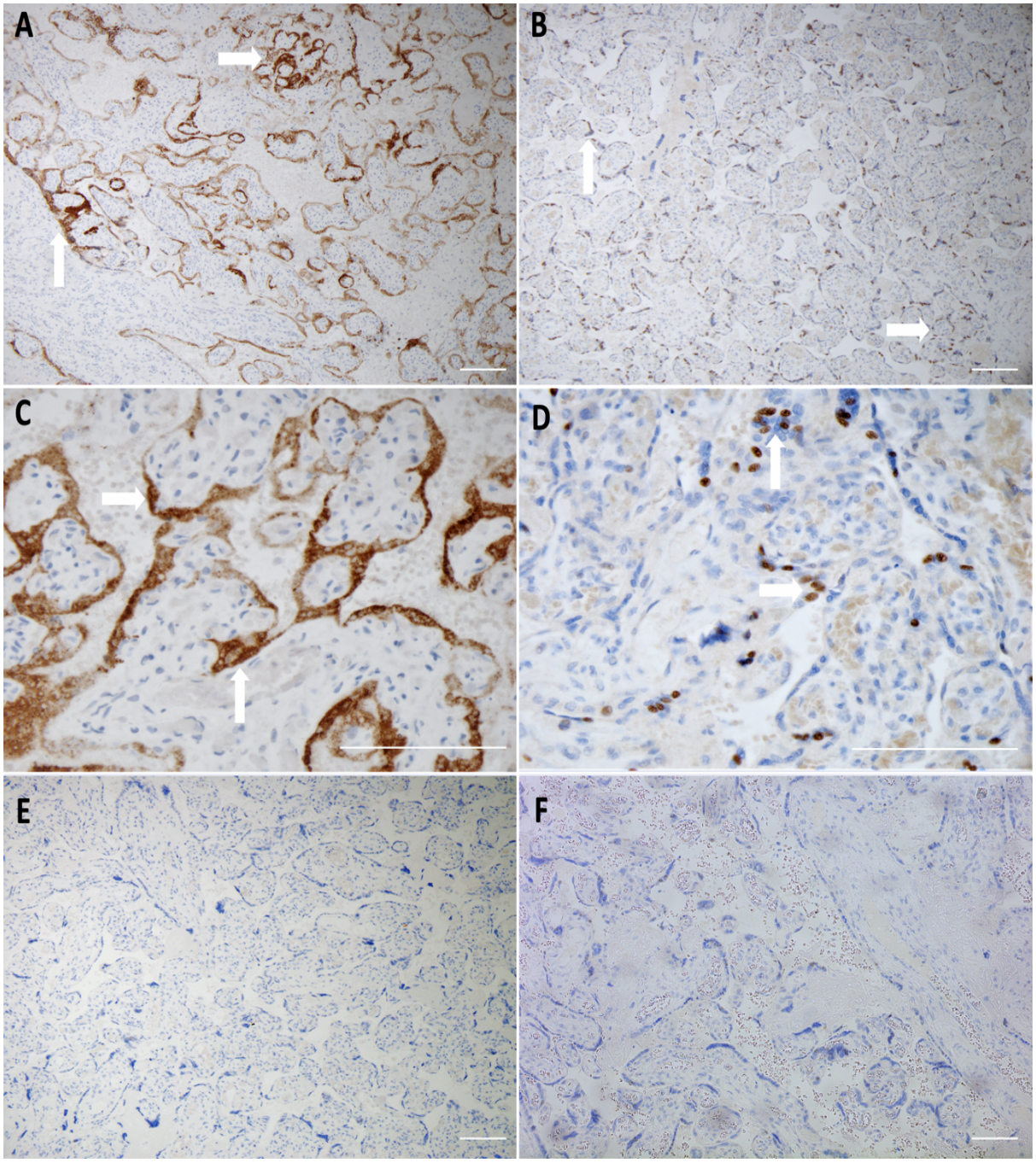

Figure 5. 5 Immunostaining for SARS-CoV-2 nucleocapsid (N) protein.

Block arrows show areas of positive immunostaining for SARS-CoV-2, scale bar represents 10μm. **A** original magnification 10X positive control sample. **B** shows positive immunostaining for SARS-CoV-2 nucleocapsid (N) protein in syncytiotrophoblast of term placental sample (SU0089). 10X original magnification. **C** positive control sample showing SARS-CoV-2 N-protein in syncytiotrophoblast, original magnification 40X. **D** patchy areas of positive immunostaining in term sample (SU0089) syncytiotrophoblasts, original magnification 40X. Unlike **C**, there is no evidence of trophoblastic necrosis in the syncytial barrier or diffuse positivity. **E** original magnification 10X negative control sample. **F** example negative immunostaining at original magnification of 10X of preterm placenta sample (SU0093).

5.4 Discussion

5.4.1 CMV and HSV-1/2

In summary, analysis of the samples in this study indicates neither CMV nor HSV-1/2 infection are associated with preterm birth in UK cohorts. A combination of histopathological and immunohistochemical evaluation of full-thickness placental samples validated the absence of CMV, HSV-1 and HSV-2 in the study cohort. On histopathological evaluation, no characteristic CMV owl's eye inclusion bodies (524, 525) were visualised in any of the thirty-four placental samples. This finding is in contrast to published studies which report the prevalence of congenital CMV infection to be 0.2% - 6.1% (median 1.1%) in newborns (481, 485, 494, 526), although the UK incidence is low at an estimated 0.3% (481). Similarly, no distinguishing features of HSV infection, such as chronic villitis, intervillositis, villous necrosis or characteristic HSV Cowdry type B viral inclusion bodies (187), were observed in any of the thirty-five placental samples included for HSV-1/2 analysis, irrespective of gestational age. Data from this study suggest no association between HSV-1/2 infection and preterm birth, contrary to a number of studies which have shown primary HSV-2 infection to adversely affect pregnancy outcome in a dose-dependent manner (527) and intrauterine infection with HSV-1 or HSV-2 to result in chronic placental insufficiency, preterm birth and even fetal death (186). In addition, the results refute those presented by Finger-Jardim *et al.* (2017) who determined a 28% and 29.9% prevalence of HSV-1 and HSV-2, respectively in placental samples collected from the basal plate (maternal side) and 12.6% and 8.3% prevalence from the chorionic plate (fetal side) of placentas delivered by asymptomatic women of whom >90% reported no history of HSV infection (462), a cohort with similar characteristics to the population enrolled to the study described in this chapter, albeit much larger (422 women).

An important factor to take into consideration is that detailed histopathological evaluation was conducted for all preterm and term placentas included in the CMV and HSV-1/2 aspects of the study. In addition, data were collected on duration of rupture of membranes (ROM) and of labour to determine if prolonged ROM or labour increased the risk of placentitis. As discussed in *Chapter 3*, women delivering preterm had membranes which had ruptured for significantly longer periods prior to labour (PPROM) than women delivering at term. In a 2020 case report described by Smith *et al*., HSV-2 infection was diagnosed in a preterm placenta

following prolonged PPROM in the absence of neonatal and maternal HSV infection or maternal history of HSV (186). An important observation by the authors was that this study was the first case of placental pathology indicating *in utero* HSV exposure prior to presentation of neonatal HSV symptoms and that this resulted in confirmatory immunohistochemical analysis, reports of findings to the neonatologist on day five of life and a favourable neonatal outcome (186). Notwithstanding morbidities of CMV such as neurosensory disabilities and learning difficulties which may not become apparent until later years (504, 528), congenital CMV infection is an underappreciated cause of sudden unexplained death in infancy (SUDI), a risk which may be increased given that CMV-infected neonates generally appear healthy at birth (529, 530). Although no CMV or HSV-1/2 viral inclusions were observed in placentas of this study population, the immunohistochemical results substantiate the placental histopathological examination and emphasise its importance in providing robust guidance to clinicians and parents or carers of neonates which may improve clinical outcomes. Whilst it is acknowledged that resources would not permit pathological assessment of placentas from all births, identification of at-risk infants, such as those exposed to prolonged PPROM and those born to mothers with a history of prior CMV and/or HSV infection, followed by ante- or postnatal screening and placental histopathological evaluation is recommended.

5.4.2 SARS-CoV-2

During the second Covid-19 wave in the UK, research focused on the effects of Covid-19 in pregnancy but was limited predominantly to symptomatic women who were positive at or close to the time of delivery. Unique to the small cohort in this study was all women had returned a negative RT-PCR test or lateral flow test as part of the inclusion criteria. Health and safety procedures, the strict use of personal protective equipment and recruitment and laboratory restrictions implemented to mitigate the risks of Covid-19 are detailed in *Chapter 2*, and it is these which resulted in a cohort of Covid-19-negative mothers enrolled in the study, and a confidence that the results observed did not occur from clinical or research staff transmission. Although understanding the contribution of SARS-CoV-2 on placental histopathology was an important area, determining the effect, if any, of prior or asymptomatic Covid-19 infection on the placenta was an underappreciated area at the time of the study presented in this chapter. Therefore, preliminary data were collected on the

presence of SARS-CoV-2 N-protein from women testing negative for Covid-19 at or close to the time of delivery. It was expected that all placentas from this cohort would also be negative for SARS-CoV-2. At the time of analysis and based on a thorough search of the obstetric and placenta-related Covid-19 literature, the finding of an immunohistologically-confirmed placental SARS-CoV-2 infection in a mother with a negative RT-PCR nasopharyngeal swab result had not been reported.

5.4.2.1 SARS-CoV-2 placentitis

SARS-CoV-2 infection in pregnancy generally resulted in mild symptoms for mothers, with limited adverse effect on their babies. However, PTB, fetal death, severe maternal illness and maternal death were also reported (515). Since the initial waves of Covid-19 in the UK, placental pathological findings have been published in several case reports where mothers have been positive for Covid-19. Sisman *et al*. (2020) described histopathological features of CHIV with associated syncytiotrophoblastic karyorrhexis and necrosis of the villous tissue; SARS-CoV-2 N-protein presence in the syncytiotrophoblast was confirmed by immunohistochemistry in this case (531). In Patanè *et al*.'s 2020 case report, the authors acknowledged the potential for vertical SARS-CoV-2 transmission to be controversial and that supporting evidence was lacking. However, their findings revealed SARS-CoV-2 RNA on the fetal side of the placenta in unison with maternal and neonatal positive RT-PCR nasopharyngeal swab results, suggesting *in utero* vertical transmission of SARS-CoV-2 from the mother to the fetus, in two cases. Moreover, histopathological evaluation of the placentas of these two women showed chronic intervillositis and $CD68^{+}$ macrophage infiltration (histiocytes representing CHIV) (532). Vivanti *et al*. (2020) reported a single case of transplacental transmission of SARS-CoV-2 infection from a symptomatic mother to her fetus. Alongside acute intervillositis and CHIV, diffuse PVFD was noted, as was intense trophoblastic cytoplasmic immunopositivity for SARS-CoV-2, demonstrating late gestation transplacental SARS-CoV-2 transmission (533). CHIV and syncytiotrophoblastic necrosis were placental pathological features confirmed in larger retrospective case studies where SARS-CoV-2 positivity was shown via immunohistochemistry, such as that described by Schwartz *et al*. (2021) (534). The presence of massive PVFD and CHIV as distinct features of SARS-CoV-2 placentitis were further defined by Stenton *et al.* (2022) in a study of fifty-nine mothers and sixty-one infants from ten tertiary centres in the UK (535).

CHIV and PFVD are not SARS-CoV-2-exclusive histopathological features, yet the incidence is reported as <1% of all pregnancies for both conditions and a combination of the two together was rarely described pre-Covid-19 pandemic (535). Positive RT-PCR nasopharyngeal swabs, immunohistochemistry or in-situ hybridisation in conjunction with CHIV or massive PVFD, as described in the case reports above, supports the suggestions that SARS-CoV-2 infection can be transmitted vertically and results in a distinct placentitis. In contrast, a case report authored by Hsu *et al.* (2021) found no remarkable placental pathology and no classic SARS-CoV-2 placentitis features following mild maternal Covid-19 disease. Focal lymphohistiocytic inflammation consistent with chronic villitis was, however, noted (536). It was suggested that third trimester exposure may explain the lack of CHIV and PVFD (536) which may also be true of Case #6 (SU0089) presented in this chapter. No RT-PCR or LFT for maternal Covid-19 was performed or reported for Case #6 between 28- and 40 weeks' gestation, but she did not self-report Covid-19 infection or symptoms during this period (information collected from clinical notes).

More severe adverse outcomes, such as extreme prematurity and fetal death, are reported to occur where pregnant women had symptomatic Covid-19 infections (537); similarly, there is a correlation between timing of Covid-19 infection and adverse obstetric outcome with first- and second-trimester infections leading to increased risk of PTB, stillbirth or a small for gestational age infant (538). SARS-CoV-2 entry to cells, including trophoblasts, is via angiotensin converting enzyme 2 (ACE2), yet expression levels are almost indiscernible in the placenta at term (538), potentially providing further explanation as to why minimal and patchy SARS-CoV-2 positive immunostaining was observed in the placental sample of Case #6 who delivered at 41 weeks. An alternative explanation is that IHC findings were a false positive in this case. Variables in immunohistochemical methodologies have previously been documented to be associated with false negatives (poor signal) or false positives (high background) (539). In this study, IHC staining for SARS-CoV-2 was performed on all samples in one staining run using an automated process, consequently, all samples were treated uniformly with respect to antigen retrieval procedures, tissue quenching, antibody concentrations and incubation periods (539). Fixation of tissue prior to IHC is also known to result in false-positive and false-negative results, but there was no delay in fixation following placenta collection and the type and pH of 10% NBF used for all samples did not differ (539)

and positive and negative controls were included to assess antibody specificity, both of which were immunopositive and immunonegative, respectively. A further possibility for the discrepancy between the negative patient RT-PCR result, the method of maternal testing in place at the time of delivery in the positive case in this cohort, and the positive immunostaining of the placenta is that the target of the immunohistochemical procedure was only the SARS-CoV-2 nucleocapsid (N) protein whereas the RT-PCR targets either the envelope (E), nucleocapsid (N) or the spike (S) proteins (540) and it was not possible to determine which protocol was in use in the NHS laboratories at the time of testing. Two studies conducted during the early stages of the Covid-19 pandemic showed N protein antibody concentrations to be higher than those for S proteins (541, 542) which may account for the negative maternal test result, although both papers focused on samples prepared for LFTs which have been shown to have lower sensitivity overall than RT-PCR (540). In the immunopositive case reported in this chapter, there were no placental pathological features, SARS-CoV-2-specific or otherwise, and no trophoblast necrosis. Focal fibrin deposition was observed on pathology, but this was deemed to be normal for the gestation by the reporting perinatal pathologist (MCC) similar to a single case presented by Ferraiolo *et al.* (2020) where all findings were in limited areas of the placenta and didn't extend beyond what was normal for the gestation (543). SARS-CoV-2 infection of the placenta has been described as patchy and irregular with viral localisation concentrated in some areas more than others (523, 544). Taking these factors into consideration along with the term gestation and no reported maternal Covid-19 infection, it is suggested that the positive case presented in this chapter was a true positive. The lack of ACE2 expression at term, coupled with the timing of a presumed mild, asymptomatic Covid-19 infection, support cases presented in the literature where a low viral load was present, and this conferred protection from placental injury (536, 545, 546).

As maternal Covid-19 infections are more widely studied and case reports published, adverse obstetric outcomes are being linked to symptomatic and asymptomatic presentations. A 2021 case report from Kalsar *et al*. described a severe postpartum haemorrhage (SPPH) in an asymptomatic Covid-19 patient (547). Graham *et al.* (2022) reported ten cases of spontaneous coagulopathy related to Covid-19 with eight women consenting to inclusion to the study (548). PPH occurred in five of the eight cases and, despite no causal association between Covid-19 and coagulopathy being described, the authors were unable to offer an

alternative aetiology for coagulopathy in all eight cases, as was the case in the Kalsar report (547, 548). The patient described in this chapter did not experience prolonged second or third stages of labour which may have increased her risk of primary PPH (549). Additional risk factors for the SPPH the patient experienced, including macrosomia, multiple pregnancy or retained placenta (550), were not present in her particular case. However, as shown in a large cohort (11,323) of South American women, the need for perineal suture is associated with SPPH (aOR:2.50; 95% CI 1.87-3.36) (550) and this may explain the patient's SPPH in this case report following the 3b tear experienced during delivery. Nonetheless, an increased risk of thromboembolism and coagulopathy have been linked to maternal Covid-19 infection (551) and PPH remains a substantial cause of maternal mortality (552). Consequently, it is imperative that pregnant women with Covid-19 associated coagulopathy are identified (548) and consideration is given to maternal adverse outcomes which may appear unrelated to Covid-19 infection.

5.4.3 Post-pandemic SARS-CoV-2 and the placenta

Whether SARS-CoV-2 is associated with vertical transmission from mother to fetus and with placental pathology remains contentious, despite research into the effects of Covid-19 infection in pregnancy continuing throughout the pandemic and into the post-pandemic era (553). Whilst some studies appear to confirm the characteristic SARS-CoV-2 placentitis described by Stenton *et al.* (535), Patanè *et al.* (532) and Vivanti *et al.* (533), others report more routine placental pathology such as MVM and decidual arteriopathy in women with Covid-19 infections (553). Encouragingly, the frequency and severity of Covid-19 cases have declined since the initial waves of the pandemic, largely due to successful vaccination and public health programmes, yet mutations in SARS-CoV-2 persist and variants of concern with high transmissibility continue to be identified (554). As discussed, placental changes seen in cases of positive maternal or placental SARS-CoV-2 infection are neither pathognomic nor consistent and do not appear to correlate directly with maternal disease severity (535, 555, 556). Here, similarities can be drawn with the progression of disease seen during the Zika virus (ZIKV) epidemic in Brazil in 2015, prior to which no pregnancy-related disease had been observed and, in the sixty years since its 1947 isolation from a sentinel Rhesus monkey in Uganda, only around twelve cases of human ZIKV disease had been reported, each with mild symptoms of fever, conjunctivitis and arthralgia (557, 558). A further parallel between these

distinct viral outbreaks includes a probable causative link between cross-species transmission and acquired pathogenicity through rapid viral adaptation (558).

Like ZIKV, SARS-CoV-2 displays placental tropism, yet research to establish the sequence of events from SARS-CoV-2 genome and mutation discovery to the effects on placental tissue morphology or maternal immunity appear to have stalled. *In vitro* models have confirmed SARS-CoV-2 productively infects syncytiotrophoblasts (STb) utilising the ACE2 receptor with an infection rate at day 1 of STb differentiation of approximately 10%. Additionally, in the presence of the virus, hCG production and the STb fusion index were shown to be reduced, factors associated with dysregulated STb differentiation, placental dysfunction and IUGR (559, 560). Studies of adverse pregnancy outcomes associated with coronaviruses were minimal prior to 2020; those available focused on the effects of Severe Acute Respiratory Syndrome (SARS-Cov) and Middle Eastern Respiratory Syndrome (MERS-CoV). However, whilst these studies reported associations with PTB, IUGR and spontaneous abortion, with one exception, all were case reports of only one patient and all PTBs were indicated due to the severity of maternal disease and admission to the intensive care unit. In the single study with twelve confirmed SARS-positive cases, maternal mortality was recorded as 25% and four women experienced a first trimester miscarriage. Of the five births, four were preterm but all placentas were negative for SARS-CoV, and none were reported with a placentitis. Three had normal histopathological features and two had avascular fibrotic terminal villi and an associated IUGR neonate, but there was no evidence this was related to maternal SARS-CoV infection (561-563). Why SARS-CoV-2 robustly infects cells of the placenta unlike SARS-CoV and MERS-CoV and despite relatively low ACE2 receptor expression in STbs is potentially explained by mutations in its spike (S) protein. Viral replication commences only after attachment and cellular entry, a process mediated via interactions between the ACE2 receptor and the S protein. Then, transmembrane serine protease 2 (TMPRSS2) cleaves the S protein to S1 and S2 domains, the former of which mediates ACE2 receptor attachment and the latter being responsible for membrane fusion. S2 is highly conserved between coronaviruses, however, utilising a genome alignment tool (clustalw), in late 2020, Hatmal and colleagues revealed the N-terminal subdomain (NTD) of S1 to be considerably less preserved (51% identity with SARS-CoV and MERS-CoV) (564). Through the same method, variances in two amino acid residues were discovered in the S1 NTD and, although neither of

these altered the polarity of the protein or its tertiary structure, a 4-fold increase in the number of atomic interactions between the SARS-CoV-2 receptor binding domain (RBD) and the ACE2 receptor was noted when compared to those of the RBD of SARS-CoV and ACE2 receptors (564). Adding to the complexity in understanding the effects of molecular differences between coronaviruses, Chen *et al*. more recently highlighted a spectrum of effects, and treatments, within SARS-CoV-2 variants through their *in vitro* study, specifically in treating Omicron variants which required a combination of anti-spike antibodies and anti-ACE2 antibodies in order to effectively prevent infection and cell death and to restore hCG secretion, unlike ancestral SARS-CoV-2 variants which required only anti-spike antibodies (560). Viral variability may be the important factor in determining disease susceptibility and severity, however, it is challenging to know which variant an individual, as with the participant with a positive placental sample presented in this thesis, has been infected with, particularly as testing becomes less routine and accessible. Nonetheless, biological variability between individuals and populations may also account for the differences in global infection rates and disease severity in pregnant women, as well as observable placental infection and tissue damage. As populations adapt to live with Covid-19, there remains a lack of knowledge of the potential of SARS-CoV-2, notably in pregnancy. As with SARS-CoV-2, the pathogenic mechanisms of ZIKV infection in the placenta have yet to be elucidated and no research appears to focus on the shift from infrequent and mild ZIKV disease symptoms to the reported association with Guillain-Barré Syndrome during ZIKV outbreaks in Yap Island, Federated States of Micronesia, and French Polynesia in 2007 and 2013, respectively (558, 565). Even following more than 4000 suspected microcephaly cases and almost 3300 confirmed ZIKV-associated child development disorders reported from Brazil between 2015 and 2018, researchers have failed to address the progression of ZIKV disease to placental tissue damage and fetal neurological complications, or why this was only observed in Brazilian women (558, 565). As with ZIKV, SARS-CoV-2 is endemic, therefore, it is essential that histopathological evaluation of the placenta continues so that effects of maternal Covid-19 on the placenta can be established, and clear public health guidance can be delivered to pregnant women to prevent the impasse situation seen in ZIKV research. Studies to better understand the significance of known and emerging SARS-CoV-2 variants, the timing of infection, maternal immune system changes, and the outcomes of placental tissue damage are also necessary.

5.4.4 Conclusions

Although no positive CMV or HSV-1/2 cases were identified in the placentas of this small cohort, given that infants with congenital CMV or HSV-1/2 infections appear healthy at birth but may later develop severe sequelae including CMV-related neurodevelopment disorders and hearing loss (528), or dermatologic, ocular and neurologic disease in the case of neonatal HSV infection (566), screening of preterm infants and placental histopathological examination following prolonged PPROM may be justified. Overall, these data support the need for larger fully-powered studies.

In the case of SARS-CoV-2, as far as was known, this was the first study to describe the presence of viral SARS-CoV-2 N-protein in the syncytiotrophoblast of the placenta from a mother who tested negative for Covid-19. These preliminary findings support the possibility of asymptomatic placental infection and of determining the timeframe for maternal SARS-CoV-2 infection and possible vertical transmission. Results presented in this chapter formed part of a grant application to further study the effects of maternal Covid-19 infection timing, placental SARS-CoV-2 viral load and the association with preterm birth (University of Sheffield Grant Code: HEIF X-014726-14). IHC methods and optimisations developed and performed by the author were shared with SCH to conduct IHC analysis on placental samples to corroborate histopathological findings described by Stenton *et al*., 2022 (535). Whilst future studies are beyond the scope of this thesis, research is ongoing to characterise placental histopathology and correlate this to the presence of SARS-CoV-2 in cohorts of women delivering preterm and at term with both negative and positive nasopharyngeal RT-PCR swab results with the aim of closing the gap in knowledge which remains on the effects of maternal Covid-19 on the placenta.

Chapter 6: Gestational Age and Placental Inflammatory Response Affect the Localisation, Abundance and Ratio of M1:M2 Phenotype Macrophages Within the Placenta

6.1 Introduction

As described in *Chapter 1: Introduction*, macrophages are an abundant cell population in the placenta comprising up to 30% of all leukocytes at the maternal-fetal interface (201, 290, 567). Polarisation of macrophages is essential in maintaining a successful pregnancy through immune tolerance of the semi-allogenic fetus and in responding to pathogenic insults (201, 290). Macrophage phenotypes are commonly categorised according to biological function and frequently described as analogous to T-helper 1/T-helper 2 cells (Th1/Th2) in that the M1 phenotype are typically pro-inflammatory, mounting robust antiviral and antibacterial responses, and the M2 phenotype are anti-inflammatory and involved in wound healing and tissue growth (201). However, a critical homeostatic role in late gestation, which may be responsible for reducing the risk of preterm labour, has more recently been described (569). Strategies employed by M1 macrophages following classical-activation by lipopolysaccharides (LPS), or Th1 cytokines such as interferon-gamma (IFN-γ), interleukin-1-beta (IL-1β) or tumour necrosis factor-alpha (TNF-α) are three-fold and result in the M1 phenotype having a functional ability to destroy pathogens (201, 570-572). Firstly, M1 macrophages produce proinflammatory cytokines, including IL-1, IL-6, IL-8, IL-12 and TNF-α, and each of these exerts direct or indirect antimicrobial properties. Secondly, M1 macrophage polarisation activates other immune cells to enhance the antimicrobial response and, finally, as reported in the placenta, M1 macrophages provide antibacterial and antiviral protection by generating a highly oxidised environment (572). Inducible nitric oxide synthase (iNOS) mediates conversion of L-arginine to produce L-citrulline, reactive oxygen species (ROS) and nitric oxide (NO); in the resultant extremely oxidative environment, M1 macrophages display potent antimicrobial properties (201, 570-572). On the other hand, glucocorticoids, immune complexes, transforming growth factor-beta (TGF-β) and Th2 cytokines IL-4 and IL-13 directly activate M2 macrophages to produce L-ornithine and urea from L-arginine by upregulating arginase-1 (Arg-1) levels (201, 570, 571, 573). This alternative-activation equips M2 macrophages with anti-inflammatory and endocrine signalling functions and the capability to scavenge apoptotic cells and repair and remodel tissue (290, 573). In addition to expression of Arg-1, M2 macrophages secrete anti-inflammatory interleukin-10 (IL-10), antimicrobial proteins CC motif ligand 17 (CCL17) and CC motif ligand 22 (CCL22), chemokines with

bacteriocidal activity against *E.Coli* and *Staphyloccocus aureus*, and CD163, a transmembrane scavenger protein expressed exclusively in monocytes and macrophages (573, 574).

Previous research has elucidated important roles for macrophages at all stages of pregnancy, although these cells exhibit different phenotypes and polarisation states at different stages of gestation. Dysregulated M1/M2 proportions in early pregnancy have been shown to contribute to deficient trophoblast invasion and spiral artery remodelling during placentation which, in turn, has been linked to preeclampsia, IUGR and PTB (201, 286, 287, 290, 570). As a highly pleiotropic signalling molecule at cellular and systemic levels, NO is known to play a critical role in cellular respiration, vascular tone regulation, cellular proliferation and apoptosis. Although it has been shown that NO is the main vasodilator in the placenta and that it generates the hypoxic environment necessary for trophoblast invasion and vasculogenesis and angiogenesis, and evidence suggests an M1 dominance in the early gravid uterine environment, the contribution of macrophage polarisation and subsequent NO production in early pregnancy is yet to be clarified (201, 575). Late in the first trimester, macrophage plasticity leads to a heterogenous M1/M2 population which persists until mid-gestation and completion of placental development (201). A transfer to M2 prevalence occurs until late gestation when decidual macrophage proportions increase, more so in placentas delivered at term following labour than either term or preterm deliveries without labour, and a return to M1 dominance is observed (151, 201, 287, 569). In late pregnancy, locally-produced cytokines recruit macrophages to the amniotic cavity, myometrium and cervix where they contribute to cervical ripening and amplification of the inflammatory cascade required for labour initiation (287, 290). Furthermore, macrophages and neutrophils infiltrate the compartments of the placenta, including the decidua and fetal membranes, potentially leading to SROM in non-complicated term labour (287).

Nonetheless, evidence as to whether the presence of placentitis alters the balance or proportions of M1/M2 macrophages is contradictory, with studies reporting decreased M1 expression in placentas with histologically-diagnosed chorioamnionitis (HCA) (213) and others describing the converse (191). Throughout gestation, these fluctuating total numbers and proportions of M1/M2 macrophages protect the fetus from the maternal environment and aid in establishing maternofetal tolerance (201, 290). In abnormal pregnancies, such as those

with preeclampsia, IUGR and preterm birth, it has been suggested that there is a premature shift towards increased numbers of classically activated M1 macrophages, notably at the maternofetal interface (decidua), and subsequent early initiation of labour processes. This increase in pro-inflammatory immune cells linked to adverse outcomes implies that M2 anti-inflammatory macrophages are primarily responsible for immunosuppression and a positive obstetric outcome (201) and moderation of M2 macrophage markers contributes to PTB pathophysiology through augmentation of inflammation (569). However, explaining macrophage plasticity extends beyond the M1/M2 paradigm; studies have revealed twenty-eight stimuli capable of activating macrophages, including high-density lipoprotein, free fatty acids and stimuli associated with chronic inflammation (576). Traditionally, only stimuli associated with acute inflammation are included in the M1/M2 bipolar model and little weight is placed on the effect of the microenvironment on macrophage polarisation (573). Though macrophages have been shown to play a critical role in maintaining pregnancy, and dysregulation has been associated with adverse obstetric outcomes, the complex immunology of pregnancy is inadequately defined. Moreover, there is a paucity of evidence of the existence of macrophages with opposing functionalities of stimulating or down-regulating inflammation and a lack of understanding of macrophage phenotype by placental compartment across gestation and how these specific microenvironments may contribute to macrophage plasticity and preterm birth .

6.1.1 Hypothesis and aims

An initial hypothesis that an abundance of pro-inflammatory M1 compared to anti-inflammatory M2 phenotype macrophages would be evident in preterm placentas versus term placentas and in those with histologically-diagnosed inflammation was proposed. A review of literature focused on M1/M2 ratios in inflammatory conditions highlighted variance in the spatiotemporal choreography of inflammation resolution, thus the hypothesis extended to suggest macrophages may translocate in the placenta to sites of infection and inflammation in order to resolve inflammation. Alternatively, it was hypothesised that aberrant macrophage polarisation and/or localisation may account for nonresolving inflammation and subsequent PTB.

The aims of this chapter were to:

a) identify M1 and M2 phenotype macrophages in term and preterm placentas and in those with and without inflammation,
b) localise immunostained M1 and M2 macrophages to the three placental compartments, chorionic plate, villous tissue and decidua basalis, in term and preterm placentas and in those with and without inflammation,
c) quantify numbers of M1 and M2 macrophages in selected fields of view for each placental compartment, using a uniform random sampling technique, and,
d) compare numbers and localisation of total, M1 and M2 macrophages in term and preterm placentas and in those with and without inflammation.

This pilot study set out to determine the feasibility of quantifying and localising macrophages in the placenta and was intended to inform appropriately powered studies. However, due to restrictions on laboratory access and reagent availability imposed by the Covid-19 pandemic, time and resources did not permit these further investigations to be completed. The results and discussion presented in this chapter therefore focus on these preliminary investigations and findings from these.

6.2 Materials and methods

6.2.1 Participant recruitment and immunohistochemical techniques

A full description of ethical approval, participant recruitment, study design, collection, processing and reporting of placental samples plus methods and reagents used in this chapter is set out in *Chapter 2: Materials and Methods*.

6.2.2 Sample selection

In total, twelve samples were selected for macrophage analyses. Samples were selected at random from placentas delivered between 21 May 2019 and 24 January 2020 where full thickness formalin-fixed paraffin embedded blocks were available, and a complete histopathological evaluation of the placenta had been performed. Six preterm and six term samples were selected, of each group three with a histologically-diagnosed placental inflammatory response and three without were included for analysis. For the purposes of this

chapter, placental samples were abbreviated depending on whether they were preterm (PT) or term (T) and whether there was presence or absence of a placental inflammatory response (PIR or No PIR) as shown in Table 6.1.

Table 6. 1 Study group characterisation and abbreviation

Study Group	Abbreviation
Preterm no placental inflammatory response	PT/No PIR
Preterm with placental inflammatory response	PT/PIR
Term no placental inflammatory response	T/No PIR
Term with placental inflammatory response	T/PIR

6.2.3 Immunofluorescence staining

To determine whether cells were positive for CD68, CD80 and CD163, triple staining on sequential slides was initially performed. Extensive investigation showed the combination of CD68, CD80 and CD163 did not give reliable results due to the microscopy difficulties in detecting distinct differences between the wavelengths of light of AlexaFluor® 405nm (CD80) and AlexaFluor® 461nm (4',6-diamidino-2-phenylindole; DAPI). Therefore, double-labelled staining was performed on each sample to ascertain if cells were positive for only CD68, a pan-macrophage marker being used in place of a specific M1 marker, or both CD68 and a cell-surface marker specific to M2 macrophages, CD163.

Primary, secondary and IgG non-immune antibodies used for immunostaining and as isotype controls are described in detail in Section 2.7 and Table 2.8 of *Chapter 2*. All immunofluorescence staining was conducted using a hand-staining technique in one single run. Negative control slides are detailed in *Chapter 2*, a positive control slide using tonsil tissue kindly provided by SCH was included when staining placental samples for this arm of the study.

6.2.4 Image acquisition

As detailed in Section 2.7.3, immunostained sections were blind-coded to limit bias and visualised using a 40X objective on an EVOS FL Auto fluorescence microscope (Life Technologies). Software on the EVOS microscope allowed for automatic grid-lining and positioning of the slide from which six random squares, representing a field of view, were selected, two within the chorionic plate, two in the villous tissue and two in the decidua basalis. Although initially selected at random, squares at the edge of the grids were not chosen to ensure boundaries of the samples were avoided as these are not representative of the whole tissue due to sampling procedures. Uniformity was ensured in that the same grid squares were selected for each sample imaged. Cell counts were performed on unenhanced images and only cells with a clearly stained nucleus were counted.

6.2.5 Statistical analyses

Statistical analyses were performed as described in *Chapter 2: Materials and Methods*. Additionally, two-tailed Student's *t*-tests were conducted to compare the mean macrophage numbers when the cohort were (i) categorised to those who delivered preterm (*n*=6) and those who delivered at term (*n*=6), (ii) reported with the presence (PIR; *n*=6) or absence (no PIR; *n*=6) of a placental inflammatory response, and, (iii) classified to M1 and M2 macrophage phenotypes. To determine significant associations between categorical variables, Fisher's exact tests were performed on 2 x 2 contingency tables and Chi-square tests were performed on 2 x 3 contingency tables. Results were considered statistically significant at a *P*-value <0.05.

6.3 Results

6.3.1 Clinical characteristics of study population

The study utilised placental samples collected as part of the wider studies and PRIME projects. Twelve placentas were selected post-histopathological evaluation for assessment of macrophage numbers, phenotype and location based on delivery preterm without PIR, preterm with PIR, term without PIR and term with PIR (*n*=3 per group). Characteristics for this cohort are shown in Table 6.2. Statistical analyses were not performed on these data.

Table 6. 2 Brief overview of clinical characteristics of study population

Characteristic	Preterm/No PIR (*n* = 3)	Preterm/PIR (*n* = 3)	Term/No PIR (*n* = 3)	Term/PIR (*n* = 3)
Maternal age (years)	20.91 ± 0.87	30.17 ± 2.98	28.95 ± 5.98	35.50 ± 6.16
BMI (kg/m^2)	30.90 ± 8.51	26.00 ± 4.36	23.33 ± 2.32	28.66 ± 9.89
Gestational age (weeks)	34.67 ± 1.80	33.80 ±4.16	39.03 ±0.87	40.0 ± 0.40
Parity (median: range)^	1: 0-3	0: 0-1	1: 0-1	1: 0-1
Rupture of membranes (n)^				
PPROM	0	1	0	0
SROM	0	0	1	2
AROM	1	2	2	1
Other	2	0	0	0
Mode of delivery (*n*) ^ CS	2	0	0	0
SVD	1	3	3	3
Duration of labour (hours)	0.4 ± 0.7	5.9 ± 4.2	4.8 ± 2.1	11.0 ± 9.9
Duration of ROM (hours)[†]	0.0 ± 0.0 (*n*=2)	1009.6 ± 1423.3 (*n*=2)	1.67 ± 1.9 (*n*=2)	11.7 ± 0.5
Birthweight (g)	1627 ± 366	2625 ± 502	3500 ± 298	3566 ± 350
Sex (*n*) ^ Male	2	2	1	1
Female	1	1	2	2

AROM; artificial rupture of membranes, *BMI*; body mass index, *CS*; caesarean section, *PIR*; placental inflammatory response, *PPROM*; premature prelabour rupture of membranes, *ROM*; rupture of membranes, *SROM*; spontaneous rupture of membranes, *SVD*; standard vaginal delivery. Values are presented as mean ± SD unless stated otherwise

6.3.2 Immunofluorescence staining can identify macrophages in placental compartments and classify macrophages by phenotype

To achieve aim (a), that is to identify and phenotype macrophages in the placenta, samples were double stained using fluorescent polyclonal secondary antibodies. As a pan-macrophage marker expressed in the cytosol, an anti-CD68 primary antibody (Cat No. ab201340; Abcam, Cambridge, MA, USA) and red fluorescing secondary antibody (Alexa Fluor® 680, Cat No. ab175775; Abcam, Cambridge, MA, USA) were used to identify the presence of *any* phenotype macrophage within the placenta. An antibody against the M2 macrophage-specific cell surface marker, CD163 (Cat No. ab106162; Abcam, Cambridge, MA, USA), was used to identify M2 phenotype macrophages by the addition of a green fluorescing secondary antibody (Alexa Fluor® 488, Cat No. ab150073; Abcam, Cambridge, MA, USA). DAPI (AlexaFluor® 461nm, Cat No. ab285390; Abcam, Cambridge, MA, USA) was added to stain nuclei blue.

It was possible to identify individual macrophage cells in all compartments of the placenta, namely the chorionic plate (CP), villous tissue (VT) and decidua basalis (DB). It was further possible to categorise macrophages by phenotype based on immunoreactivity; M1 phenotype macrophages, specifically those which were $CD68^{+}/CD163^{-}$ and fluoresced only red, and M2 phenotype macrophages, as indicated by both red and green fluorescence and CD68/CD163-positivity, were observed within the cohort. A representative photomicrograph of an area of the chorionic plate from each of the term/No PIR (A), term/PIR (B), preterm No PIR (C) and preterm/No PIR (D) groups is shown in Figure 6.1. Total numbers and proportions of M1 and M2 macrophages varied between samples, as highlighted by the images, however, macrophages were observed in at least one of the selected fields of view for all twelve samples. Figure 6.1A shows a potential single M1 macrophage in the image; it is not possible to distinguish clear DAPI nuclear staining, so this cell was not counted as a macrophage for study purposes. The image is included to demonstrate the variability in the samples. More cells overall are visible in the preterm placental samples (Figure 6.1C and D) compared to the term placental samples (Figure 6.1A and B), yet there is also variability in the presence of M1 and M2 macrophages and proportions of the two phenotypes.

Figure 6.2 shows representative examples of macrophage presence in villous tissue of the four groups, labelled A-D as above. Again, the presence and proportions of M1 and M2 macrophages is variable between the groups. In images 6.2A and C, M1 macrophages are shown in the maternal space outside the villi where they would be circulating in maternal blood. As described in *Chapter 2: Materials and Methods*, due to issues with red blood cells autofluorescing and affecting the signal from both M1 and M2 macrophages, samples were quenched with Vector® TrueVIEW® Autofluorescence Quenching reagents (Cat. No. SP-8400-15, Vector Laboratories, Peterborough, Cambridgeshire, UK) resulting in the appearance of an empty space in the images. M2 macrophages primarily reside in the villi, close to the syncytiotrophoblasts and this is also evident in Figure 6.2A-D. A single M1 macrophage is shown in Figure 6.1B within a villous (highlighted by a narrow white arrow); this is an unexpected finding as placental macrophages, or Hofbauer cells, are generally considered to be of an M2 phenotype which do not polarise to M1 (209). A clear example showing the difference in expression, and therefore staining, of CD68 and CD163 is highlighted by a wide yellow arrow (Figure 6.2D). Here, the blue DAPI stained nucleus is surrounded by the red CD68-stained cytosol which is then enclosed by the cell surface marker CD163 which fluoresces green. Variability in decidua basalis images is likely to have arisen due to gestational age differences, delivery methods and sampling procedures. In Figure 6.3A and C, villi can be seen and macrophages within (A and C) and around these (A) are clearly visible. However, in the both the term and preterm samples with PIR (B and D), there are minimal numbers of any cell types in the selected field of view, although small numbers of M1 macrophages are present.

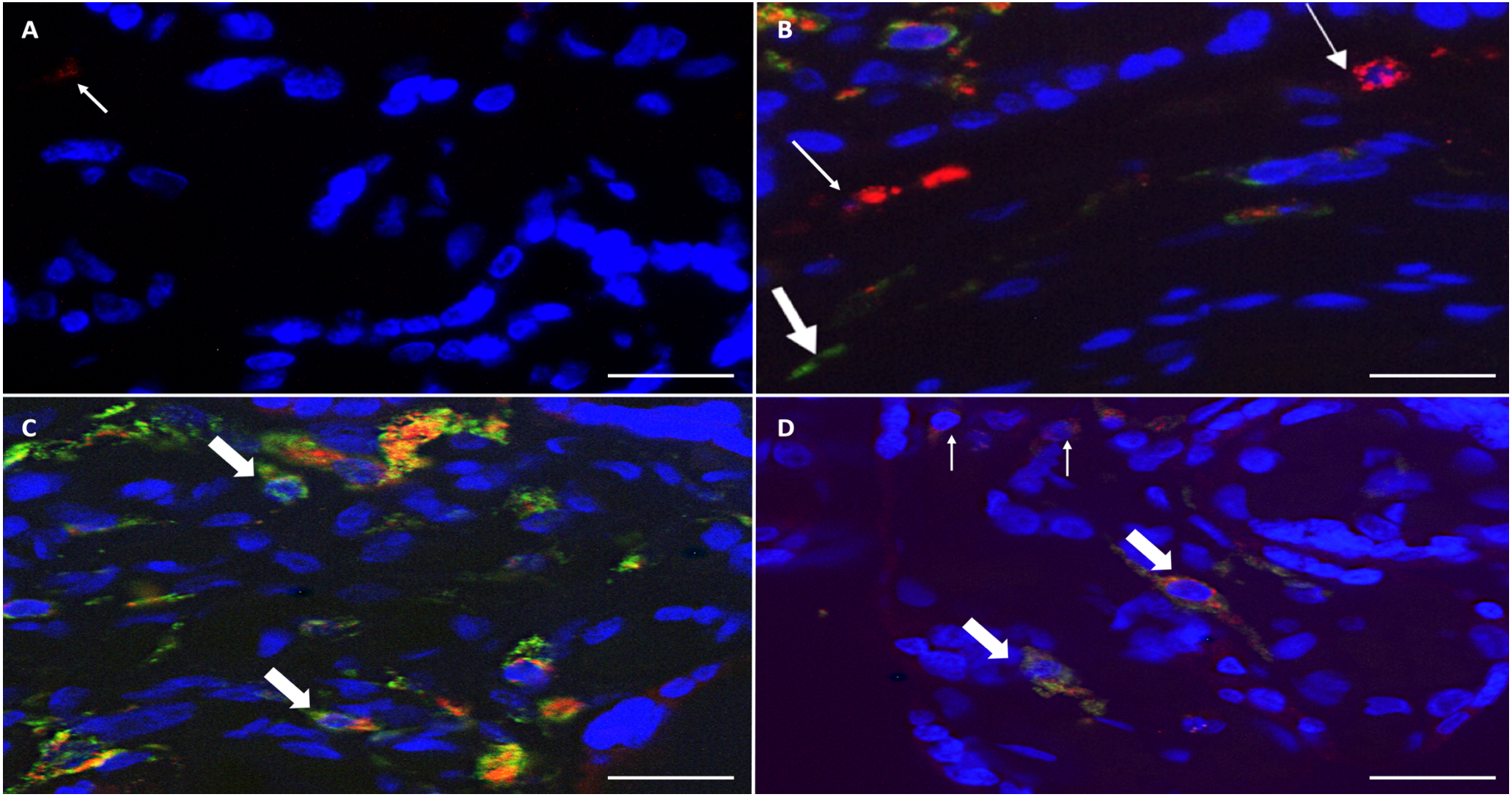

Figure 6. 1 Representative photomicrographs of M1 and M2 phenotype macrophages in the chorionic plate from each of the four study groups. Original magnification 60X.
Immunofluorescence staining was able to detect both M1 $CD68^{+}/CD163^{-}$ (red stain, narrow arrows) and M2 $CD68^{+}/CD163^{+}$ (red and green stain, wide arrows) cells in all samples, but images highlight variability in number of macrophages present. **A** Term/No PIR. One possible $CD68^{+}/CD163^{-}$ macrophage can be seen to the top left of the image although nuclear staining with DAPI is not visible so this would not have been included in cell counts. **B** Term/PIR. Presence of both $CD68^{+}/CD163^{-}$ and $CD68^{+}/CD163^{+}$ is highlighted in this sample. **C** Preterm/No PIR. More cells are visible in the sample, however, only $CD68^{+}/CD163^{+}$ macrophages were observed. **D** Preterm/PIR. Presence of both $CD68^{+}/CD163^{-}$ and $CD68^{+}/CD163^{+}$ is highlighted within this sample. Nuclei are stained blue with DAPI. Scale bar represents 10µm.

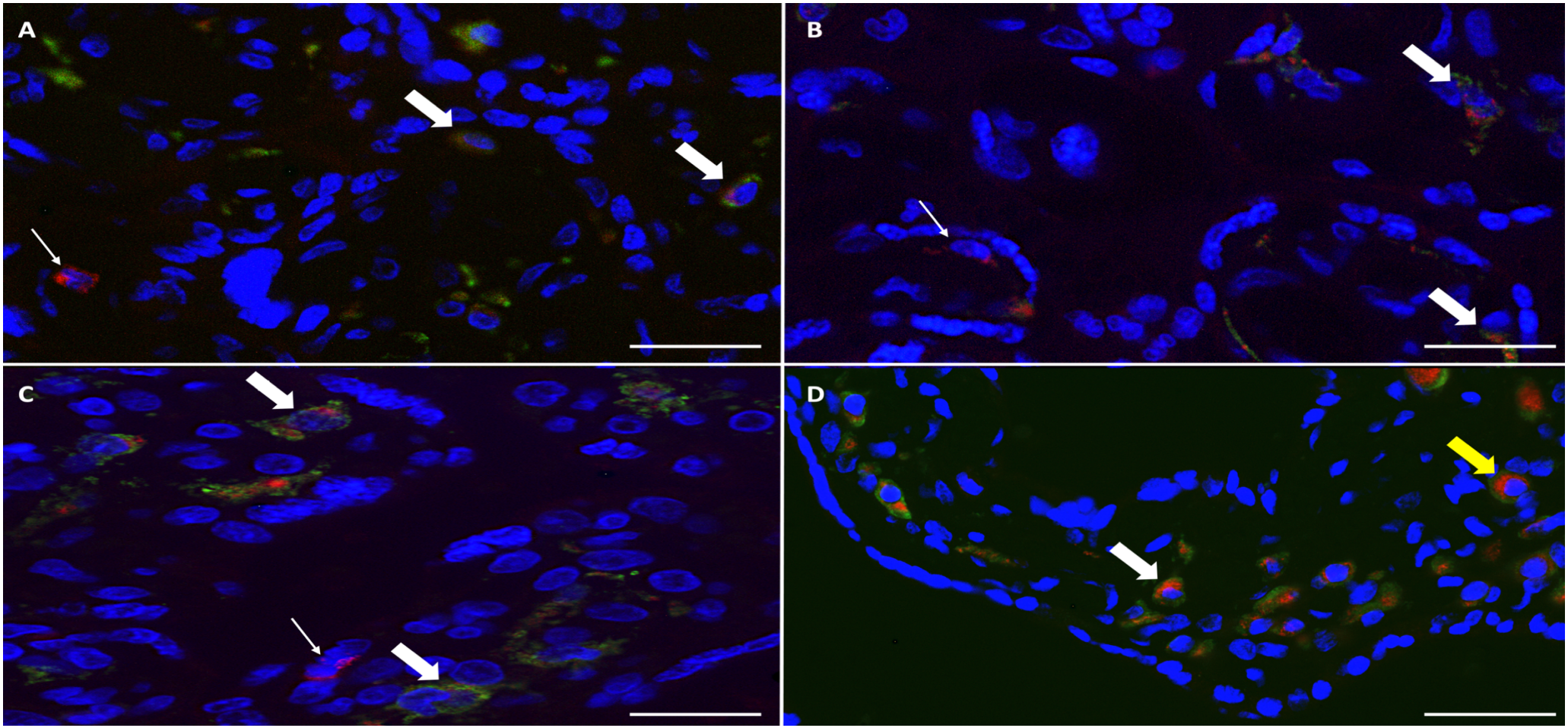

Figure 6. 2 Representative photomicrographs of M1 and M2 phenotype macrophages in the villous tissue from each of the four study groups. Original Magnification 60X.

Immunofluorescence staining was able to detect both M1 $CD68^{+}/CD163^{-}$ (red stain, narrow arrows) and M2 $CD68^{+}/CD163^{+}$ (red and green stain, wide arrows) cells in all samples, but images highlight variability in number of macrophages present. **A** Term/No PIR. Presence of both $CD68^{+}/CD163^{-}$ and $CD68^{+}/CD163^{+}$ is highlighted in this sample. M1 $CD68^{+}/CD163^{-}$ (narrow arrow) cell is observed in the maternal space outside the villi whereas M2 $CD68^{+}/CD163^{+}$ are observed within the villi close to the syncytiotrophoblastic barrier. **B** Term/PIR. $CD68^{+}/CD163^{-}$ and $CD68^{+}/CD163^{+}$ present in the sample; M1 $CD68^{+}/CD163^{-}$ unusually seen within the villi. **C** Preterm/No PIR. M2 $CD68^{+}/CD163^{+}$ cells seen within the top left villi. Arrows bottom centre show $CD68^{+}/CD163^{-}$ and $CD68^{+}/CD163^{+}$ separated by syncytiotrophoblasts. **D** Preterm/PIR. No $CD68^{+}/CD163^{-}$ were observed in this field of view for the sample, however, the presence of $CD68^{+}/CD163^{+}$ macrophages is highlighted. The yellow arrow shows a clear example of the difference in expression location of cytosolic CD68 and CD163 which is found on the cell surface. Nuclei are stained blue with DAPI. Scale bar represents 10µm.

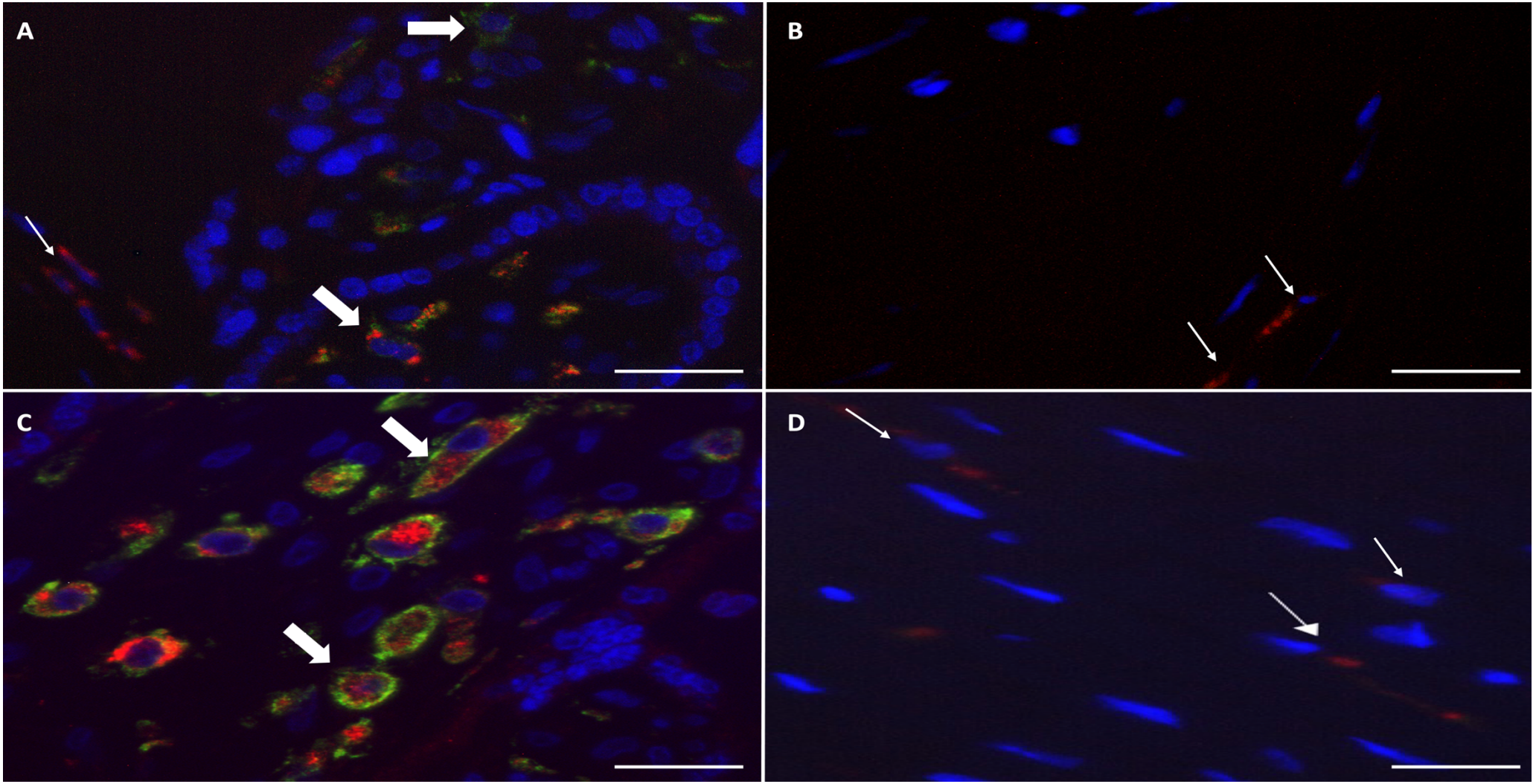

Figure 6. 3 Representative photomicrographs of M1 and M2 phenotype macrophages in the decidua basalis from each of the four study groups. Original magnification 60X.

Immunofluorescence staining was able to detect both M1 $CD68^{+}/CD163^{-}$ (red stain, narrow arrows) and M2 $CD68^{+}/CD163^{+}$ (red and green stain, wide arrows) cells in all samples, but images highlight variability in number of macrophages present. **A** Term/No PIR. Presence of both $CD68^{+}/CD163^{-}$ and $CD68^{+}/CD163^{+}$ is highlighted in this sample with $CD68^{+}/CD163^{-}$ cells located in the maternal space and $CD68^{+}/CD163^{+}$ cells within the villi present in the tissue. **B** Term/PIR. Minimal numbers of any cells were seen in this field of view; however, $CD68^{+}/CD163^{-}$ were present although $CD68^{+}/CD163^{+}$ were not observed. **C** Preterm/No PIR. More cells are visible in the sample, however, as with the villous tissue from this sample, only $CD68^{+}/CD163^{+}$ macrophages were observed. **D** Preterm/PIR. Villi are generally absent from the decidua basalis, as seen in this sample, no $CD68^{+}/CD163^{+}$ were observed in this field of view, although $CD68^{+}/CD163^{-}$ macrophages were present. Nuclei are stained blue with DAPI. Scale bar represents 10µm.

6.3.3 Total numbers of macrophages do not differ between preterm and term placentas, or those with and without a reported placental inflammatory response

The total number of macrophages counted in full thickness placental tissue was classified according to preterm and term births and by the presence or absence of a histologically-diagnosed placental inflammatory response (PIR) to determine any differences between study groups. In the whole cohort, total numbers of immunostained macrophages did not significantly differ between the preterm and term-delivered placentas (388 vs. 310; 55.6% vs. 44.4%, *P*=0.4843). Over half (53.6%) of all macrophages were counted in placentas reported with no PIR, but Student's *t*-test revealed no statistically significant effect for the presence or absence of a PIR (*P*=0.6564). There was, however, a significantly higher proportion of M2 macrophages compared to M1 macrophages in the whole cohort (*P*=0.0002). Of the 698 macrophages counted from twelve samples, three-quarters (*n*=569; 75.8%) were immunopositive for both CD68 and CD163 markers and thus considered to be an M2 phenotype.

Categorising placentas to four study groups showed no significant differences in the total number of macrophages per group when analysed by one-way ANOVA (*P*=0.3239). More macrophages were counted in the preterm with no PIR (32.8%) group than in the term with PIR (23.6%), preterm with PIR (22.8%) and the term with no PIR groups (20.8%) as shown in Figure 6.4. However, when a one-way ANOVA was performed to compare the effect of gestational age group (preterm or term) and PIR status (present or absent) on macrophage counts, this revealed there to be no significant difference (*F*(3,68)=[0.7304]; *P*=0.3239).

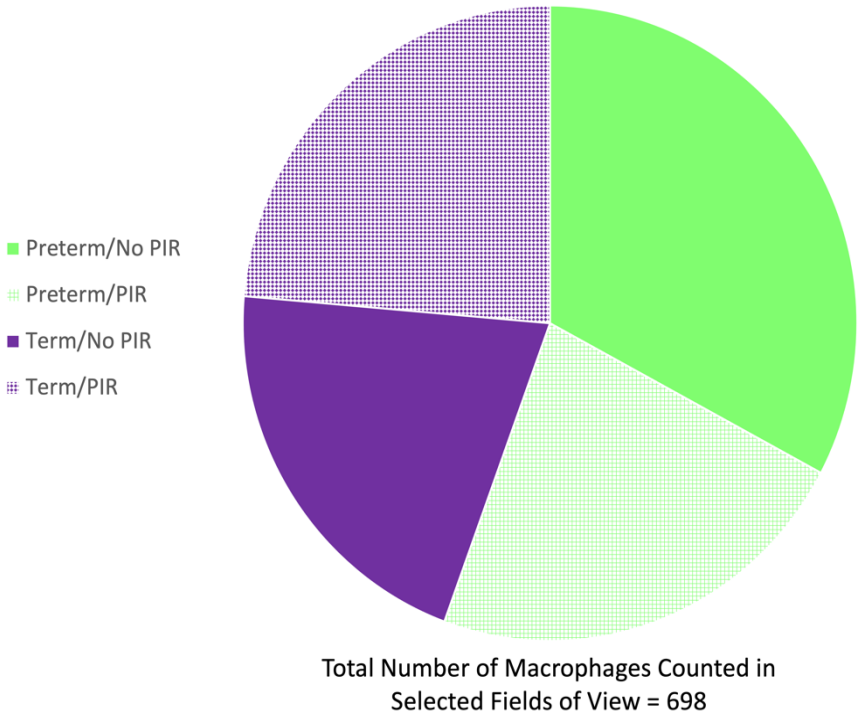

Figure 6. 4 Proportions of total macrophages counted in each study group

A total of 698 macrophages were counted across all samples (*n*=12). Although preterm placentas with no PIR had more macrophages overall than any of the other groups (*n*=229; 32.8%), this was not a significant difference (*P*=0.3239). *PIR;* placental inflammatory response.

6.3.4 Increased proportions of macrophages are significantly associated with placentas delivered preterm with no reported placental inflammatory response

Macrophage numbers were counted and totalled for placentas delivered preterm and those delivered at term (gestational age group) and again for those with PIR and those without (PIR status). In the preterm cohort, 41.0% (*n*=159) of macrophages were counted in placentas with a reported PIR compared to 53.2% (*n*=165) in those delivered at term with PIR. Fisher's exact test determined a statistically significant association between gestational age group and PIR status (*P*=0.0013) with the greatest proportion of macrophages counted in preterm placentas with no PIR (59.0%) (Table 6.3).

Table 6. 3 Total counts and proportions (%) of macrophages by gestational age group and reported placental inflammatory response status

	Term (*n* = 6) Macrophage Count (%)	**Preterm (*n* = 6) Macrophage Count (%)**	***P* Value***
PIR (*n* = 6)	165 (53.2)	159 (41.0)	0.0013
No PIR (*n* = 6)	145 (46.8)	229 (59.0)	

Proportion (%) of total count for group by column. *P;* probability value. *P*<0.05 considered to be significant.

6.3.5 There is a significant association between proportions of M2 phenotype macrophages and preterm delivered placentas and with placentas with no inflammatory response

M2 macrophages were the most abundant phenotype observed in placentas delivered either preterm or at term gestations, accounting for 84.8% (*n*=240) of all macrophages counted in the preterm cohort and 77.4% (*n*=329) in the term cohort. This association between preterm gestational age group and increased proportions of M2 macrophage phenotype was shown to be statistically significant by application of a Fisher's exact test (*P*=0.0141) (Table 6.4).

Likewise, irrespective of whether placentas were reported to have PIR or not, M2 macrophages were the prevalent phenotype. In placentas with PIR, 77.8% (*n*=252) of macrophages were polarised to an M2 phenotype compared to 84.8% (*n*=317) in the cohort reported with no PIR. Fisher's exact test was used to determine a statistically significant association between PIR status and macrophage phenotype with a greater proportion of M2 macrophages observed in placentas with no reported PIR (*P*=0.0191) (Table 6.4).

Table 6. 4 Total counts and proportions (%) of M1 and M2 phenotype macrophages by gestational age group and by reported placental inflammatory response status

	Term (*n* = 6) Macrophage Count (%)	Preterm (*n* = 6) Macrophage Count (%)	*P* Value*	No PIR (*n* = 6) Macrophage Count (%)	PIR (*n* = 6) Macrophage Count (%)	*P* Value*
M1 Phenotype (*n* = 6)	70 (22.6)	59 (15.2)	0.0141	57 (15.2)	72 (22.2)	0.0191
M2 Phenotype (*n* = 6)	240 (77.4)	329 (84.8)		317 (84.8)	252 (77.8)	

Proportion (%) of total count for group by column. *P;* probability value. *PIR; placental inflammatory response. P*<0.05 considered to be significant.

6.3.6 Reduced M1 macrophage proportions are significantly associated with an absence of inflammatory responses in placentas delivered preterm but not at term

Further analyses were conducted to identify if the proportion of M1/M2 macrophages was associated with PIR status in the preterm and term cohorts separately. The proportion of M1 macrophages in preterm placentas with no PIR was almost half that of term placentas with no PIR (11.4% vs. 21.4%), an association also shown to be statistically significant (*P*=0.0117). However, in the preterm and term with PIR cohorts no such difference was observed (M1 21.4% vs. 23.6%) and no statistically significant relationship was revealed (*P*=0.5935) (data not shown).

When considering the PIR status of the preterm cohort only, a statistically significant association was shown between PIR status and macrophage phenotype (*P*=0.0142) with a predominance of M2 macrophages in both the preterm with PIR and without PIR groups, but most notable in the no PIR cohort where 88.6% (*n*=203) of macrophages were an M2 phenotype compared to 79.2% (*n*=126) in the preterm with PIR group (Table 6.5). There was no statistically significant association between PIR status and macrophage phenotype in the term cohort when assessed by Fisher's exact test (*P*=0.6840). M2 macrophages were observed more frequently than M1 macrophages, but this was broadly equal in both regardless of the absence (78.6%; *n*=114) or presence of PIR (76.4%; *n*=126) (Table 6.5). Overall these results demonstrate an association with increased proportions of M2 phenotype macrophages in preterm placentas with no PIR.

Table 6. 5 Associations between total counts and proportions (%) of M1 and M2 phenotype macrophages in the preterm with and without a placental inflammatory response and in the term with and without a placental inflammatory response cohorts

	Preterm/No PIR (*n* = 6) Macrophage Count (%)	**Preterm/PIR (*n* = 6) Macrophage Count (%)**	***P* Value***	**Term/No PIR (*n* = 6) Macrophage Count (%)**	**Term/PIR (*n* = 6) Macrophage Count (%)**	***P* Value***
M1 Phenotype (*n* = 6)	26 (11.4)	33 (20.8)	0.0142	31 (21.4)	39 (23.6)	0.6840 ns
M2 Phenotype (*n* = 6)	203 (88.6)	126 (79.2)		114 (78.6)	126 (76.4)	

Proportion (%) of total count for group by column. *ns;* not significant, *P;* probability value, *PIR;* placental inflammatory response. *P*<0.05 considered to be significant.

6.3.7 Proportions of macrophages across placental compartments are not significantly associated with gestational age group

As discussed previously, proportions of M1/M2 macrophages have been suggested to fluctuate across gestation and in the presence of infection or inflammation, therefore, in order to meet the aim to localise macrophages in compartments of the placenta, analyses were conducted on macrophage numbers in the three defined compartments of the chorionic plate (CP), villous tissue (VT) and the decidua basalis (DB). Macrophages were counted in individual compartments as described previously and Chi-square tests were performed to assess whether there were any associations between macrophage counts in these placental compartments and gestational age group, PIR status and macrophage phenotype.

Villous tissue contained more macrophages than either the chorionic plate or the decidua basalis in both the preterm and term cohorts accounting for 45.6% and 51.0% of the total macrophage counts, respectively. In both groups, the chorionic plate contained the lowest proportion of total macrophages with similar values of 23.5% of the total counted in the preterm cohort CP and 22.9% in the term CP. Approximately one-third of macrophages were located in the decidua basalis of the preterm cohort (30.9%) compared to slightly more than a quarter of those counted in the term cohort (26.1%). No statistically significant association between placental compartment and gestational age group was shown when a Chi-square test was performed ($\chi^2_{df=2}$ = 2.428; P<0.2970) (Table 6.6).

6.3.8 There is no significant association between proportions of macrophages in each placental compartment and the presence or absence of a placental inflammatory response

PIR status was not statistically significantly associated with placental compartment ($\chi^2_{df=2}$ = 4.138; P<0.1263) (Table 6.6). No greater proportions of macrophages were observed in any placental compartment in association with a reported PIR; approximately half of all macrophages were counted in the villous tissue of placentas with no PIR (52.3%) and in those with PIR (44.7%). The chorionic plate contained the lowest proportion of macrophages in both cohorts (20.7% no PIR vs. 25.1% PIR) and approximately one third of macrophages were located in the decidua basalis of both the no PIR (27.0%) and the PIR (30.1%) cohorts.

6.3.9 Proportions of M1 and M2 phenotype macrophages are not significantly associated with their location within the placenta

Given that data have been published showing variance in the proportions of M1 and M2 macrophages in placental compartments, it was expected that there would be a statistically significant association between macrophage phenotype and placental compartment, however, when a Chi-square test was performed, no statistically significant association was shown ($\chi^2_{df=2}$ = 0.7586; P<0.5525) (Table 6.6). Unexpectedly since evidence suggests M1 macrophages are most abundant in the decidua basalis, 47.3% of counted M1 phenotype macrophages were located in the villous tissue, in proportion to M2 macrophages (48.2%) which are generally considered to be more abundant in this compartment. These balanced ratios of M1/M2 phenotype were replicated across the chorionic plate (M1 25.6% vs. M2 22.7%) and the decidua basalis (M1 27.1% vs. M2 29.1%) (Table 6.6).

Table 6. 6 Total counts and proportions (%) of macrophages in the three placental compartments with associations between macrophage location and gestational age group, placental inflammatory response status and ratio of M1 and M2 phenotype

	Term Macrophage Count (%)	Preterm Macrophage Count (%)	*P* Value*	No PIR Macrophage Count (%)	PIR Macrophage Count (%)	*P* Value*	M1 Phenotype Macrophage Count (%)	M2 Phenotype Macrophage Count (%)	*P* Value*
Chorionic Plate	71 (22.9)	91 (23.5)	0.2970 ns	63 (20.7)	99 (25.1)	0.1263 ns	33 (25.6)	129 (22.7)	0.5525 ns
Villous Tissue	158 (51.0)	177 (45.6)		159 (52.3)	176 (44.7)		61 (47.3)	274 (48.2)	
Decidua Basalis	81 (26.1)	126 (79.2)		82 (27.0)	119 (30.1)		35 (27.1)	166 (29.1)	

Proportion (%) of total count for group by column. *ns;* not significant, *P;* probability value. *PIR;* placental inflammatory response. *P*<0.05 considered to be significant.

6.4 Discussion

As key modulators of pregnancy (190), the stimulation of pro- and anti-inflammatory macrophages is understood, however, the contribution of the complement and localisation of polarised macrophages and their effects on placental homeostasis and prevention of immunogenic rejection remains to be defined (201). Consequently, this chapter sought to determine the feasibility of assessing macrophage abundance, phenotype and location within the placenta using an immunofluorescence technique and to understand if the presence of specific macrophage subsets may be associated with preterm birth or placental inflammation.

6.4.1 Macrophage polarity can be determined by immunofluorescence staining and reveals a significant difference in M1 and M2-polarised macrophages in the placenta

M1 and M2 polarised macrophages have been found to play vital roles in vascular remodelling and trophoblast invasion in early pregnancy, protection of the fetus throughout gestation and cervical ripening and membrane rupture before parturition (190, 201, 287, 290). Furthermore, evidence shows even minor fluctuations in macrophage numbers or polarisation states can result in poor pregnancy outcomes such as intrauterine growth restriction, pre-eclampsia, and preterm labour (190, 191). It is therefore critical to understand the composition of macrophage phenotype during gestation and the factors which may influence macrophage plasticity. Immunofluorescence staining techniques performed in this study successfully determined expression of protein markers for both M1 and M2 subpopulations. Furthermore, it was possible to orientate these macrophages across the three placental compartments. All macrophages abundantly express the heavily glycosylated glycoprotein CD68, therefore, its immunopositivity confirmed the presence of macrophages in the placenta (577); the combinatory expression of the macrophage-specific haemoglobin scavenger receptor, CD163, indicated an alternatively activated phenotype characterised as M2 polarisation (196, 578). Once it had been established that it was possible to study macrophage populations utilising immunofluorescence, total populations and phenotypic populations were examined within placentas delivered following spontaneous term labour and spontaneous preterm labour. In addition, the effects on macrophage populations in placentas with a reported inflammatory response (PIR) were compared to those with no PIR. In the present cohort, it was found that there was no significant difference in total macrophage numbers where placentas had been delivered preterm compared to at term.

Likewise, there was no difference between macrophage abundance in placentas with or without PIR. However, when gestational age group and PIR status were assessed for independence, macrophage numbers were significantly associated with gestational age and PIR status in that overall macrophage proportions were highest in preterm placentas with no reported PIR (32.8%). Interestingly, total counted macrophages were observed in similar proportions in preterm placentas without a reported PIR (22.8%) to the proportions seen in term placentas irrespective of whether PIR was present (23.6%) or absent (20.8%). Whether macrophage numbers in the preterm with no PIR group were elevated or those in the preterm with PIR group were reduced is not immediately clear. Likewise, conflicting evidence exists as to if and how macrophage numbers change in response to PIR, or in a temporal manner across gestation. If the data presented in this study are accepted as macrophage numbers being reduced in the presence of PIR, this is supported by studies authored by Ben Amara *et al*. and Vinnars *et al*. who reported significantly reduced expression of $CD68^+$ macrophages in placentas with histologically-diagnosed chorioamnionitis (HCA) compared to control placentas (213, 579), although there was variation in the number of fields of view selected for macrophage counting between both studies and the number described in this thesis (thirty, five and six, respectively) which may account for some of the variation. In contrast, studies which used a similar manual count and number of fields of view to those presented in this chapter, as authored by Hung *et al*., Toti *et al*. and, more recently, Bae *et al*., all report a significant increase in the number of $CD68^+$ macrophages in placentas with HCA, notably at term in the first two studies, in conflict with the findings of this chapter (580-582).

6.4.2 M2 phenotype macrophages are more abundant than M1 phenotype, but M1 proportions are increased in preterm placentas with no reported inflammatory response

Macrophage function is associated with phenotype; M1 macrophages exhibit a proinflammatory state whereas M2 macrophages display an anti-inflammatory state (201). It was found that M2-polarised macrophages predominated in the total cohort (75.8%), as has been previously reported following spontaneous labour (196). M2 populations were also significantly associated with preterm birth and an absence of PIR, and, in this group, there was a significant decline in the proportion of M1 macrophages (11.4%) in comparison to the proportions seen in preterm placentas with PIR (20.8%) even though the preterm with no PIR group had more macrophages overall than the preterm with no PIR group (59.0% vs. 41.0%).

M1 macrophages primarily secrete proinflammatory cytokines and chemotactic agents, such as IL-1β, IL-6, TNF-α, and IL-8, which have been shown to be significantly increased in gestational tissues during preterm and labour, so it was unexpected that their numbers were reduced (583, 584). As with overall numbers, it may be that rather than a decline in M1 numbers being observed in preterm placentas with no PIR, the results presented demonstrate in increase in M1 macrophages in preterm placentas with PIR. A recent study by Shan and colleagues appears to support this hypothesis in that the research showed a differential expression pattern of M1-expressed proinflammatory mediators in a mouse model with elevated expression of IL-1β, IL-6 and TNF-α observed in the myometrium in preterm and term labour. However, in preterm labour there was also significantly increased expression in the decidua and placenta which was not seen in term samples, and a simultaneous shift to an M1 macrophage dominance. Shan *et al.*'s study did not, however, observe any difference in pro-inflammatory cytokine expression between the PIR-induced and progesterone withdrawal-induced (to represent no PIR) models used in the study which would suggest the differential expression pattern is related only to the difference in gestational age at delivery (585). Nonetheless, it is known that, regardless of gestational age, expression of these inflammatory cytokines and chemokines is regulated by NFκB signalling (586) which follows parallel actions of functional withdrawal of progesterone and immunosuppressive glucocorticoids and activation of oestrogen, the result of which is stimulation of cyclooxygenase-2 (COX-2) and placental macrophage secretion of NFκB-induced inflammatory cytokines. Changes to the placental microenvironment and increased proinflammatory stimuli, in turn, classically activates placental macrophages to polarise to an M1 phenotype, a shift suggested to be involved in labour initiation (585, 587). In contrast to Shan *et al*.'s findings, M1 and M2 proportions did differ between placentas with PIR and those without in the present study. This suggests a variation in the upstream stimulus activating NFκB signalling and activation of proinflammatory genes, such as that described in Section 1.2.1.2, whereby endogenous damage-associated molecular pattern molecules (DAMPs) are released in response to cellular stress or tissue damage, potentially due to placental hypoxia or ischemia from vascular malperfusion which is unrelated to inflammatory lesions, as has been observed frequently in preterm births in this study cohort. The subsequent release of the alarmin IL-1α from necrotic cells and its binding to the pattern recognition receptor (PRR)

IL-1R then results in the same downstream effects as those seen in LPS-induced macrophage secretion of IL-1β, ultimately leading to labour initiation (65, 73). Nonetheless, it appears that a more prolonged and intense response to proinflammatory cytokine release occurs in preterm placentas with PIR than those without which promotes further recruitment of macrophages, polarisation to M1 phenotype and sustained expression of proinflammatory proteins in a positive feed-forward loop (585, 588).

6.4.3 Conclusions

Immunofluorescence is a valuable method for examining placental macrophage numbers and phenotypes, however, it is not without its limitations. Some researchers suggest expression of M1- and M2-specific macrophage markers is only measurable at specific trimesters of gestation whilst others state CD163 alone is not a reliable generic marker for M2 macrophages *in vivo* (196, 597). However, in this small cohort, both CD68 and CD163 were detectable, and all macrophages classified to an M2 phenotype displayed immunopositivity to both CD68 and CD163 suggesting a) they were macrophages, and b) in this case, staining had been able to distinguish between M1 and M2 phenotypes. Future work should focus on larger sample sizes taken from a wider range of gestational ages to minimise the effects of temporal changes in marker expression. Additionally, to ensure specificity in macrophage marker expression and provide consideration for macrophage protein expression fluctuations across gestation, more suitable co-expressed markers, for example hypoxia-inducible factor-1 alpha (HIF-1α) and signal transducer and activator of transcription-3 (STAT3), transcription factors known to regulate M1 and M2 polarisation respectively (597), should be included in immunohistochemical or immunofluorescence studies. Data presented in this chapter revealed a significant association between macrophage abundance in preterm placentas with no PIR and in macrophage polarisation states. The differences observed in proportions of macrophages and, specifically, the increased abundance of M1 phenotype macrophages in preterm placentas with no reported PIR suggests the mechanisms underlying preterm labour in cases of PIR differs to that of preterm labour with no PIR. Whether this stems from differences in upstream stimuli, recruitment and activation of immune cells other than macrophages or a differential response to secreted proinflammatory cytokines remains unclear. Studies assessing macrophage functional activity and immune cell composition are therefore necessary.

Chapter 7: The role of Natural Antimicrobial Proteins and Inflammation-Associated Cytokines and Chemokines in Preterm Birth

7.1 Introduction

Mediation of the immune system is critical for maintaining pregnancy and initiating timely labour (598). A bias towards an anti-inflammatory T helper cell (Th2) environment and the subsequent engagement of cytokine-signalling pathways promotes maternofetal tolerance and prevents aberrant cellular behaviour (248, 279). Labour is a known inflammatory event characterised by leukocytic infiltration to the cervix, myometrium, placenta, fetal membranes and maternal peripheral blood (263, 599, 600). Data from rat and human pregnancies suggests placental mitochondrial DNA and syncytiotrophoblast membrane microparticles are released in inflammatory environments and that these contribute to parturition-associated inflammation through induction of cytokine secretion (601). Nonetheless, there has been a long-held belief that different conditions are necessary to initiate preterm and term labour (602) and that a more robust response to infiltration of leukocytes to the fetal membranes is observed in preterm births compared to term (603). Alongside a tightly-regulated immune balance, endogenous antimicrobial proteins (AMPs) of the cathelicidin, defensin and whey acidic protein (WAP) motif-containing-proteins families contribute to the broad-spectrum protection against intra-uterine infection during pregnancy (224). The range of antimicrobial activity exhibited by these natural antimicrobials is extensive and comprises Gram-negative and Gram-positive bacteria, mycobacteria, fungi and viruses (224, 236). In addition to their antimicrobial actions, AMPs represent a promising therapeutic option given their anti-inflammatory, immunomodulatory and wound-healing properties, coupled with their ability to bind and neutralise bacterial endotoxins (217, 218). With that in mind, the aim of this chapter was to determine variations in selected cytokine and AMP expression in preterm versus term placentas and in placentas with and without a PIR. Initial hypotheses were that altered expression of pro-inflammatory cytokines and their regulation of natural AMPs is associated with preterm labour and increasing grades of PIR.

Following extensive review of the literature, a select panel of pro- and anti-inflammatory cytokines were chosen to determine their contribution to timing of labour and influence on PIR status, specifically, these included interferon-gamma (IFN-γ), interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10). Additionally, two AMPs were selected, firstly human cathelicidin antimicrobial protein (also known as human cationic antimicrobial protein) (hCAP18) was chosen due to the lack of understanding of its role in pregnancy and

parturition and a general paucity in research of this cathelicidin. Secondly, secretory leukocyte peptidase inhibitor (SLPI) was selected as it is secreted in abundance from neutrophils, the immune cells examined to determine grade and stage of PIR (103). Further, SLPI is increased by inflammatory stimuli (226) and has been associated with PPROM, MIAC and labour at both preterm and term gestations (233). The zinc- and calcium-dependent matrix metalloproteinase MMP-9 was included in the immune protein profile given its role in physiological and pathological membrane rupture in addition to the known inhibition of MMP-9 by SLPI (604-606).

7.1.1 Hypothesis and aims

The hypothesis for this chapter was that expression levels of target antimicrobial and inflammatory proteins would differ between preterm and term placentas. The aims of this chapter were to:

a) examine levels of target AMPs, cytokines, chemokines and MMPs, specifically hCAP18, SLPI, IFN-γ, IL-6, IL-8, IL-10 and MMP-9, in the placenta, and
b) investigate whether expression levels varied at preterm and term gestations or between placentas with and without a histologically-diagnosed inflammatory response.

7.2 Materials and methods

7.2.1 Participant Recruitment and Study Design

A full description of ethical approval, participant recruitment, study design and collection, processing and reporting of placental samples is set out in *Chapter 2.*

7.2.2 Sample Selection

Eight term and eight preterm full thickness placental samples were selected for molecular biological study from snap-frozen tissue previously stored at -80°C as part of PRIME studies. PIR status was determined based on histopathological evaluation as detailed in *Chapter 2: Materials and Methods*. Samples selected for this aspect of the study and their categorised group are shown in Table 7.1.

Table 7. 1 Placental samples grouped by outcome and placental inflammatory response status for ELISA analysis

Unique ID	Outcome	PIR Status	Group
SU0006	Preterm	PIR	PT/PIR
SU0022	Term	None	T/No PIR
SU0029	Preterm	PIR	PT/PIR
SU0030	Preterm	None	PT/No PIR
SU0034	Preterm	None	PT/No PIR
SU0035	Term	None	T/No PIR
SU0041	Preterm	PIR	PT/PIR
SU0043	Term	PIR	T/PIR
SU0045	Preterm	None	PT/No PIR
SU0049	Preterm	PIR	PT/PIR
SU0052	Term	None	T/No PIR
SU0054	Term	PIR	T/PIR
SU0059	Term	PIR	T/PIR
SU0069	Term	None	T/No PIR
SU0070	Term	PIR	T/PIR
SU0072	Preterm	None	PT/No PIR

PIR; placental inflammatory response, *PT*; preterm, T*;* term

7.2.3 Enzyme Linked Immunosorbent Assay (ELISA) Validation and Optimisation

Validation experiments were performed in accordance with the manufacturer's instructions to determine optimal sample dilutions for each assay. For each antibody, validation assays included one set of standard solutions, one blank sample and one sample each from the preterm with and without a histologically-diagnosed placental inflammatory response (PIR) and term with and without PIR groups. Samples were diluted in 1X PBS (pH 7.4) (Thermo Fisher Scientific; DLdevelop assays) or assay diluent (Assay Biotechnology; Omnikine assays) to concentrations of 0.2 mg/ml and 2 mg/ml before standard curves were constructed and sample measurements calculated. Assays were validated by performing serial dilutions to confirm that when absorbance was plotted a linear response parallel to the standard curve was obtained.

7.2.4 ELISAs for quantification of concentrations of proteins of interest

Taking into consideration results of the validation ELISAs and placental homogenate volumes, hCAP18, IFN-γ, IL-8, MMP-9 and SLPI assay samples were diluted 1:2 in assay diluent (Assay Biotechnology and Wuhan Fine Biotech Co.). IL-6 assay samples were diluted 1:10 in 1X PBS (Thermo Fisher Scientific) and IL-10 assay samples were analysed undiluted due to diluted IL-10 levels in validation ELISAs being at the very low end of the range of the standard curve (31.25 – 2000 pg/ml). Since the mass of total protein extracted per placental tissue sample and the volume of solution added to the ELISA were recorded, all results were converted from ELISA reported concentrations (pg/ml) to picogram of target protein per milligram of total protein (pg/mg). Reporting results in reference to the total protein from each placental tissue sample subsequently allowed for comparisons between ELISAs performed with differing dilutions factors. All target protein raw data is located in Appendix IV.Va.

7.2.5 Data and statistical analyses

GraphPad Prism 9.1.1 (GraphPad Software Inc., San Diego, CA, USA) and Excel (Microsoft Corp., Redmond, WA, USA) were used for all statistical analyses. Coefficient of variability (CV) values were calculated from triplicate OD values for each standard and sample. CV values >20% were excluded from subsequent analyses in order to improve accuracy of results. Standard curves were constructed in GraphPad Prism before protein concentrations in placental homogenates were calculated by multiplying the values interpreted from the standard curve by the assay's applicable dilution factor (where required). Target protein concentrations were normalised to total protein levels. In addition to unpaired Student's *t*-tests or Mann-witney *U* tests to compare groups, Spearman correlations were performed on data collected for this chapter to test relationships between expression levels of target proteins in preterm and term cohorts and in placentas with and without PIR. A two-sided *P* value of <0.05 was considered to be statistically significant for all analyses.

7.3 Results

7.3.1 Characteristics of study population

The study utilised placental samples collected as part of the wider studies and PRIME projects. Sixteen placentas were selected post-histopathological evaluation for assessment of levels of specific antimicrobial proteins (AMPs), matrix metalloproteinases (MMPs), chemokines and cytokines. Samples were categorised as being delivered preterm (<37 weeks; *n*=8) or term (≥37 weeks; *n*=8) for initial analysis and as having at least one reported placental inflammatory response (PIR; *n*=8) or no PIR (*n*=8) for subsequent analyses. Characteristics for this cohort are shown in Table 7.2.

Table 7. 2 Brief overview of clinical characteristics of study population

Characteristic		Preterm (*n* = 8)	Term (*n* = 8)	*P Value
Maternal age (years)		28.96 ± 7.14	30.28 ± 8.12	0.6966 ns
BMI (kg/m^2)		24.77 ± 2.65	24.74 ± 5.45	0.9922 ns
Gestational age (weeks)		34.01 ± 2.55	39.66 ± 0.92	n/a
Parity		0.5 ± 0.5	0.6 ± 1.0	0.7703 ns
Rupture of membranes (n)^				0.0188
	PPROM	4	0	
	SROM	0	3	
	AROM	3	5	
	Other	1	0	
Mode of delivery (*n*) ^	CS	1	0	0.7333 ns
	SVD	6	7	
	IVD	1	1	
Duration of labour (hours)		5.7 ± 3.6	7.2 ± 6.7	0.5624 ns
Duration of ROM (hours)†		433.0 ± 885.2 (*n*=5)	6.0 ± 5.6 (*n*=7)	0.0177
Birthweight (g)		2001 ± 565.7	3526 ± 247.7	n/a
Sex (*n*) §	Male	2	4	0.6084 ns
	Female	6	4	

AROM; artificial rupture of membranes, *BMI*; body mass index, *CS*; Caesarean section*, IVD; instrumental vaginal delivery, PIR*; placental inflammatory response, *PPROM*; premature prelabour rupture of membranes, *ROM*; rupture of membranes, *SROM*; spontaneous rupture of membranes, *SVD*; standard vaginal delivery. Values are presented as mean ± SD unless stated otherwise. *Student's *t*-test, §Fisher's exact test, ^Fisher's exact test with Freeman Halton extension, †Mann-Whitney *U* test. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant. *n/a* not analysed.

As detailed in Table 7.2, there was no statistically significant difference in maternal age, BMI at booking or parity between women delivering preterm and those delivering at term (*P*=0.6966, *P*=0.9922 and *P*=0.7703, respectively). Data were collected on whether women in the study presented with PPROM, SROM ≥37 weeks, AROM or Other. No women in the term group presented with PPROM before then delivering ≥37 weeks, however, 50% of women delivering preterm experienced PPROM. The duration of rupture of membranes was significantly longer before preterm births when compared to term births (*P*=0.0177), yet the duration of labour (DoL), determined as being from the time of ≥4cm cervical dilation as recorded in clinical notes (established labour), did not differ significantly between the two groups (*P*=0.5624). There were more females than males born (6:2) in the preterm group and, although in the term group there was an equal proportion of female to male neonates (4:4), the difference between groups was not statistically significant (*P*=0.6084), potentially due to the small sample sizes in the study (Table 7.2).

7.3.2 Target protein levels do not differ between preterm and term placentas

The greatest mean protein expression was observed for the AMP hCAP18 term samples (152.4 ± 54.34 pg/mg) and the lowest expression was recorded for the anti-inflammatory cytokine IL-10, also in the term group (1.258 ± 0.3282 pg/mg). Mean expression levels of all target proteins as measured by commercial 96T sandwich ELISA kits, did not significantly differ between preterm and term placentas as shown in Table 7.3. Furthermore there was no identifiable trend of non-significant increase or decrease of any of the proteins between preterm and term cohorts.

Table 7. 3 Mean concentration (pg/mg) of specific target proteins in preterm and term placentas

Protein	Mean Protein Concentration (pg/mg)		*P V*alue
	Preterm (*n* = 8)	Term (*n* = 8)	
hCAP18	148.4 ± 54.52	152.4 ± 54.34	0.8844 ns
IFN-γ[†]	2.598 ± 1.140	2.640 ± 1.149	0.5737 ns
IL-6	11.34 ± 9.028	11.19 ± 3.999	0.9666 ns
IL-8[†]	5.804 ± 8.399	1.895 ± 2.386	0.2786 ns
IL-10	1.396 ± 0.5391	1.258 ± 0.3282	0.5473 ns
MMP-9[†]	66.73 ± 34.22	78.78 ± 40.86	0.4418 ns
SLPI	50.48 ± 19.30	42.81 ± 12.73	0.3639 ns

hCAP18; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *SLPI*; Secretory Leukocyte Peptidase Inhibitor. Student's *t* test, except [†]Mann-Whitney *U* test. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

Mean total protein concentration in placentas delivered following a preterm birth was 13.52 ± 1.772 mg/ml and this did not differ significantly from placentas from term births where the mean total protein concentration was 14.07 ± 2.737 mg/ml (Figure 7.1A; *P*=0.6387). When preterm and term samples were combined and compared based on their inflammatory status (PIR or no PIR), there was no significant difference in the mean total protein concentration in placentas with PIR (13.28 ± 2.479 µg/ml) versus those with no PIR (14.30 ± 2.016 µg/ml) (Figure 7.1B; *P*=0.3827).

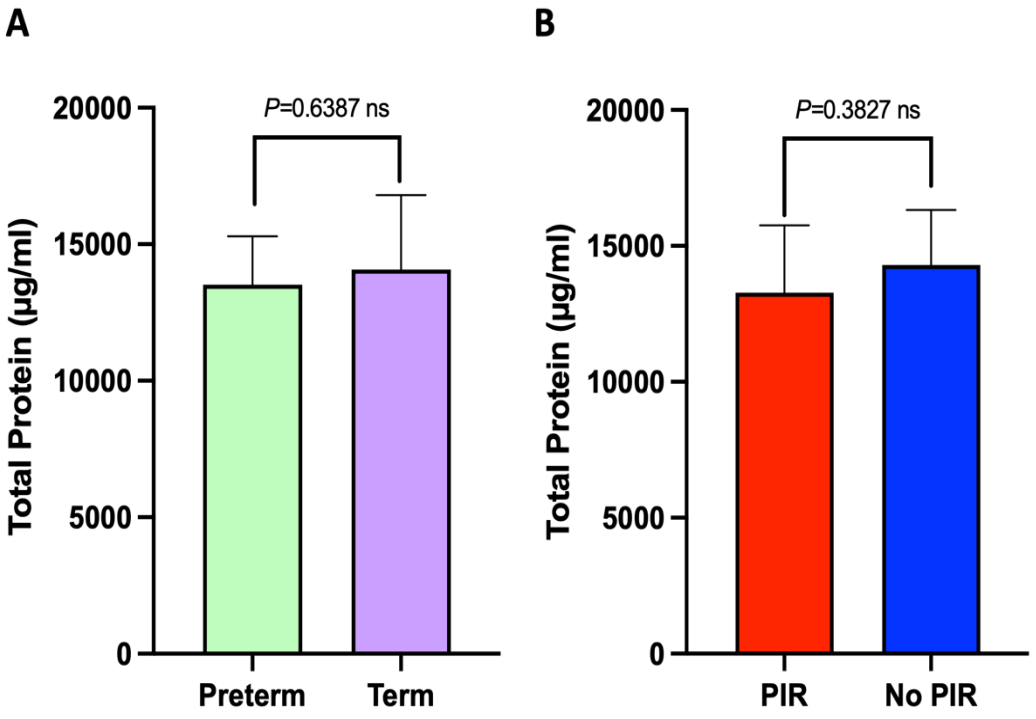

Figure 7. 1 Total Protein Concentration in Preterm versus Term Placentas (A) and in Placentas with PIR and without (B)

Samples were categorised to preterm or term and then with or without PIR for comparison of total protein concentrations. No significant differences in total protein concentration were found between delivery outcome or placental inflammatory status when assessed by Student's *t* tests.

7.3.3 Expression levels of the natural antimicrobial protein SLPI but not hCAP18 are significantly increased in placentas with a histologically-diagnosed inflammatory response

To assess differences in AMP expression between placentas with and without histologically-diagnosed PIR, Student's *t*-tests were performed on collected target protein concentration data. hCAP18 was detected in all eighteen placental samples, at higher concentrations than any of the other assessed proteins, but to varying levels (range: 77.80 - 238.6 pg/mg). The mean concentration ± SD of hCAP18 in placentas with a histologically-diagnosed PIR (175.5 ± 56.20 pg/mg) was slightly higher than that of those with no PIR (125.3 ± 36.53 pg/mg) but this was not statistically significant (*P*=0.524) (Figure 7.2A).

SLPI was also expressed in each of the full thickness placental tissue homogenates at concentrations between 28.93 and 88.51 pg/mg. As expected since neutrophils highly express SLPI and it is the number, confluence and location of neutrophils which is used to report PIRs, mean SLPI concentrations were significantly higher in placentas with PIR (55.06 ± 18.35 pg/mg) than in those with no PIR (38.23 ± 8.338 pg/mg; *P*=0.0332) (Figure 7.2B).

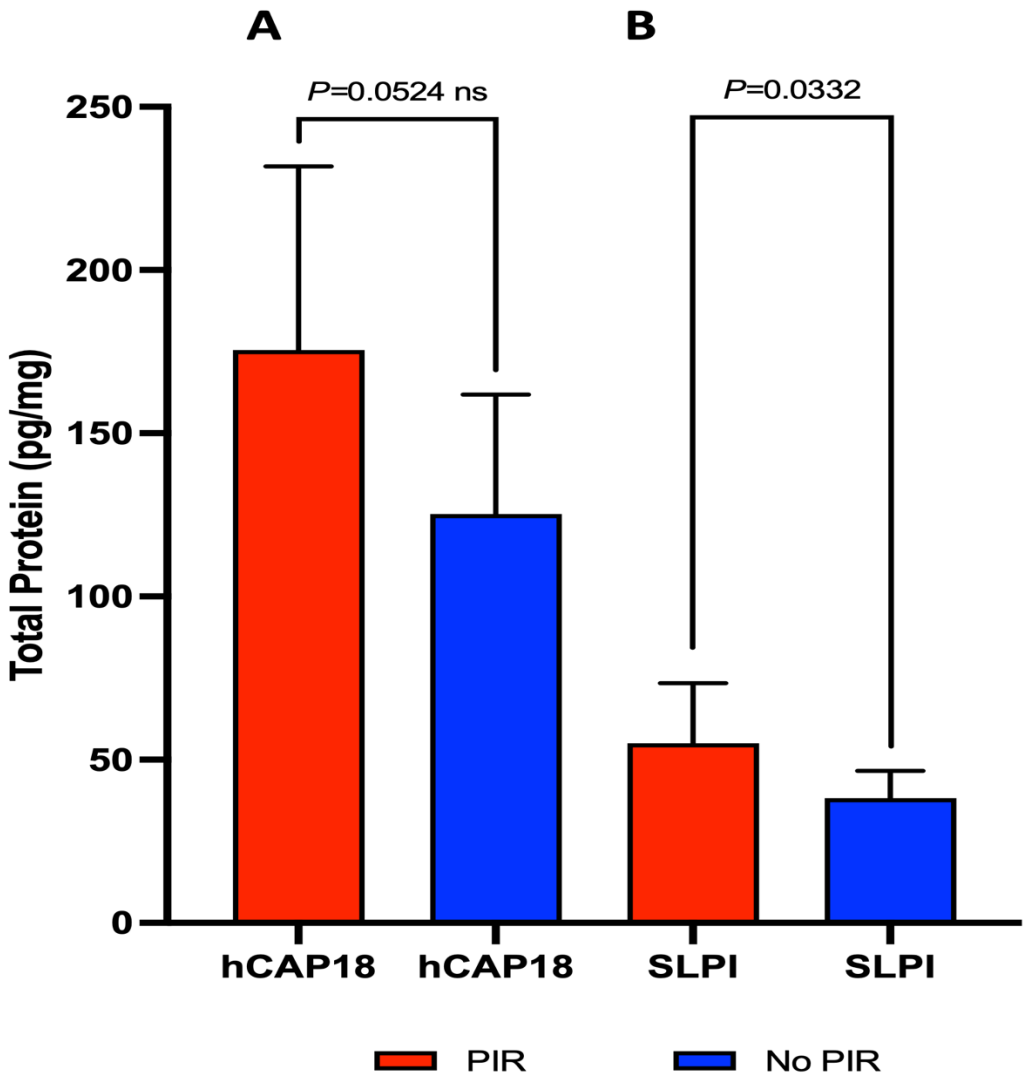

Figure 7. 2 Mean protein concentrations of the natural antimicrobial proteins hCAP18 (A) and SLPI (B) evaluated by ELISA in placentas with PIR vs. placentas with no PIR.

No difference in hCAP18 expression levels was found between placentas with or without a PIR when assessed by Student's *t* test (*P*=0.0524). SLPI concentrations, however, were significantly higher in placentas with a histologically-diagnosed PIR compared to those with no PIR (*P*=0.0332).

7.3.4 Matrix metalloproteinase-9 levels are not significantly different in placentas with an inflammatory response compared to those without

Although MMP-9 was detectable in all samples (range: 38.16 – 158.7 pg/mg), there was no significant difference (*P*=0.2540) between the mean concentration of MMP-9 whether PIR was present (84.50 ± 46.35 pg/mg) or absent (61.02 ± 21.44 pg/mg) (Figure 7.3).

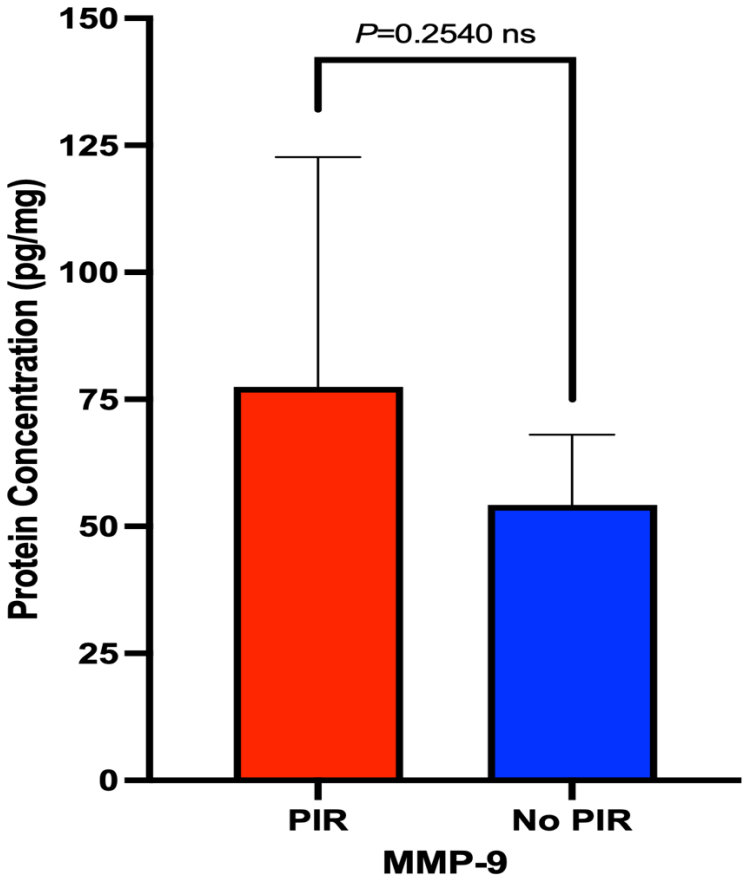

Figure 7. 3 Mean protein concentrations of MMP-9 evaluated by ELISA in placentas with PIR vs. placentas with no PIR.

A commercial 96T sandwich ELISA kit was utilised to determine the protein concentration of MMP-9, a matrix metalloproteinase involved in cervical ripening and membrane rupture, in full thickness placental tissue samples. When evaluated by Student's *t* test, there was no statistically significant difference in MMP-9 concentrations between placentas with a histologically-diagnosed PIR and those with no PIR (*P*=0.2540).

7.3.5 Interleukin-6 expression is significantly higher in placentas with an inflammatory response compared to those with no inflammatory response

To determine significant differences between expression levels of selected inflammatory chemo- and cytokines, placental homogenates were analysed by ELISA for IFN-γ, IL-6, IL-8 and IL-10. All four inflammatory mediators were detectable in each sample. Ranges and mean concentrations for placentas with PIR and those with no PIR are shown in Table 7.4.

Table 7. 4 Mean concentration (pg/mg) of cytokines in placentas with PIR and placentas with no PIR

Protein	Range (pg/mg)	Mean Protein Concentration (pg/mg)		*P V*alue
		PIR (*n* = 8)	No PIR (*n* = 8)	
IFN-γ	1.697 - 5.366	3.024 ± 1.445	2.213 ± 0.3975	0.1481 ns
IL-6	3.009 - 30.08	14.76 ± 7.200	7.772 ± 4.212	0.0327
IL-8	0.374 - 24.88	6.547 ± 8.239	1.152 ± 50.07	0.0861 ns
IL-10	0.7273 - 2.268	1.514 ± 0.5109	1.140 ± 0.2618	0.0872 ns

IFN-γ; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10*, PIR;* placental inflammatory response. Student's *t* test. *P*; probability value. Two-Tailed *P*<0.05 considered to be statistically significant, *ns*; not significant.

Chemokine (IL-8) and cytokine ranges were lower than those detected for natural AMPs and MMP-9, with IL-10 having the lowest concentration of all the analysed proteins. Although recorded concentration values were higher in placentas with PIR compared to those with no PIR, there was no statistically significant difference between PIR and no PIR IFN-γ expression levels (*P*=0.1481). IL-8 protein concentrations in the PIR group were higher (6.547 ± 8.239 pg/mg) than the no PIR group (1.152 ± 50.07 pg/mg), however, as for IFN-γ this was not found to be significantly different (*P*=0.0861).

Levels of the anti-inflammatory cytokine IL-10 were low in the total cohort and, although not statistically significantly different (*P*=0.0872), placentas with PIR were recorded to have higher levels than those without (1.514 ± 0.5109 vs. 1.140 ± 0.2618 pg/mg). In contrast, IL-6, a cytokine known to be abundantly expressed during inflammation, was statistically significantly higher in placentas with PIR (14.76 ± 7.200 pg/mg) compared to placentas with no PIR (7.772 ± 4.212 pg/mg) where levels were measured at almost half of those recorded for PIR samples (*P*=0.0327) (Table 7.5 and Figure 7.4).

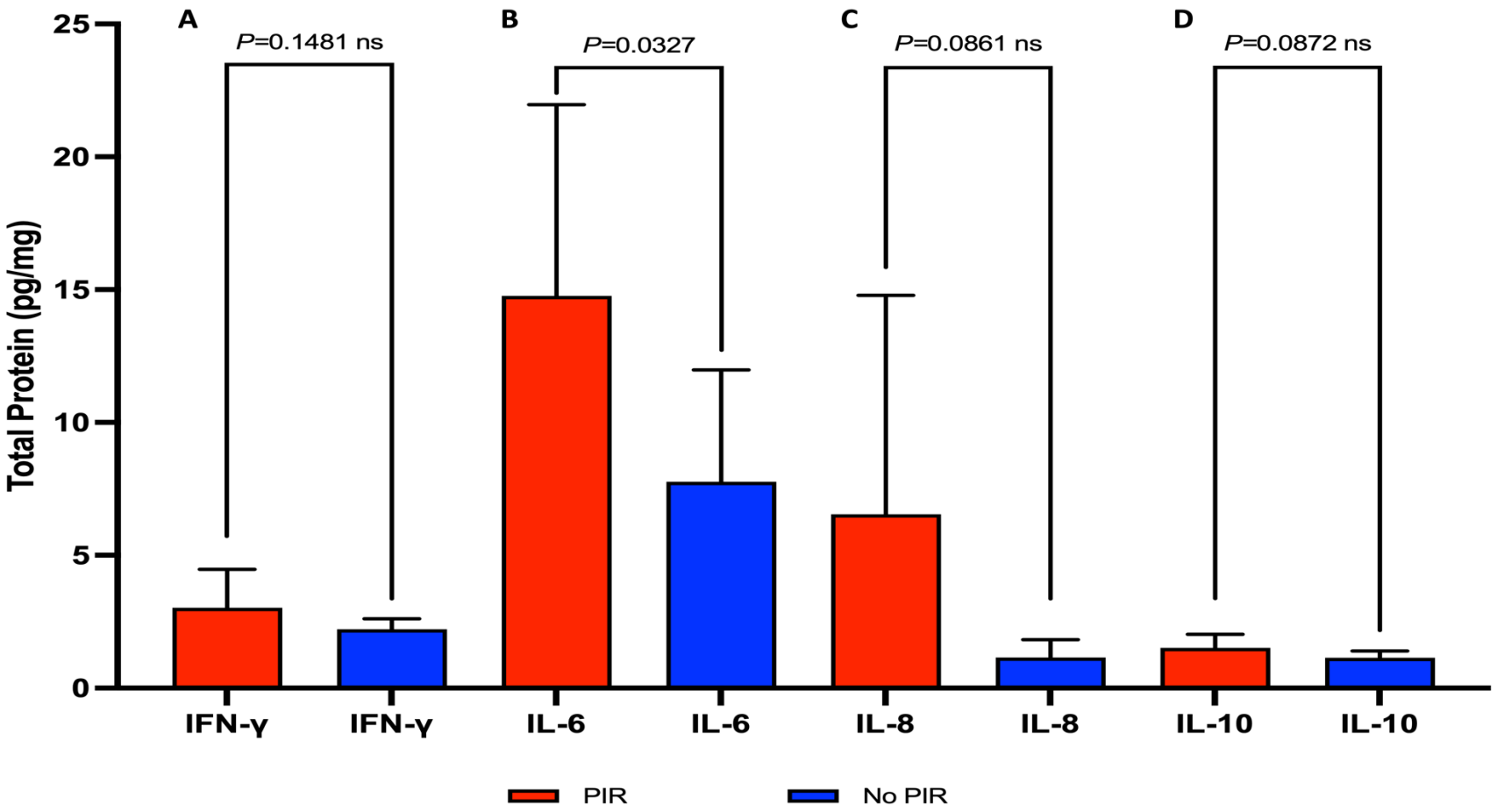

Figure 7. 4 Mean protein concentrations of pro- and anti-inflammatory chemokines and cytokines in placentas with PIR compared to placentas with no PIR.

Commercial 96T sandwich ELISA kits were utilised to determine the protein concentrations of the pro-and anti-inflammatory cytokines, IFN-γ, IL-6, and IL-10 and the chemokine, IL-8, in placental homogenates. No differences in IFN-γ, IL-8 and IL-10 expression levels were found between placentas with or without a PIR when assessed by Student's *t* test (*P*=0.1481, *P*=0.0861 and *P*=0.0872, respectively). In contrast, IL-6 concentrations were significantly higher in placentas with a histologically-diagnosed PIR compared to those with no PIR (*P*=0.0327).

7.3.6 Antimicrobial protein, matrix metalloproteinase and cytokine expression levels positively correlate in preterm versus term placentas

To evaluate linear relationships existing between individual target protein expression levels in preterm compared to term placentas, Spearman's correlations were performed for each target protein between the preterm and term groups. However, as birth is multifaceted and unlikely to be correlated to a change in a single protein, the importance of relationships between expression levels of each of the different target proteins was also assessed. By examining the relationships between pairs or groups of proteins, the aim was to also elucidate whether any patterns emerged, for example, an increase in only proinflammatory protein signalling, an increase in proinflammatory mediators resulting in a concomitant decrease in anti-inflammatory protein signalling, or a reduction in anti-inflammatory protein expression alone.

To understand these associations, firstly, the Spearman's correlation coefficient, Rho (ρ), and probability (*P*) values were calculated to assess whether changes in specific target proteins were related to delivery preterm or at term, for example, hCAP18 preterm samples were compared against hCAP18 term samples. Then, all preterm- or term-specific relationships were considered and ρ and *P* values were determined for relationships between target proteins in preterm placentas and separately in term placentas, that is, hCAP18 preterm versus IFN-γ preterm, IL-6 preterm, etc., followed by hCAP18 term versus IFN-γ term, IL-6 term and so on. Finally, protein expression levels were analysed between each target protein in preterm samples and relationships determined between all other target proteins in term samples, for example, hCAP18 preterm versus IFN-γ term, or IL-6 preterm compared to MMP-9 term expression levels. All evaluated correlations between protein expression levels are displayed as a matrix (Figure 7.5) with Spearman's ρ values quantified between -1 and +1; each value is presented to 2 decimal places. Positive relationships, i.e., as one protein expression level increases, so does the second protein expression level, are denoted by blue coloured boxes. Negative relationships, i.e., as one protein expression level increases, the second protein expression level decreases, are highlighted by red coloured boxes with deeper colour representing a stronger relationship in either direction. Significant relationships were considered to be perfect if the correlation coefficient was +1 or -1, very strong if ≥ +/-0.80, moderate if ≥ +/-0.60 but < +/-0.80, fair if ≥ +/-0.30 but < +/-0.60 and poor if > 0 but < +/- 0.30 as accepted in medical research studies (609).

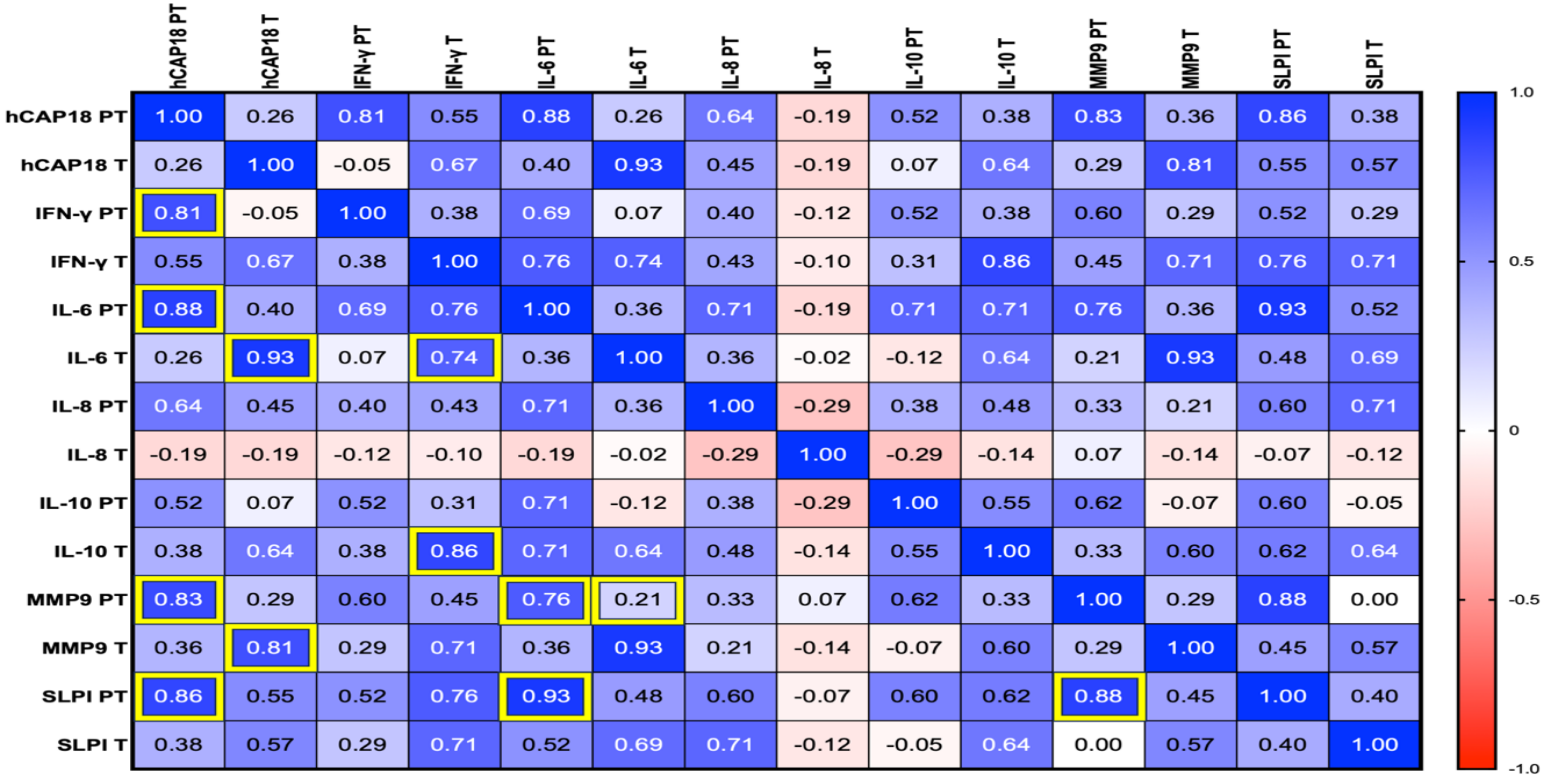

Figure 7. 5 Spearman's rank correlations between preterm and term specific target protein expression levels and between all analysed proteins

hCAP18; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *SLPI*; Secretory Leukocyte Peptidase Inhibitor. No significant correlations were reported between the preterm and term concentrations of any individual target protein. However, significant relationships were revealed between expression levels of target proteins within the preterm cohort and within the term cohort, individually. Only two significant correlations between target proteins in the preterm versus term cohorts were revealed, specifically IFN-*γ* at term was significantly correlated with IL-6 and SLPI expression levels in preterm samples. All significant correlations, as highlighted by yellow boxes and detailed in Table 7.6, were strongly positive, that is, as the expression level of one protein increased, the second target protein expression level increased in parallel (e.g. hCAP18 and IL-6 in preterm placentas; ρ=0.88, *P*=0.0218).

No significant correlations between preterm and term concentrations were shown for any individual target protein (Table 7.5) suggesting that gestational age alone is not related to changes in expression level of any of the analysed proteins.

Table 7. 5 Correlation coefficient and probability values for specific target protein expression levels when compared between preterm and term placentas

Target protein preterm vs. term	ρ *V*alue	*P V*alue
hCAP18	0.2619	0.5364 ns
IFN-γ	0.3809	0.3599 ns
IL-6	0.3571	0.3893 ns
IL-8	-0.2857	0.5008 ns
IL-10	0.5476	0.1710 ns
MMP-9	0.2857	0.5008 ns
SLPI	0.4047	0.3268 ns

hCAP18; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *SLPI*; Secretory Leukocyte Peptidase Inhibitor*. ns;* not significant, ρ; Spearman's rho value, *P;* probability value. Two-sided *P* value <0.05 considered to be significant.

7.3.6.1 There are very strong significant correlations between selected target proteins in preterm placentas

When relationships were assessed between proteins within the preterm group individually and again in the term group, very strong positive correlations were shown between a number of the target proteins as detailed in Table 7.6. hCAP18 had the largest number of significant correlations with other proteins (hCAP18 significantly associated with four proteins) and expression levels in preterm placentas showed the most significant very strong positive correlation to be between hCAP18 and IL-6 (*P*=0.0072), followed by hCAP18 and SLPI (*P*=0.0107), hCAP18 and MMP-9 (*P*=0.0154) and hCAP18 and IFN-γ (*P*=0.0218) (Table 7.6). The direction of these correlations showed that in preterm placentas, as hCAP18 protein expression levels increase, concentrations of the four stated target proteins increase, however, it is not possible to determine which of these proteins may increase first.

Along with the above reported statistically significant very strongly positive associations, preterm IL-6 concentrations were also strongly positively correlated with MMP-9 (*P*=0.0368) and SLPI (*P*=0.0022) expression levels in preterm placentas (Table 7.6). Finally in the preterm group, MMP-9 concentrations showed a very strong positive and significant association with expression levels of the antimicrobial protein SLPI (*P*=0.0007) (Table 7.6).

7.3.6.2 There are very strong significant correlations between selected target proteins in term placentas

At term, hCAP18 concentrations very strongly positively and significantly correlated with IL-6 (*P*=0.0022) and, less significantly with MMP-9 (*P*=0.0218) concentrations but not with any other cytokine or antimicrobial protein (Table 7.6). Expression levels of the pro-inflammatory cytokine IFN-γ very strongly positively correlated with the proinflammatory cytokine, IL-6 (*P*=0.0458), but more significantly with the anti-inflammatory cytokine IL-10 (*P*=0.0107) in term placentas (Table 7.6).

As was observed preterm, at term, there was a significant very strong positive correlation between IL-6 and MMP-9 expression levels (*P*=0.0022). Significant and very strongly positive correlations were also observed between term placental IFN-γ protein expression levels and preterm placental expression levels of IL-6 and SLPI (Table 7.6).

Table 7. 6 Correlating protein expression levels in preterm and term placentas

Correlating Variable 1	Correlating Variable 2	ρ *V*alue	*P V*alue
hCAP18 Preterm	IFN-γ Preterm	0.8095	0.0218
hCAP18 Preterm	IL-6 Preterm	0.8810	0.0072
hCAP18 Preterm	MMP-9 Preterm	0.8333	0.0154
hCAP18 Preterm	SLPI Preterm	0.8571	0.0107
hCAP18 Term	IL-6 Term	0.9286	0.0022
hCAP18 Term	MMP-9 Term	0.8095	0.0218
IFN-γ Term	IL-6 Term	0.7381	0.0458
IFN-γ Term	IL-10 Term	0.8571	0.0107
IFN-γ Term	IL-6 Preterm	0.7619	0.0368
IFN-γ Term	SLPI Preterm	0.7619	0.0368
IL-6 Preterm	MMP-9 Preterm	0.7619	0.0368
IL-6 Preterm	SLPI Preterm	0.9286	0.0022
IL-6 Term	MMP-9 Term	0.7619	0.0022
MMP-9 Preterm	SLPI Preterm	0.8810	0.0007

hCAP18; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *SLPI*; Secretory Leukocyte Peptidase Inhibitor. ρ; Spearman's rho value, *P;* probability value.

7.3.7 Antimicrobial protein, matrix metalloproteinase and cytokine expression levels positively correlate in placental cohorts with and without inflammatory responses

To examine linear relationships existing between individual target protein expression levels in placentas with PIR compared to those with no PIR, samples were regrouped from preterm and term cohorts to PIR and no PIR cohorts and Spearman's correlations performed for each target protein between the PIR and no PIR groups. As previously, relationships were also assessed between expression levels of each of the different target proteins in each cohort and between cohorts.

Spearman's correlation coefficient, Rho (ρ), and probability (*P*) values were calculated to assess whether changes in specific target proteins were related to the presence or absence of PIR, for example, hCAP18 PIR samples were compared against hCAP18 no PIR samples. Then, all PIR- or no PIR-specific relationships were considered and ρ and *P* values were determined for relationships between target proteins in the PIR cohort and separately in the no PIR cohort, e.g., hCAP18 in the PIR group versus IFN-γ in the PIR group, etc., followed by hCAP18 in the PIR cohort versus IFN-γ in the no PIR cohort. Finally, protein expression levels were analysed between each target protein in the PIR group and relationships determined between all other target protein in the no PIR group, i.e., hCAP18 with PIR versus IFN-γ with no PIR.

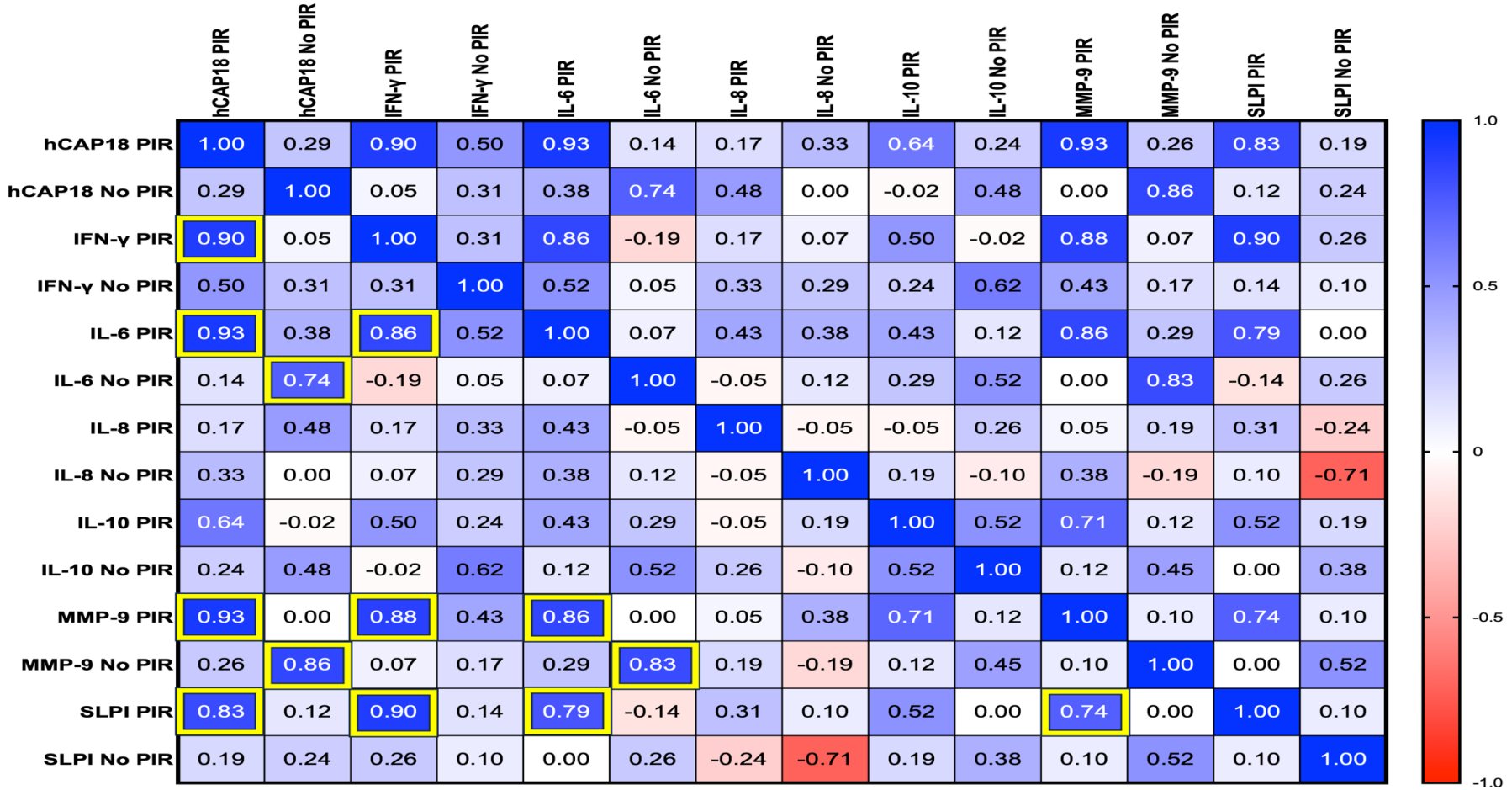

Figure 7. 6 Spearman's rank correlations between specific target protein expression levels and between all analysed proteins in placentas with PIR and those without

hCAP18; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *SLPI*; Secretory Leukocyte Peptidase Inhibitor. No significant correlations were reported between the PIR and no PIR concentrations of any individual target protein or between different proteins in the PIR and no PIR groups. However, significant relationships were revealed between expression levels of target proteins within both the PIR and no PIR cohorts. All significant correlations, as highlighted by yellow boxes and detailed in Table 7.8, were strongly positive, that is, as the expression level of one protein increased, the second target protein expression level increased in parallel (e.g. hCAP18 and IL-6 in placentas with PIR; ρ=0.93, P=0.0022).

No significant correlations between individual target protein expression levels were observed between the PIR and no PIR cohorts (Table 7.7) which is likely due to no single cytokine or antimicrobial protein being involved in inflammatory responses linked only to infection rather than those also being involved in normal labour.

Table 7. 7 Correlation coefficient and probability values for specific target protein expression levels when compared between PIR and no PIR cohorts

Target protein PIR vs. no PIR	ρ *V*alue	*P V*alue
hCAP18	0.2857	0.5008 ns
IFN-γ	0.3095	0.4618 ns
IL-6	0.0714	0.8820 ns
IL-8	-0.0476	0.9349 ns
IL-10	0.5231	0.1966 ns
MMP-9	0.0952	0.8401 ns
SLPI	0.0952	0.8401 ns

hCAP18; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *SLPI*; Secretory Leukocyte Peptidase Inhibitor. *ns;* not significant, ρ; Spearman's rho value, *P;* probability value. Two-sided *P* value <0.05 considered to be significant.

7.3.7.1 There are very strong significant correlations between selected target proteins in placentas with a histologically-diagnosed inflammatory response

Examining relationships between proteins within the PIR group and then separately in the no PIR group, very strong positive correlations were shown between a number of the target proteins as detailed in Table 7.8. Very strong positive and significant relationships in placentas with PIR and those with no PIR roughly mirrored associations seen between preterm and term placentas in that one of the most significantly very strongly positive correlations was seen between hCAP18 and IL-6 expression levels, although this was more significant in the PIR cohort (*P*=0.0022) than the preterm cohort, but equally significant to the association seen at term. In the PIR cohort, hCAP18 and MMP-9 were more significantly very strongly positively correlated than had been observed in either the preterm or term cohorts (*P*=0.0022) (Table 7.8).

Similarly, the association seen between hCAP18 and IFN-γ (*P*=0.0046) was more significant in the PIR cohort than in the preterm cohort, although it remained very strong and in the same direction. In contrast, and somewhat unexpectedly since SLPI is expressed in abundance by neutrophils, the very strong positive correlation between hCAP18 and SLPI in the PIR cohort was less significant than the relationship seen in the preterm group (*P*=0.0154) (Table 7.8).

IFN-γ expression levels were slightly different to those seen when evaluated in the preterm and term cohorts in that IFN-γ was most significantly very strongly positively correlated with MMP-9 (*P*=0.0007), a relationship not seen when assessing cohorts by gestational age category. IFN-γ concentrations were also significantly very strongly correlated with the proinflammatory cytokine IL-6 expression levels (*P*=0.0107) and the antimicrobial protein, SLPI (*P*=0.0046) in placentas with PIR (Table 7.8). Notably, these correlations were more significant than when seen in the term cohort or between the preterm and term cohorts.

IL-6 expression levels were significantly very strongly positively associated with both MMP-9 and SLPI expression levels, as had been observed in preterm placentas. However, in placentas with PIR, the correlation between IL-6 and MMP-9 was more significant (*P*=0.0107), whereas the relationship between IL-6 and SLPI was much less significant (*P*=0.0279). In addition to the above relationships, MMP-9 expression levels in placentas with PIR showed a very strong positive and significant association with expression levels of the antimicrobial protein SLPI in the PIR group (*P*=0.0458), although this was noticeably less significant than the relationship seen in preterm placentas (*P*=0.0007) (Table 7.8).

7.3.7.2 There are very strong significant correlations between selected target proteins in placentas with no histologically-diagnosed inflammatory response

Only three significant correlations were observed between specific target proteins in the no PIR cohort, although all were very strongly positive. As seen in all other groups, hCAP18 concentrations very strongly positively and significantly correlated with IL-6, although much less significantly than had been observed in the preterm, term or PIR cohorts (*P*=0.0458). hCAP18 and MMP-9 were significantly very strongly positively associated (*P*=0.0218), yet it was noted this relationship was less significant to that observed in the preterm or PIR groups, but equal to the correlation observed in term placentas. A significant relationship consistently

observed when all groups were analysed was that between IL-6 and MMP-9. In placentas with no PIR, this correlation was also found to be significantly very strongly positive which was more significant than the correlation seen in placentas with PIR (*P*=0.0154) (Table 7.8).

Table 7. 8 Significantly correlating protein expression levels in placentas with PIR and no PIR

Correlating Variable 1	Correlating Variable 2	ρ *V*alue	*P V*alue
hCAP18 PIR	IFN-γ PIR	0.9048	0.0046
hCAP18 PIR	IL-6 PIR	0.9286	0.0022
hCAP18 PIR	MMP-9 PIR	0.9286	0.0022
hCAP18 PIR	SLPI PIR	0.8333	0.0154
hCAP18 No PIR	IL-6 No PIR	0.7381	0.0458
hCAP18 No PIR	MMP-9 No PIR	0.8571	0.0107
IFN-γ PIR	IL-6 PIR	0.8571	0.0107
IFN-γ PIR	MMP-9 PIR	0.8810	0.0007
IFN-γ PIR	SLPI PIR	0.9048	0.0046
IL-6 PIR	MMP-9 PIR	0.8571	0.0107
IL-6 PIR	SLPI PIR	0.7857	0.0279
IL-6 No PIR	MMP-9 No PIR	0.8333	0.0154
MMP-9 PIR	SLPI PIR	0.7381	0.0458

hCAP18; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *PIR;* placental inflammatory response, *SLPI*; Secretory Leukocyte Peptidase Inhibitor. ρ; Spearman's rho value, *P;* probability value.

7.3.8 Antimicrobial protein, matrix metalloproteinase 9 and cytokine levels do not differ with increasing grade of maternal or fetal inflammatory response

Placentas with evidence of inflammation were graded and staged as described previously in in *Chapter 2* and according to the Amsterdam Placental Workshop Group Consensus Statement published as Khong *et al*., 2016 (103). As noted in preceding chapters, for the purposes of this study an absence of PIR was graded and staged as zero. Inflammatory lesion diagnoses and corresponding grades and stages for placentas sampled for the study are detailed in Table 7.9 below.

Table 7. 9 Inflammatory lesion diagnoses and maternal and fetal inflammatory response stages and grades in placentas delivered preterm and at term

Sample ID	Outcome (T/PT)	Primary PIR	Additional PIR	MIR Stage	MIR Grade	FIR Stage	FIR Grade
SU0006	PT	Acute Chorioamnionitis		2	1	1	1
SU0029	PT	Acute Chorioamnionitis	Acute Phlebitis	2	2	2	2
SU0041	PT	Acute Chorioamnionitis		2	1	1	1
SU0043	T	Acute Chorioamnionitis		1	1	0	0
SU0049	PT	Acute Chorioamnionitis	Acute Funisitis	2	2	3	2
SU0054	T	Acute Chorioamnionitis		1	1	1	1
SU0059	T	Acute Chorioamnionitis	Acute Phlebitis	3	1	3	2
SU0070	T	Acute Chorioamnionitis	Acute Funisitis	1	1	1	1

FIR, fetal inflammatory response*; MIR,* maternal inflammatory response*; PIR,* placental inflammatory response*; PT,* preterm*; T,* term. Grades and stages reported according to the Amsterdam Placental Workshop Group Consensus Statement (Khong *et al.,* 2016).

Mean concentrations of all analysed proteins were highest where there was presence of maternal and/or fetal inflammatory responses at either Grade 1 or 2 compared to absence graded as 0, however, none of these associations were statistically significant when assessed by a Kruskal-Wallis test with Dunn's post-hoc analyses. Additionally, there was no difference in protein concentrations when comparing between grade 1 and grade 2 MIR and FIR (data presented in Appendix IV, Figure AIV.VII) (Table 7.10).

Table 7. 10 Mean protein concentrations by maternal and fetal inflammatory response grade

Protein	Mean Protein Concentration (pg/mg)					
	MIR Grade 0	**MIR Grade 1**	**MIR Grade 2**	**FIR Grade 0**	**FIR Grade 1**	**FIR Grade 2**
hCAP18	125.3	164.3	194.3	125.3	173.2	158.2
IFN-γ	2.213	2.861	3.417	2.213	3.296	2.122
IL-6	7.772	11.96	9.43	7.772	15.15	13.47
IL-8	1.152	2.818	12.76	1.152	7.513	6.996
IL-10	1.140	1.322	1.833	1.140	1.423	1.507
MMP-9	61.02	84.94	83.76	61.02	87.22	56.13
SLPI	38.23	47.27	68.04	38.23	58.12	53.03

FIR; Fetal Inflammatory Response, *hCAP18*; Human Cationic Antimicrobial Peptide 18*, IFN-γ*; Interferon-gamma*, IL-6*; Interleukin-6, *IL-8*; Interleukin-8, *IL-10*, Interleukin-10, *MMP-9*; Matrix Metalloproteinase 9, *MIR;* Maternal Inflammatory Response, *SLPI*; Secretory Leukocyte Peptidase Inhibitor.

7.4 Discussion

Previous studies and hypotheses, dating back to the mid-1980s, have shown successful pregnancy outcomes rely on immune tolerance delivered by a T helper type 2 (Th2) bias and, in cases where this balance is disturbed, adverse obstetric outcomes, including preterm birth, are reported (216, 279). As discussed in *Chapter 6*, macrophages are key modulators in pregnancy and their expression of pro- and anti-inflammatory proteins is vital for maintaining maternofetal tolerance throughout gestation as well as contributing to cervical remodelling and membrane rupture prior to delivery (190, 201, 287). However, research on the immune cells of the placenta which are responsible for releasing these parturition-associated cytokines, chemokines, antimicrobial proteins and matrix metalloproteinases, in addition to whether they differ in term and preterm labour, appears to have diminished over time. Consequently, there remains uncertainty about the protein milieu contributing to the inflammatory cascades involved in labour, irrespective of gestational age (603, 610-612).

Cytokines have been suggested to be involved in the transition from myometrial quiescence to contractility (613) and evidence indicates innate immune cells such as macrophages and neutrophils release pro-inflammatory cytokines, chemokines and matrix metalloproteinases which are involved in the mediation of labour (151). Furthermore, although both hCAP18 and SLPI are antimicrobial proteins involved in immune responses, it has been shown that SLPI concentrations are elevated prior to term labour and myometrial cathelicidin-expressing cells increase in uncomplicated term labour with studies postulating they play a key role in labour initiation and progression (614, 615). Whether placental proteins contribute to these labour-initiation processes remains open for debate. It was, therefore, hypothesised that in placentas with a histologically-diagnosed inflammatory response, antimicrobial proteins, MMP-9 and the pro-inflammatory cytokines IFN-γ, IL-6 and IL-8 would increase. Additionally, it was postulated that the anti-inflammatory cytokine IL-10 would decrease, that these alterations in protein concentrations would be proportional to the increased grade, or severity, of the inflammatory response and that these variations in protein concentrations would contribute to preterm labour and birth. In the assessed cohort, there were no significant differences in mean total protein concentrations from the preterm versus term and the PIR versus no PIR placentas which demonstrates the reliability and validity of the QuBit® Protein Assay Kit (Life Technologies, California, USA) and QuBit® Fluorometer (Life Technologies, California, USA)

methods used to extract and then measure protein. As such, variation in the samples was minimised when assessing each individual participant and the analysed proteins by standardising results and expressing the amount of protein of interest in picograms as a proportion of total placental protein in milligrams (pg/mg) for all analyses. Although all proteins were detected in each of the sixteen samples, no significant differences in individual expression levels of any of the proteins between preterm- or term-delivered placentas were found. This was surprising given that previous studies have shown SLPI increases in cervical mucus and cervical epithelial cells before preterm labour (615, 616) and hCAP18, IL-6 and IL-8 concentrations increase at term, as reviewed in a number of studies (214, 601, 614). Elevated MMP-9 levels have also been described in term gestational tissues (617) whilst IFN-γ and IL-10 concentrations either do not alter or decrease as gestational age increases (282, 601) as described in a 2018 review by Keelan (72). Nonetheless, it is plausible that these reported changes in antimicrobial protein, matrix metalloproteinase and cytokine expression levels are only evident in tissues other than the placenta, for example, hCAP18 expression increases in fetal membranes and MMP-9 and SLPI concentrations are described as increasing in the cervix before preterm labour (214, 615, 616), but at term are only elevated in the myometrium after the onset of labour (618). Equally, there is the potential that the placenta is more effective at controlling inflammation than the fetal membranes or the cervix. This study is, however, the first to report the specific inflammatory protein profile including antimicrobial proteins hCAP18 and SLPI, matrix metalloproteinase-9, the chemokine IL-8 and pro- and anti-inflammatory cytokines, IFN-γ, IL-6, IL-10, in the placenta.

7.4.1 Antimicrobial proteins in the placenta

Of all analysed proteins, hCAP18, a small cationic polypeptide with the ability to destroy or inhibit the action of microorganisms (224) was found in the highest concentrations. Antimicrobial proteins, including hCAP18, have been shown to play a critical inflammatory role in the pathogenesis of conditions such as inflammatory bowel disease (IBD) (619) and asthma (620) as well as predicting severity of infectious disease when concentrations are low in cases of paediatric bronchiolitis (621). Coupled with evidence that antimicrobial proteins are present in multiple areas of the female reproductive tract, it was anticipated that hCAP18 levels would be elevated in placentas with a histologically-diagnosed inflammatory response (PIR) and, although there was a tendency for higher protein expression levels in the placentas

with PIR this was not statistically significant. However, hCAP18 is a precursor protein which has the ability to induce chemotactic agents of IL-8 and attract neutrophils to sites of inflammation at which time it is enzymatically cleaved to the active cytotoxic peptide LL-37 to initiate its antimicrobial properties (226, 622, 623). The similarity in levels of hCAP18 between placentas with PIR and those without PIR found in this study may be accounted for by elastase-, cathepsin G- or proteinase 3-linked exocytotic cleavage of hCAP18 to LL-37 in neutrophils, a protein not included in the target protein analyses. As such, these findings may in fact demonstrate declining hCAP18 levels as PIR grade and stage increase due to greater numbers of neutrophils migrating to inflammatory sites across the placental compartments and more cleavage of hCAP18 to release LL-37. Alternatively, a deficiency or overexpression of hCAP18 may not be a consequence of placental infection and subsequent inflammation but rather a reflection of the normal labour process or a beneficial pro-inflammatory response required to clear bacterial infections (614). In this study, all enrolled women had experienced spontaneous labour, and this potentially explains why hCAP18 concentrations did not differ significantly regardless of whether placental levels were compared between preterm and term or PIR and no PIR samples. Lim *et al.,* (2015) demonstrated high hCAP18 protein expression in fetal membranes of labouring women, predominantly in the amnion epithelium, cytotrophoblasts and decidua, as well as in myometrial longitudinal and transverse muscle fibres. The study also found increased hCAP18 expression, albeit to a lesser extent, in leukocytes (221) which corroborates not only the findings of high hCAP18 protein expression levels in all samples being most likely a product of labouring tissue, but also confirms that the same patterns of hCAP18 expression are observed in placental tissue as other reproductive tissues.

A significant increase in SLPI protein expression levels in placentas with PIR compared to those without was identified, but there was no such difference between placentas delivered preterm and those delivered at term. SLPI is a known natural inhibitor of neutrophil elastase (NE) and is found in cervical mucus, fetal membranes and amniotic fluid with studies showing links between dysregulated expression and parturition, PPROM and tissue destruction, potentially through stimulation of proteases such as MMP-9 which are associated with membrane rupture, and as a driver of inflammation and initiation of labour pathways (233). Reviews showing SLPI protein expression increases in response to inflammatory molecules

such as bacterial products and cytokines (236, 604, 624, 625), or non-infectious stimulants including alarmins from cell stress or apoptosis (437) validate the findings reported in this chapter. Nonetheless, the focus of previous studies has been on mucosal tissues (604), the uterus, term amnion and chorion and the cervical mucus plug (236) with little description of SLPI's role in the placenta or contribution to PIRs and potentially preterm birth which require further exploration.

Histological diagnosis of PIR relies on assessment of the number, confluence and location of neutrophils in placental compartments (103) and, as principal SLPI-secreting leukocytes, it is not unexpected to observe higher SLPI concentrations in placental samples with a reported inflammatory response. Neutrophils secrete proteases in order to facilitate their migration through tissue extracellular matrices and it is this which stimulates a responsive SLPI protein expression from leukocytes and epithelial cells to counteract tissue damage (236, 604). SLPI concentrations and grade of PIR were not associated with either PPROM or spontaneous SROM (data not shown) despite an increase in neutrophilic proteases, particularly NE, being demonstrated in PPROM in previous studies with a concomitant reduction in SLPI protein expression levels, reduced inhibition of MMPs and rupture of fetal membranes (236, 604). Further, King *et al.,* (2007) described fetal membrane and amniotic fluid decreases in SLPI as being the driver of increased protease activity and elevated MMPs which results in ruptured membranes (236). A difference in the reproductive tissue included in this study could account for these conflicting results and this highlights a need for further investigation which should include samples from the fetal membranes, umbilical cord and placental disc to ascertain where imbalances in SLPI concentrations have a direct effect on membrane rupture.

Notwithstanding the difficulties in demonstrating SLPI's effect on membrane rupture, this study found no significant difference in SLPI concentration with advancing maternal or fetal inflammatory response, rather it only identified that SLPI protein expression levels were higher in the presence of PIR versus the absence of PIR. Alongside SLPI's antiprotease activity, it is involved in tissue remodelling and wound repair (236, 626) and has the ability to induce bacterial lysis (624) and neutralise the endotoxic effects of LPS, a cell wall product of Gram-negative bacteria (626). A further feature of SLPI is the inhibition of apoptosis in neutrophils, but, if apoptosis does occur and macrophages are exposed to these apoptotic cells they have

the ability to increase production and secretion of SLPI to minimise further tissue damage and promote repair (604). These properties of SLPI could explain the observation of increased SLPI in placentas with PIR but an absence of correlation between increased PIR grades and increased SLPI. Since SLPI is able to inhibit NFκB-induced responses in monocytes and macrophages by competitively binding to the p65 NFκB consensus binding site (627), it is also possible that there is a saturation of binding which accounts for no further SLPI expression or difference in concentration with increasing PIR severity. Observationally, in the samples with the highest PIR (Stage 3 FIR and/or MIR), no correlation with the greatest increase in SLPI concentrations was shown, although SLPI expression was elevated. Interestingly, one of these placental samples corresponded to a prolonged (>12 weeks) period of PPROM and a rarely-seen case of higher FIR stage than MIR stage, whilst the second was seen in a term placenta with neutrophilic attenuation of the endothelial layer in the umbilical vessel walls. It is possible local SLPI levels in these samples were at high enough concentrations to inhibit apoptosis and protease activity thus limiting PIR progression to necrotising chorioamnionitis and karyorrhexis (142, 604). Conversely, or possibly concurrently, SLPI may have limited NFκB activation preventing further tissue destruction (604) and a less severe stage and grade of PIR in placentas in this cohort but this needs confirming through larger cohort studies. On the other hand, the effects seen may be independent of SLPI and result from activation of NFκB, stimulation of neutrophilic apoptosis and efferocytosis by macrophages which lead to decreased expression of proinflammatory cytokines, increased expression of the anti-inflammatory cytokine IL-10, and resolution of inflammation (628, 629).

7.4.2 Matrix metalloproteinase 9 in the placenta

It was hypothesised that placentas from PPROM or SROM deliveries would have higher concentrations of matrix metalloproteinase 9 (MMP-9) on account of this protein being associated with collagen degradation (268) and normal and pathological rupture of membranes (271, 630). However, of the sixteen participants in this arm of the study, over 60% required assisted rupture of membranes despite expected elevated concentrations of MMP-9. Additionally, higher MMP-9 expression levels in preterm placentas than term placentas was not observed, contrary to findings reviewed by Geng, Huang and Jiang (271) and reported more recently by Sundari *et al.* (631). There is a paucity of research into MMP-9 levels in the placenta, particularly in relation to preterm birth, even though MMP-induced

extracellular membrane (ECM) degradation is known to be crucial for successful detachment of the placenta (269). There are a number of potential explanations for MMP-9 levels not being differentially elevated in PPROM or preterm placentas in the study cohort, firstly, as noted, much of the research into the role of MMP-9 in these pathologies focuses on MMP-9 concentration in the myometrium, cervix (617) and amniotic fluid (632) in contrast to the presented assessment of the placental MMP-9 protein expression. A second factor contributing to this chapter's findings is the mode of delivery; with the exception of one Caesarean section, all women delivered vaginally following spontaneous onset of labour. The absence of significantly increased MMP-9 levels between the preterm and term cohorts differs from results presented by other authors which showed MMP-9 concentrations to be elevated in preterm placentas but only following spontaneous vaginal delivery (269, 276). Without elective Caesarean section control placentas, it is not possible to draw conclusions on any contrasting contribution of MMP-9 on membrane rupture and cervical remodelling between preterm and term deliveries.

Finally, a crucial function of MMP-9 is modulation of the uterine and vascular alterations necessary for successful placentation and uterine expansion throughout gestation (268, 633). It has been postulated that decreased MMP-9 expression and/or activity results in collagen impairment and aberrant uterine ECM and spiral artery remodelling which may lead to microcirculatory ischaemia, reduced uterine perfusion pressure and obstetric complications such as preeclampsia (268, 633). Within the heterogenous population three women were reported to have preeclampsia toxaemia and there was a further queried case; none of these placentas were diagnosed with PIR. In two cases there were obvious decreases in MMP-9 expression compared to placentas from the same group, conversely the other two placentas showed increased expression in comparison to MMP-9 values in their respective group. To add to the lack of comparability in placental MMP-9 values in this cohort, two women had recorded gestational diabetes mellitus (GDM) which has been linked to upregulated expression of MMP-9 in trophoblasts (634). Whilst the obstetric complications make aberrant MMP-9 expression unlikely to be the direct cause of preterm birth in this diverse cohort, it does not exclude altered MMP-9 concentrations being a response to the release of pro-inflammatory cytokines or antimicrobial agents or the presence of infectious pathogens.

7.4.3 Cytokines in the placenta

Expression levels of IFN-γ, IL-6, IL-8 and IL-10 were measured in placental homogenates to determine differences in concentration at preterm and term and in relation to the presence or absence of PIR. Normal term parturition is considered to be an inflammatory event (599, 600, 635) and, as such, most studies exclude anti-inflammatory cytokines in their analysis yet it is speculated that the maintenance of pregnancy relies on a balance between pro- and anti-inflammatory proteins (214). Consequently, IL-10, a recognised anti-inflammatory cytokine, was included in the selected panel of inflammatory proteins to establish whether any changes in its expression were observable between term and preterm cohorts. All placental cytokine and chemokine protein expression levels were considerably lower than those observed for antimicrobial proteins and MMP-9, however, IFN-γ, IL-6, IL-8 and IL-10 were detected in all samples. In this study, there were no significant differences in cytokine and chemokine concentrations between the preterm and term groups, as has been an area of conflict for other researchers in the past (262, 263). When evaluating differences between placentas with PIR and those without, there was a significant increase in IL-6 in placentas with PIR which corroborates findings describing IL-6 increases in gestational tissues in the presence of inflammation (149, 260, 636). On the other hand, there was no significant difference in IL-6 expression levels with increasing MIR or FIR or any association between IL-6 levels and rupture of membranes (data not shown) further highlighting the challenges in using IL-6 as a predictive marker of a specific pathology or of its severity.

There were no significant differences in IFN-γ expression levels between preterm and term placentas or when comparing placentas with and without PIR. In a mouse model, IFN-γ signalling was shown to be critical for spiral artery remodelling with dysregulation mediating abnormal vascularisation of the placental labyrinth in the presence of malaria infection (248, 282). This vital signalling role requires IFN-γ in abundance, a state seen in early human pregnancy, yet, by term, this was reversed and IFN-γ concentrations were found to be low (282) as demonstrated in the placental samples in this study. Wilke *et al.,* described decreasing IFN-γ concentrations as the placenta developed and that there were low expression levels in both the second and third trimester of women who delivered preterm and those who delivered at term (266) corroborating findings of Hanna *et al.,* (637). Wilke's

study demonstrated a marginal increase in IFN-γ expression levels during contractions, which the authors speculated was related to increased prostaglandin concentrations, but a significant decrease in spontaneous recurrent abortion. Whilst there was no difference in IFN-γ concentrations in placentas delivered preterm or at term, as in the data presented in this chapter, Wilke *et al.,* found the critical early increases of IFN-γ concentrations were absent in women who later went on to deliver preterm. Notably, these IFN-γ concentrations were analysed from antenatal maternal peripheral blood samples and postpartum decidual samples compared to the present study's postpartum full thickness tissue samples (266). Assessing the placenta may be the underlying reason for not observing changes in IFN-γ expression levels in preterm and term births, therefore, future studies are proposed to sample cervicovaginal fluid (CVF) at mid- and late-trimester timepoints to assess whether these reported temporal changes in peripheral blood samples are observed in CVF and whether they too may be a predictive marker of preterm birth. With regards to placentas with a reported inflammatory response, it was hypothesised that increased IFN-γ expression levels would be recorded due to IFN-γ's influence on macrophage activation, defence against microbial pathogens and inflammation (638). The study results corroborate previous findings that IFN-γ concentrations do not differ in placentas with histologically-diagnosed PIR and those with no PIR (639, 640), however, a recent study demonstrated significantly elevated IFN-γ expression levels in venous blood taken from preterm neonates with sepsis (641). Therefore, whilst there is little to no IFN-γ molecular signature related to preterm birth or histologic chorioamnionitis in the postpartum placenta, assessing maternal peripheral blood, and potentially CVF samples, in early to mid-pregnancy could highlight those at risk of delivering preterm. Moreover, following preterm birth, evaluating IFN-γ concentrations in neonatal venous blood may be a viable predictive marker of neonatal sepsis, a condition with high morbidity and mortality rates and one which is increasingly difficult to diagnose (641).

Although IL-8 is the primary chemotactic agent for neutrophils, stimulating their migration to sites of inflammation and subsequent activation (642), there was no significant difference in IL-8 expression levels in placentas with PIR compared to those with no PIR. Furthermore, there was no difference in IL-8 expression level between grades of PIR. This was unexpected considering previous studies have shown IL-8 concentrations in the placenta to increase

following LPS-induction (642). This conflict may be explained by the neutrophilic infiltration processes described in detail by Giaglis *et al.* (2015), who showed local increases in IL-8 led to neutrophilic infiltration to the myometrium during term labour but that this generally only occurred post-partum. This study also showed moderate numbers of neutrophils were observed in the fetal membranes in the absence of inflammation whereas in the presence of infection or inflammation, vast numbers of neutrophils were recruited to the fetal membranes (596). A further finding which aligns with data presented in this chapter is that in cases of infectious preterm labour, IL-8 expression is elevated in amniotic fluid which recruits neutrophils to the chorion before progressing through the placental compartments (596). There were no significant differences in the grades and stages of the MIR and FIR and, overall, severity (as determined by the grade of PIR) was low (preterm MIR mean 1.75 ± 0.50 and term MIR mean 1.00 ± 0.00). As amniotic membrane and umbilical cord samples were not included in the placental homogenates evaluated for cytokine levels, but these are where low grade and stage PIR were reported from, it is suggested that it is not practicable to measure IL-8 concentrations in placental chorionic, villous and decidual tissue and compare these to histologic findings which do not fully reflect the full placenta. Future studies should consider IL-8 expression levels in amniotic membranes as this is more likely to elucidate the effects of increased local IL-8 expression levels which may be dysregulated in preterm labour and/or in cases of histologic chorioamnionitis.

In contrast to many studies which focus only on pro-inflammatory, Th2, cytokines, IL-10 was included in the panel of investigated proteins. This anti-inflammatory, immune-regulatory cytokine (265, 643) was not significantly different between preterm and term placentas, nor was there any difference in IL-10 expression levels when comparing placentas with PIR to those with no PIR. An important role of IL-10 is that of controlling pregnancy pathologies induced by inflammation, such as preterm birth (644) and, when administered alongside LPS, IL-10 is effective in preventing endotoxin-induced preterm labour in a rat model (645). Additionally, IL-10 expression levels decrease both at term and in preterm placentas with an associated PIR (602, 646) and, as such, it is plausible that attenuated IL-10 concentrations observed in preterm placentas and those with PIR are expected values which demonstrate initiation of different pathways necessary for preterm and term labour.

7.4.4 Influence of antimicrobial proteins, MMP-9 and cytokines on each other in placentas with PIR and in placentas with no PIR

Although aberrant regulation of antimicrobial proteins, MMP-9 and selected cytokines was considered in this thesis, it is important to consider the interactions between these proteins and the influence they have on each other. Pregnancy and labour are dichotomous states whereby a shift to a T helper cell 2 (Th2) bias is necessary for successful pregnancy maintenance. Conflicting evidence has previously been reported on when the return to a non-pregnant T helper cell 1 (Th1) bias occurs with some studies indicating this to be a feature in labour and others describing normal values up to four weeks postpartum (reviewed in (279)). There was no significant difference between preterm and term placentas for all evaluated proteins, nor was there a significant shift to Th1 or Th2 prevalence in this cohort which is understandable considering the inconsistences reported in the literature. What is considered to be accurate though, is that no single cytokine or inflammatory protein acts as a 'switch' for labour initiation, either preterm or at term, and that there are varying cytokine networks responsible for preterm delivery compared to term births (602, 647).

Consideration was given to divergent protein signalling, consequently, the influence selected proteins had on each other was examined. Very strong positive correlations were observed between preterm hCAP18 concentrations and preterm IFN-γ, IL-6, MMP-9 and SLPI concentrations, associations which were replicated in placentas with PIR. Conversely, in term placentas and those with no PIR, hCAP18 concentrations were only significantly correlated with IL-6 and MMP-9 expression levels, target proteins which were also significantly associated with each other in all cohort combinations. Although it is not possible to state which protein is influencing the other, only that positive correlations mean both increase in parallel, it is known that IFN-γ mediates hCAP18's antimicrobial effects (648) and hCAP18 and SLPI are released in vast quantities from neutrophils as described above. This may explain the correlations seen in placentas with PIR but does not elucidate the reasons why these significant positive correlations were observed in preterm placentas and not in term placentas other than to suggest these may be normal physiological responses to, or effects of, labour.

There was a strong positive correlation with preterm placental IL-6 concentrations and preterm MMP-9 and SLPI concentrations but in term placentas IL-6 correlated only with MMP-9. Again, this was replicated in the PIR versus no PIR placentas. Associations between pro-inflammatory molecules and elevated SLPI concentrations have been described in detail above with these studies corroborating our observed correlations of IL-6 and SLPI expression levels being increased in parallel in placentas with PIR (236, 604, 624, 625). Why preterm placentas without PIR would share the same associations between IL-6 and SLPI remains unclear. A final interesting set of correlations were those in term placentas where there was a strong positive relationship between IFN-γ and IL-6 and IFN-γ and IL-10 concentrations, relationships not seen in preterm placentas or when cohorts were compared based on placental inflammatory response status. The importance of a pro-inflammatory milieu in labour initiation has been discussed, as has the term environment most likely being different to that seen in preterm labour (602, 647); considering this specific profile was also not seen in either the PIR or no PIR placentas it is suggested that the relationship between IFN-γ, IL-6 and IL-10 in the study population is indicative of normal term labour and that a decrease in anti-inflammatory IL-10 expression may be a contributory factor in preterm labour.

Despite only observing a significant difference in two of the evaluated protein expression levels, namely IL-6 and SLPI, and only when comparing placentas with PIR and those with no PIR, additional analysis elucidated cohort-specific correlations between protein concentrations. This further supports the evidence that interactions between proteins are critical for maintaining pregnancy and initiating labour at term plus highlights the importance of assessing a complete protein expression profile of term labour, preterm labour, PIR and no PIR in future studies.

7.4.5 Conclusions

Difficulties arise in using the placenta to predict preterm birth and/or the presence and severity of placental inflammatory lesions. Although it is possible to detect molecular biomarkers of preterm birth, such as IL-6 and SLPI, in placental tissue samples, it is only viable and ethical to collect these postpartum by which time some cytokines and proteins of interest have degraded to low levels. In addition, many of the proteins which may present opportunities to predict preterm birth or histologic chorioamnionitis are only demonstrable in alternative gestational tissues such as the myometrium or cervix. Including samples taken from the umbilical cord, fetal membranes and amniotic fluid would, however, increase the range of proteins able to be assessed to understand whether these values are representative of maternal peripheral blood, cervicovaginal fluid or saliva samples. Once this is established, it may be possible to utilise these markers as prognostic indicators of preterm birth or for prediction of placental inflammatory responses which may inform treatment and management of at-risk women.

Chapter 8: General Discussion

The 2015 Amsterdam Placental Workshop Group Consensus Statement (Amsterdam Criteria) is a powerful tool used by pathologists to chronicle placental lesions according to an agreed-upon placental sampling protocol. Uniform sampling criteria, gross and microscopic descriptors, terminology of observed pathologies and diagnostic criteria were defined to include, amongst others, patterns of ascending intrauterine infection and maternal and fetal vascular malperfusion lesions (103). Refining the clinical significance of these defined lesions was highlighted as an area for ongoing development to support clinical and scientific studies in comparing placental lesions with adverse pregnancy outcomes, and this was an important overarching aim of this thesis. The studies presented in this thesis highlight limitations in current placental examination and reporting methodology, but also revealed novel significant associations between preterm birth, antepartum haemorrhage and preterm prelabour rupture of membranes (PPROM) and suggested a reversal to the order in which these adverse obstetric events complicate pregnancy and result in preterm birth. Gross morphological evaluation of placentas demonstrated a relationship between abnormal placental shape and extremely preterm birth and a potential underlying mechanism for a unique association of PPROM and incomplete membranes. Analysis of placental lesions identified specific phenotypes for World Health Organisation (WHO)-defined subcategories of preterm and term births and predictive models of phenotype by gestational age week which may be of value to neonatologists in assessing neonatal health on admission. Despite Covid-19 pandemic restrictions, immunohistochemical and immunofluorescence methodologies were optimised to identify the absence of target viruses in the placenta and, unexpectedly, identify a positive placental SARS-CoV-2 case from a negative mother. In addition these staining techniques validated the feasibility of immunofluorescence to determine macrophage polarisation and abundance in preterm and term cohorts as well as in placentas with and without histologically-diagnosed inflammatory responses. Finally, enzyme-linked immunosorbance assays were performed using a panel of antimicrobial proteins, chemokines, cytokines and matrix metalloproteinases not previously explored in the placenta which revealed significant associations between proteins unique to preterm and term placentas and in those with and without histologically-diagnosed inflammatory responses.

Basic demographic data, such as maternal age, body mass index (BMI), ethnicity, and number of previous preterm births (PTB), were retrospectively collected for women included in the study, either from clinical notes or from placental examination referral forms. In routine practise, these data would not be analysed; nevertheless, no significant differences were observed between women delivering preterm or at term in respect of adolescent or advanced maternal age, decreased or elevated BMI or reported previous PTBs or first- and mid-trimester losses, despite each of these being risk factors for PTB (26, 356, 357). A further unexpected finding in the cohort demographics was that White ethnicity was significantly associated with PTB, contrary to published literature which shows Black women to be 1.4 – 1.7-times more likely to deliver preterm than White European women (43, 649). Retrospectively collecting data proved problematic; discrepant recording of BMI was a recurrent feature in clinical notes, for example, when BMI was within the normal range a variety of records were observed, such as 24.9 kg/m^2, < 30 kg/m^2 to represent normal, or, simply, 'normal', which made analysis of this variable complex. In some cases, BMI was not recorded at booking, but later in pregnancy when values are not valid, or BMI was recorded pre-pregnancy from patient recollection of their pre-pregnancy, self-reported body mass. Likewise, ethnicity was often absent from patient records (20.4% of cases) resulting in a relatively small sample size. Coupled with the predominance of White British ethnicity in the local population and evidence that Black women are less likely to access specialist maternity care (44, 366), it is possible that a limitation was introduced during participant selection and recruitment or these data reflect an artefact of the demographics of the cohort. What has been highlighted is the need for larger cohort studies which include comprehensive analysis of demographic data collected through a study visit and questionnaire rather than a reliance on retrospective collection from incomplete clinical notes and a suggestion to extend this further to consider a broader scope of socioeconomic factors.

Despite multiple research studies discussing associations between PPROM and PTB, correlations generally focus on PPROM and histologically-diagnosed chorioamnionitis (HCA), latency periods or neonatal outcomes (369, 650, 651). Multiple linear regression revealed a novel and significant inverse relationship between PPROM and gestational age in the study cohort and a 7.5-times increased likelihood of spontaneous PTB following PPROM. A higher-than-expected number of women experienced PPROM prior to delivery, potentially due to

the centre in which the study was conducted being a specialist referral centre or due to selection bias since some women were recruited from wards where they had been admitted solely for the clinical management of PPROM. Additionally, the regression model demonstrated novel significant negative relationships between PTB and combinatory factors of PPROM and antepartum haemorrhage (APH), PPROM and preeclampsia (PE), PPROM and HCA, and PPROM, APH and HCA. The collective effect of these conditions on PTB risk have not been described before, yet they show involvement of the fetal membranes, placenta and decidua, plus highlight that vascular malperfusion, infectious inflammatory stimuli and sterile inflammation may all be involved in amplification of signalling necessary to initiate preterm labour. Findings presented in this thesis indicate that in some cases of PPROM, particularly where APH is a factor, the order of events is reversed to the current opinion that APH causes inflammation within the amniotic cavity, and this leads to PPROM. Rather, it is suggested that microfractures, tears or small holes in the amniotic membranes occur early in gestation due to aberrant collangenolysis and this permits undetected microbial invasion of the amniotic cavity (MIAC), as described by Richardson *et al.* (368). An array of proinflammatory mediators is then expressed in response to MIAC and HCA and these act on the decidua basalis to weaken it, which, in some cases, leads to APH before, eventually PPROM. In other cases, no APH may be evident, but PPROM will follow. Further research is vital to, firstly, retrospectively analyse cases of PPROM and APH, particularly where HCA or PE are also present, to confirm these associations in larger cohorts as they present an opportunity for timely PTB risk assessment in cases where women experience APH in early gestation. Furthermore, expectant management may be a negative course of action in this cohort of women and may actively contribute to HCA, weakening of the decidua and APH in some cases. However, it must be noted that the number of women where these combinatory obstetric conditions were observed in the present study was small, therefore, a larger cohort study is required to corroborate these preliminary data.

Another novel finding related to amniotic membranes was uncovered in the study cohort presented in this thesis. As part of the Amsterdam Criteria sampling and definitions (103), membrane completeness is recorded based on whether total or partial peripheral membranes have been submitted along with the placenta and umbilical cord, yet no further analysis is conducted and there is limited data on the clinical significance of reporting

membrane completeness (103, 144, 319). In this study, PPROM was significantly associated with the presence of incomplete membranes at placental examination. Placenta accreta spectrum (PAS) disorders, in which the placenta morbidly adheres to the myometrium, commonly lead to haemorrhage or retained products of conception (RPoC) and may account for incomplete, ragged or fragmented placental discs, peripheral membranes or umbilical cords (652). In a 2015 observational descriptive study, Khong, Cramer and Heller described a novel condition they termed chorion laeve accreta (ChLA). In this study, the authors reported fifty-two cases of smooth muscle presence in peripheral membrane decidua which they postulated may explain findings of retained membranes (652). This was not reflected in participants included in studies presented in this thesis; there was no evidence of traditional PAS, ChLA or RPoC in the cohort with the exception of one case (SU0036). An assumption was therefore made that increased numbers of women presenting with PPROM, and the number of placental samples submitted with incomplete or ragged membranes were a potential pathological finding explained by the association between PPROM, HCA and APH through gestational microfractures, tears and holes as discussed above. Alternatively, it is proposed that the relationship between PPROM and incomplete membranes may be due to inherent abnormalities in structural formation or modification throughout gestation and a resultant reduction in mechanical integrity. It is equally as plausible, however, that membrane incompleteness and damage is a residual effect of a prolonged latency period necessary for expectant management of PTB and, as such, any perceived damage is not, in fact, pathological. As this study is the first to report this association, caution in interpretation is advised and further studies are necessary to corroborate or refute these relationships.

Abnormal placental shape has been linked to vascular malperfusion disorders and placental insufficiency (99), APH and PTB (324). In agreement with current literature, circumvallate placentas were significantly associated with extremely preterm births (EPT) (324, 382), however, as is often also described and was true for all circumvallate reports in this study, this pathoanatomical finding generally goes undetected until postpartum gross examination of the placenta. Furthermore, data are inconsistent as to the clinical significance of circumvallation beyond an association with hypoxia and a potential increased risk of PPROM and APH. Recently, it has also been shown that there are no adverse obstetric or neonatal effects linked to circumvallate placentas (653). EPT placentas were also significantly

associated with another abnormality of placental shape, specifically those reported as succenturiate (bilobate). This abnormality is considered rare and primarily incidental, consequently, the association with EPT placentas could not be established as having been previously reported. As an infrequent phenomenon, further research into the effects of succenturiate shape and preterm birth initiation are necessary, nonetheless, it is possible that delayed or disordered angiogenesis of placental vasculature may contribute to placental dysfunction and initiation of preterm labour through hypoxic tissue damage activation of NFκB signalling and downstream activation of proinflammatory genes.

All placental evaluation and sampling described in this thesis was performed in accordance with the Amsterdam Criteria as this follows standard clinical practise at Sheffield Children's Hospital, however, as discussed in *Chapter 1,* there is no predefined standard for referral of a placenta for histopathological analysis beyond recommendations considered 'desirable' made by the Royal College of Pathologists (RCPath) (136). Moreover, this is subject to frequent change dependent on current resourcing and, as was the case during this PhD project, local, national and global factors such as the Covid-19 pandemic where the preterm gestational age advised for histopathological examination was lowered considerably (to <32 weeks' gestation) unless there was an additional obstetric complication or diagnosed maternal Covid-19 infection. A strength of this study was that it did not exclude any of the moderate to late preterm births (MPT) during these pandemic restriction or lockdown periods and so placentas from preterm births of 32 - < 37 weeks' gestation continued to receive a full placental histopathological examination. Consistent with published literature (162, 403, 654), vascular malperfusion lesions were the most common findings in MPT placentas and were 31-times more likely to be observed in preterm placentas of any gestation than at term. Malperfusion lesions are associated with severe adverse neonatal outcomes, such as intraventricular haemorrhage, which can induce poor long-term neurodevelopmental outcomes (452, 655). Maternal vascular malperfusion (MVM) lesions and fetal vascular malperfusion (FVM) lesions represent hypoxic-ischaemic damage to the placenta and, when singularly present, MVM lesions are associated with adverse pregnancy outcomes and maternal hypertension (656) and FVM lesions are linked to fetal death and increased risk of long-term neonatal neurodevelopmental problems (447). MVM lesions were observed less frequently in the entire preterm cohort than reported by other researchers, but FVM lesion

prevalence was similar to those recently described (453, 660), although this did not significantly increase the odds of PTB unlike MVM lesions where their presence showed PTB to be 15-times more likely than delivery at term. A previously unreported vasculopathy was described in the study cohort and this significantly increased the odds of preterm birth. Previous studies have reported MVM and FVM lesions in isolation, yet there were six cases of combined maternal and fetal vascular malperfusion (M/FVM) lesions in the study cohort, all of which were delivered at preterm gestations. In contrast to MVM, however, this vascular malperfusion phenotype predominated at extremely preterm gestations (18.2%) suggesting potentially greater adverse neonatal outcomes due to the global placental hypoxic effects. Nonetheless, MVM lesions were unexpectedly present in nearly half of placentas delivered in the moderate to late preterm cohort, a group currently only indicated as being a 'desirable' referral for placental examination (348).

Abundant evidence exists that hypoxia-induced placental oxidative stress leads to expression of proinflammatory cytokines and cytotoxic and vasoactive mediators which result in stabilisation of hypoxia inducible factor-1 alpha (HIF-1α) and preeclampsia symptoms (657, 658). The pathophysiological and aetiological mechanisms specifically influencing MVM and FVM are not completely understood (449), but emerging evidence extending correlations of hypoxic conditions with MVM, FVM and adverse obstetric conditions (659) highlights the need for earlier detection of MVM and FVM lesions. Although not entirely specific for vascular malperfusion lesions, the ratio of placental growth factor (PlGF) and soluble FMS-like tyrosine-kinase-1 (sFlt-1) is currently used as a classical biomarker for placental insufficiency, preeclampsia and IUGR, whereby ratios of ≤38, 39-84 and ≥ 85 categorise women respectively to low, moderate and high risk for development of preeclampsia or spontaneous preterm birth (661, 662). However, this has recently been shown to only be predictive of spontaneous PTB when individual sFlt-1 and PlGF expression levels are measured and reported as reduced, when the ratio of the two markers is considered together with individual values and where assays are performed on extracellular vesicles-exosomes rather than maternal plasma (663). HIF-1α has a κB motif within its promoter region and is therefore capable of inducing activation of NFκB in hypoxic conditions, releasing NFκB to induce proinflammatory gene expression and initiate premature labour onset, as reviewed by Cookson and Chapman in 2010 (665). Nevertheless, limited work has been done more recently to firm up the link

between hypoxia and the multiple cross-over of mechanisms which activate NFκB to induce downstream processes; consequently this is an area which requires further exploration. Whilst earlier identification of the effects of vascular malperfusion could be established by the measurement of angiogenic and antiangiogenic factors in maternal blood, such as PlGF and sFlt-1 and their ratio, a more precise hypoxia marker remains an important consideration since this would also indicate the increased risk observed in this cohort of spontaneous PTB. Postpartum identification of HIF-1α levels in the placenta would be a positive and progressive initial step to establish if these are elevated in the presence of M/FVM lesions and to determine whether HIF-1α could be a viable biomarker of vascular malperfusion disorders which contribute to PTB. An absence of current reliable antenatal diagnostic techniques to definitively identify and classify vascular malperfusion lesions, in conjunction with the data presented in this thesis, also demonstrate that it remains critical to perform examination of placentas from moderate to late preterm births even in the absence of specific maternal or obstetric conditions to monitor for adverse neonatal outcomes associated with placental vascular malperfusion lesions.

Distinct placental phenotypes were also identified in each of the remaining three gestational age groups, a characterisation not previously conducted for each of the WHO-defined preterm and term groups. It is frequently stated that acute inflammatory responses are the most commonly occurring lesions in extremely preterm placentas (162), in disagreement with the findings in this thesis of a previously unreported placental phenotype of acute PIR and vascular malperfusion lesions combined (PIR/VM) predominating in extremely preterm births (45.5%). A simple linear regression model revealed likelihoods at 24 weeks' gestation of 57.8% of the presence of PIR/VM lesions and 42.5% of vascular malperfusion (VM) lesions. There was a limited likelihood of no pathological lesions in extremely preterm placentas (5.7%). Conversely, at term (40 weeks' gestation) the chance of no pathology was 42.6%, VM lesions 13.3% and combined PIR/VM lesions only 4.8%. Surprisingly, the predicted chance of acute PIR being the only reported pathology at 24 weeks' gestation was 15.6%, whereas at term, this was greater than the chance of no pathology at 43.7%. In further support of these results, when maternal and fetal inflammatory responses (MIR and FIR, respectively and collectively PIR) were staged and graded, that is the lesions were categorised by location and progression into the placental compartments and neutrophilic abundance and confluence were assessed

(103), acute MIR and FIR overall were equally as prevalent in term placentas as in preterm placentas, as was the most commonly reported MIR (acute chorioamnionitis; HCA). Nonetheless, these findings were in contrast to published studies which report HCA as only occurring in 3-5% of term placentas (142), but subcategorising PTBs did reveal acute MIR and FIR lesions to occur more frequently in EPT placentas. Further, necrotising chorioamnionitis and acute funisitis, the most advanced and severe MIR and FIR, respectively, primarily occurred in the EPT subgroup. Overall, the study identified a preponderance for high stages and grades of MIR and FIR at preterm gestations compared to term gestations and that in the case of FIR, stage 3/grade 2 was reported more often than less severe and advanced responses. Regression models demonstrated gestational age to be an independent predictor of MIR and FIR stage and FIR grade, with MIR stage at 23.9 weeks' gestation (EPT) twice that at term (39.9 weeks' gestation), FIR stage almost 5.5-times greater and FIR grade 3.5-times higher at the same EPT versus term gestations. Previous research findings support the results in this thesis of exaggerated PIR, particularly FIR, occurring at earlier gestations resulting in an intense, robust and prolonged response ultimately leading to PTB (407); however, no mechanism of action is suggested. During sterile inflammation neutrophils play an essential role in clearance of cellular debris and a reversion to tissue homeostasis, when inflammation persists or there is continued exposure to pathogens and proinflammatory agents, such as LPS and IL-8, neutrophils can release neutrophil extracellular traps (NETs) which exacerbate tissue damage (667). There is a lack of evidence as to NET formation in PIR, but based on evidence that excessive NET formation induces hyperinflammation and tissue damage in a variety of acute and chronic diseases, including necrotising enterocolitis, sepsis, Covid-19-related lung injury, chronic obstructive pulmonary disease and cystic fibrosis (667, 668), it is hypothesised that high stage and grades of MIR and FIR are reflective of a chronic process whereby there is a sustained influx of neutrophils to placental compartments and persistent NET release. The resultant tissue damage is observed as necrosis and reported as necrotising chorioamnionitis and acute funisitis. Emerging evidence on the role of NETs in sterile inflammation, autoimmune disorders and malperfusion adds further credence to this hypothesis in that research has shown that neutrophils also inappropriately or overexpress NETs under conditions neutrophils perceive to be a threat, such as in the case of vascular malperfusion disorders (669). This may explain the increased proportions of combined acute

PIR and vascular malperfusion disorders observed in placentas from extremely preterm births.

Although as stated previously all placentas were examined according to the Amsterdam Criteria, the observation of almost equal prevalence of acute PIR lesions in preterm and term placentas highlights an issue with subjectivity in the methodology. The presence of neutrophils does not provide data on functionality and, although still poorly understood, physiological labour is considered to be an inflammatory process involving multiple upstream stimuli such as expressed inflammatory mediators, uterine stretch, oxidative damage or intrauterine infection. The resultant NFκB activation, upregulation of prostaglandin synthase 2 (PTSG2) and COX-2 and repression of progesterone receptor isoforms leads to increased inflammatory cytokine expression activating the NFκB pathway, further increasing inflammation and assisting in the downstream processes of cervical remodelling, switching of myometrial smooth muscle cells from a quiescent to a contractile state and membrane rupture (624, 670), as shown in Figure 8.1. As neutrophils are expressed in abundance during sterile tissue injury, primarily to return the tissue to a homeostatic state (644), it has been suggested that they may be involved at targeted points in these normal labour process, although there remains limited understanding as to their role beyond hypothesising dysregulation of phenotype contributing to inflammation or a loss of immunoregulatory function (671). It is possible that the results presented in *Chapters 3* and *4* show neutrophilic infiltration of the amniotic membranes representative of normal labour, particularly at term. This highlights the need for adjustments to the Amsterdam Criteria, for example, to only consider reports of stage 1, grade 1 acute chorioamnionitis to be clinically significant if observed in conjunction with other pathologies (including preterm delivery), or for immunohistochemical staining methods to be performed on samples following a protocol recently described by Muñiz-Buenrostro *et al.* (672) to specifically stain NETs with nitro-blue tetrazolium-safarin. This cost-effective method allows visualisation of NETs under light microscopy and would potentially allow discrimination of pathological and physiologic neutrophilic presence in placental samples as well as a more objective quantification of MIR and FIR stage and grade.

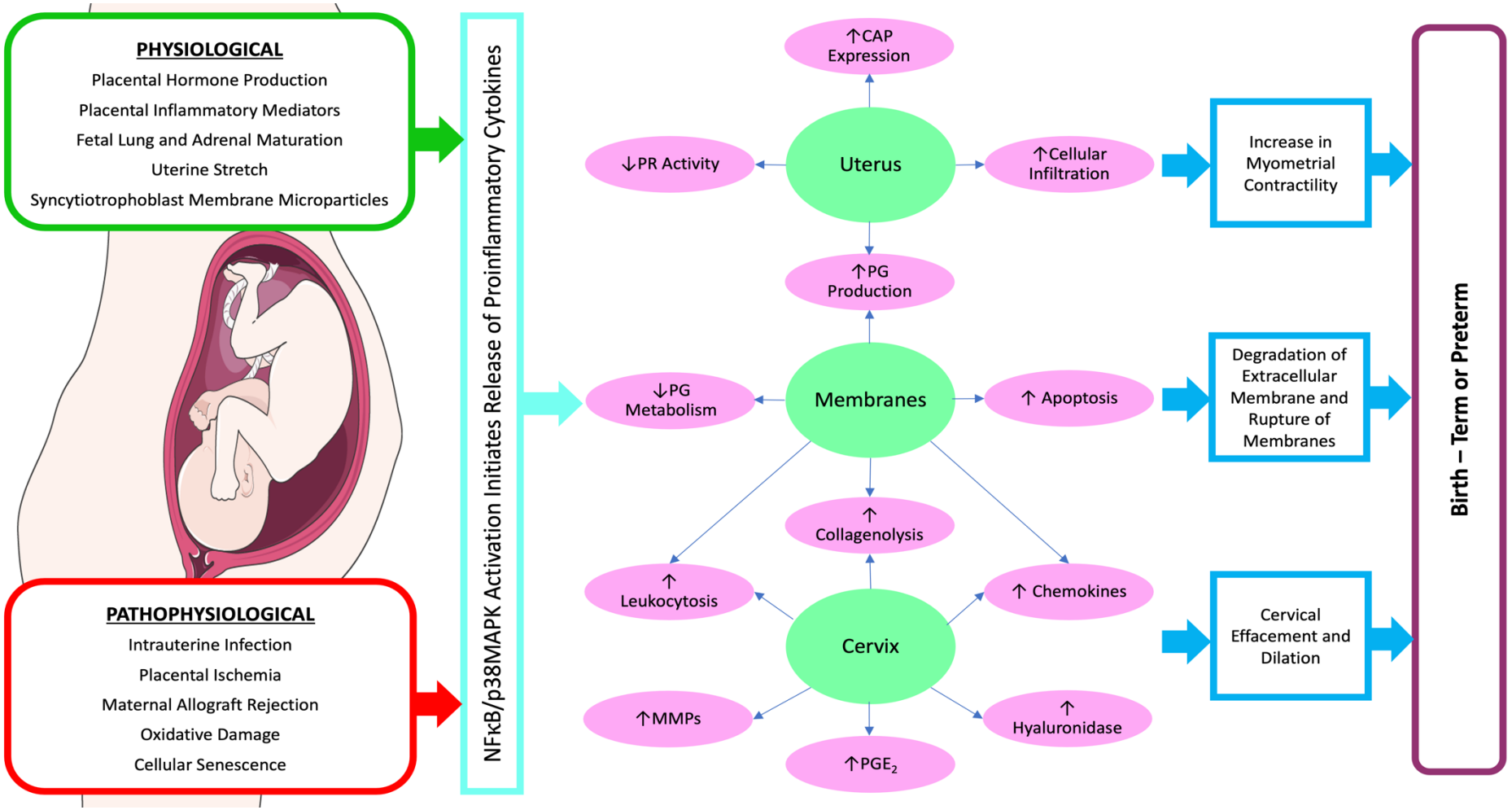

Figure 8. 1 Schematic representation of parturition as an inflammatory event.

A number of physiological processes activate NFκB and p38MAPK pathways, which, in turn, initiate the release of proinflammatory cytokines. Coordinated responses occur in the uterus, membranes and cervix, ultimately leading to myometrial contractility increasing, membranes rupture and cervical effacement and dilation. In preterm births, pathophysiological processes may contribute to activation of the common pathway seen in term labour. Fetus in utero image created with permission from Elsevier SMART images. *CAP*; contractile-associated proteins, *MMP*; matrix metalloprotease, *PG*; prostaglandins, *PGE_2*; prostaglandin E2, *PR*; progesterone receptor.

Immunohistochemical (IHC) analyses found no evidence of cytomegalovirus (CMV) and herpes simplex virus 1 and 2 (HSV-1/2), viruses previously linked to chronic villitis (170, 673) and PTB (178, 674). Currently, primary placental cells such as extravillous trophoblasts (EVT), immortalised cell lines including human invasive proliferative extravillous cytotrophoblast (HIPEC) or JEG-3 (human choriocarcinoma reflective of trophoblasts), and/or explants are used in the majority of studies of CMV (494). However, results obtained from experimentation with these cell lines do not necessarily translate to the clinical scenario (494). IHC was selected as the technique of choice to study CMV and HSV-1/2 prevalence due to its recognised advantages and because automated IHC staining is routinely performed for both viruses in the Department of Histopathology at Sheffield Children's Hospital (SCH). As described by Furrer *et al.* (675), IHC and IF, as was performed to examine macrophage phenotype and distribution (*Chapter 6*), are relatively inexpensive techniques and both allow for localisation of target antigens which was a primary aim for both *Chapters 5* and *6*. In addition, IHC staining results in permanency which permits long-term storage, delayed analysis and future work to be conducted on previously collected samples (675). As became a critical aspect in this study, IHC also provides a safe method of studying potentially harmful biological specimens (676). One third of the cohort samples were stained for the presence of CMV and HSV-1/2, with the majority of these being matched participant samples. The results do not fit with the evidence that global CMV incidence is 0.2% to 2.5% of all live births (185, 674, 677) and that HSV-1 or HSV-2 is present in almost 30% of placentas from asymptomatic women, primarily with no history of HSV infection (462), although CMV and HSV-1/2 prevalence in the UK are reported to be much lower at 0.3% (481) and 2%-3% (678), respectively, which may account for the negative findings. Additionally, research suggests CMV and HSV infection may be linked to a greater risk, albeit still low, of spontaneous abortion and stillbirth rather than PTB, and, as these outcomes were excluded from the study, it was not possible to determine the prevalence of viral inclusions in placentas from cases with these adverse obstetric outcomes (494, 679, 680). What the findings do suggest, however, is that current screening techniques, clinical management of maternal CMV and HSV-1/2 and placental histopathological examination contribute positively to the low incidence of these viral infections and, consequently, to a decreased risk of resultant PTB in UK cohorts. During the early stages of the Covid-19 pandemic, as with the majority of institutions, all research was suspended at the University of Sheffield. However, the aforementioned benefits of IHC methodologies,

primarily the ability to analysis stored samples in a safe way, presented an opportunity to identify the presence of SARS-CoV-2 antigen in full thickness placental samples collected from women who had a negative nasopharyngeal swab result. Once appropriate permissions were granted by departmental heads of the University of Sheffield and SCH, IHC analyses of six term and six preterm placental samples were performed as described in *Chapter 6*. In opposition to expected results, SARS-CoV-2 immunopositivity was recorded in one term placenta (SU0089) collected from a mother with a negative lateral flow test on admission and no reported Covid-19 infection during pregnancy. Infection with SARS-CoV-2 Alpha and Delta variants of concern (VOC) were associated with MVM, reduced placental weight and accelerated villous maturation in a study described by Shanes *et al.* (681), features which were not seen in the SARS-CoV-2 positive case despite these being the VOCs at the time of delivery (682). A recent study of fifty-two placentas from women testing positive for SARS-CoV-2 showed elevated inflammatory marker expression and extensive maternal immune cell infiltration in SARS-CoV-2-infected placentas along with a greater risk of adverse outcome in the presence of the Alpha VOC (683). The positive case presented in this thesis demonstrated SARS-CoV-2 infection of the syncytiotrophoblast, cells shown to highly express ACE2 receptors necessary for viral entry (684). However, no maternal infection was reported nor was any neonatal infection suspected. Potential explanations for this anomaly are that the original SARS-CoV-2 strain was the infective agent in this case. In the early pandemic era, limited placental SARS-CoV-2 infections were observed and, when reported, there were no concomitant increases in pathological findings (685). Alternatively, as placental entry receptors for SARS-CoV-2 are highly expressed in early- and mid-gestation, if infection occurred shortly before birth it is plausible infiltration of maternal immune cells and increased expression of inflammatory markers had not yet reached observable levels or that the placenta was acting merely as a reservoir of the virus (683). The results of this study highlight the need for ongoing research, even as the world exits the Covid-19 pandemic and moves to an endemic phase. Evidence of rapid SARS-CoV-2 viral adaptation demonstrates cross-species transmission and acquired pathogenicity, and much like Zika virus (ZIKV), shows placental tropism (559, 560). From the published literature it appears that the timing of maternal SARS-CoV-2 infection is important, as is the viral variant. Further work is necessary to establish how the underlying mechanisms of these temporal and variant effects are associated with

variability in disease severity seen in infected individuals and which appear to be replicated in the placenta.

The localisation, abundance and ratio of macrophage phenotypes were assessed using indirect immunofluorescence (IF), which is highly specific and is capable of excellent signal amplification and resolution (700) and proved to be a reliable method in this study. Although it was not possible to complete all of the intended studies of macrophages planned pre-pandemic, some interesting observations were noted. Despite the small cohort size, quantitative analysis using IF revealed the highest proportion of total macrophages to be in preterm placentas with no placental inflammatory response diagnosed (no PIR). Despite this finding being supported by some studies (213, 579), others have reported increased macrophage numbers in the presence of PIR (582) thus the effect of HCA or other PIRs on macrophage numbers remains undefined. As discussed, neutrophilic infiltration to the chorionic plate characterises HCA (103) and this predominantly results from ascending or intrauterine infection (402, 408). Bacterial lipopolysaccharide (LPS) induces the M2 surface marker CD163 in cultured cells (701) and, *in vivo*, high CD163 expression levels are observed following induction by IL-6 and IL-10 (702). Consequently, it was hypothesised that M2 macrophage numbers would be increased in placentas with HCA in response to microbial invasion of the amniotic cavity (MIAC) and LPS expression with the purpose of downregulating inflammatory responses (703). M2 phenotype macrophages were the primary phenotype in all samples, in agreement with published literature (196), yet proportions of these anti-inflammatory immune cells were not increased in conjunction with reported HCA or other PIRs as hypothesised; indeed, the converse was seen and proportions of M2 macrophages were significantly associated with preterm placentas with no PIR. Despite a recent study suggesting only gestational age rather than PIR status to influence a shift to M1 polarisation (585), the results presented in this thesis show significant associations between macrophage polarisation state (M1 or M2) and PIR status, specifically that higher proportions of M1 and lower proportions of M2 macrophages are present in placentas with PIR than those with no PIR in the total sampled cohort. However, when the preterm and term groups were analysed separately, these findings were only significant in the preterm cohort in line with results published by Shan and colleagues (585). As discussed previously, it may be that preterm placentas mount a more robust response to the elevated expression of M1 proinflammatory

mediators or that in some cases there are combinatory stimuli from pathogens and endogenous damage-associated molecular pattern molecules (DAMPs) due to hypoxia-related tissue damage and this results in further M1 macrophage secretion of NFκB-induced inflammatory cytokines and preterm labour initiation. Alternatively, observed increased proportions of M2 macrophages may represent an appropriate M2 response, downregulation or resolution of inflammation and a return to homeostasis in the placenta. Despite the elevated M1 proportions seen in placentas with PIR, M2 macrophages were still the most abundant phenotype in all samples, as noted above. The cell surface marker CD163 used to detect M2 macrophages in the study has been identified as a biomarker of disease severity in sepsis and acute kidney injury (AKI), further, it can be detected in serum, urine and cerebrospinal fluid as, upon activation, it is cleaved from the surface of the macrophage and continuously shed in a soluble form (sCD163) (701, 702). No data currently exist on measurement of sCD163 in cervicovaginal fluid (CVF) or as a discrete biomarker of HCA, but one study in 2005 did reveal serum sCD163 levels to be significantly higher in women delivering preterm (704). The potential value of sCD163 as a biomarker of HCA in CVF, serum or urine is an area for further investigation. If these findings were to be replicated in a larger, adequately-powered study of sCD163 as a clinical biomarker of HCA, the next logical steps would be to determine the predictive value of staging and grading placental inflammatory responses prepartum.

Although markers of inflammation have been shown to be increased in gestational tissues during pregnancy and in relation to normal term parturition, studies primarily focus on the amniochorionic membranes. Consequently, the number of studies analysing inflammatory biomarkers in the placenta are limited (214). Moreover, within these studies, sample sizes have a tendency to be smaller than those for the amnion, chorion, decidua and myometrium (214). As described in *Chapter 7*, a panel of inflammatory and antimicrobial peptides were selected for investigation with two potential biomarkers elucidated. Despite the relatively small sample size, interleukin-6 (IL-6) and secretory leukocyte protease inhibitor (SLPI) were significantly increased in placentas with PIR. Assessment of an IL-6 rapid bedside test revealed a 98.6% sensitivity and 94.7% specificity in detecting chorioamnionitis from cervicovaginal secretions in a recent study conducted by El-Ghazaly *et al.* (705). The authors present a 97.3% overall accuracy in predicting chorioamnionitis in the 110 women in the study, all of whom

had experienced PPROM. The findings of significantly increased IL-6 in the presence of PIR presented in this thesis are encouraging in extending El-Gazaly *et al.*'s study to predict chorioamnionitis in women with threatened preterm labour without PPROM, although they do not indicate the likelihood of PTB occurring. IL-6 concentrations presented in this thesis were obtained from full thickness placenta samples which included chorionic plate, villous tissue and decidua basalis compartments in contrast to El-Ghazaly *et al.*'s study in which CVF was analysed (706). A limitation of this sampling technique is the uncertainty of whether amniotic fluid has contaminated the CVF sample where women have experienced PPROM, a factor not considered problematic when using full thickness tissue samples. Adequately powered cohort studies to evaluate the accuracy of rapid bedside tests for IL-6 are required to determine if IL-6 concentration in CVF is a true predictor of chorioamnionitis and if these findings can be replicated before PPROM occurs. These studies would require confirmatory histopathological examination of the placenta and control samples of elective Caesarean section deliveries to ensure the test apparatus is not reporting normal inflammatory processes of labour. In contrast to findings presented in this thesis, SLPI concentrations are reported to decrease in some pathologic pregnancy conditions, for example, PPROM (236). The roles of SLPI in labour processes and in the placenta are undefined, yet its presence in normal term syncytiotrophoblast, amnion epithelium, chorion trophoblast and decidua basalis and parietalis may suggest functions related to inflammatory regulation and MIAC prevention (236). In addition to regulation of infectious inflammation, SLPI modulates cell differentiation and apoptosis in wound healing and provides a homeostatic function through regulation of neutrophil extracellular traps (NETs) (231). Decreased SLPI concentrations in PPROM are reported from amniotic fluid samples and are believed to be due to the reduced anti-protease activity, a resultant protease activity and subsequent activation of matrix-metalloproteinases and membrane rupture (236), yet the findings from placental tissue do not appear to reflect this mechanism. In the preterm cohorts with and without PIR, all but one participant had experienced PPROM. Conversely, in the term cohorts, only three of the eight participants experienced spontaneous rupture of membranes. It appears in placental tissue, SLPI concentrations are unrelated to its anti-protease function. No studies describing elevated SLPI in placental inflammation could be sourced, therefore its function in this context remains elusive. However, SLPI is expressed in human myometrium, is localised to the nuclei of myocytes and has been associated with NFκB activation and labour onset (618). It is

proposed that placental SLPI concentrations are increased in response to inflammatory stimuli, either from infectious agents, sterile tissue injury or hypoxia, and that this is further elevated due to labour inflammatory processes to provide optimal SLPI antimicrobial activity during the period when the amniotic cavity and placenta are most susceptible to infection, as was suggested to occur in the myometrium (618). Further work to establish which placental cells express SLPI and participate in the regulation of immune response is, however, necessary.

Correlations of placental target proteins revealed very strong positive relationships highlighting specific signatures at term and preterm gestations. In all cohorts, hCAP18 was very strongly significantly positively correlated with IL-6 and MMP-9, that is as one protein concentration increased, so did the other, although determining if one *causes* a rise in the other is not possible using the technique employed in this thesis. Previous studies support increased hCAP18 expression in amniotic membranes and myometrium following spontaneous labour and show the cleaved C-terminal peptide of hCAP18, LL-37, to induce IL-6 secretion in these gestational tissues and MMP-9 secretion in amniotic membranes via the NFκB pathway. Furthermore, hCAP18 acts as an inducible antimicrobial during labour and is elevated in the presence of HCA and PPROM (221). As a range of studies have shown hCAP18 to be induced by inflammation and during infection in amniotic membranes and myometrium, it is therefore not unreasonable to assume that the same mechanisms underly the significant positive associations observed in placental tissue in this study in all groups. Likewise, IL-6 and MMP-9 were very strongly positively correlated in the present study; it is suggested this is a relationship linked via hCAP18 or other antimicrobial protein as described. Multiple studies highlight that no single protein is responsible for labour, either at term or preterm, it is as unlikely that a combination of only two proteins would be responsible either.

Preterm and PIR cohorts showed similar protein correlations, as highlighted in green and red, respectively on Table 8.1. The exception to this being the absence of significant associations with IFN-γ observed in the PIR group. M1 macrophage proportions were increased in placentas with PIR by almost 50% compared to those without in this study. Classical activation and polarisation to M1 phenotype occurs following stimulation by IFN-γ, M1 macrophages

then express this cytokine in abundance (195, 199, 200). Increased proportions of IFN-γ-expressing macrophages likely accounts for the significant associations seen in placentas with PIR which were absent from the preterm signature. Likewise, proportions of M1 macrophages were increased in term placentas in comparison to preterm placentas, in almost identical ratios, which may account for the elevated IFN-γ concentrations. Contrary to other studies, a positive correlation was shown between IFN-γ and IL-10 an anti-inflammatory, pro-pregnancy cytokine (708). IL-10 expression is a key indicator of resistance to preterm labour (644), therefore, it is possible that the association is an observation of 'normal', that is IL-10 downregulating proinflammatory cytokines and increasing in response to increasing IFN-γ concentrations. Why a similar profile was not observed in placentas with no PIR, however, is not clear and requires further investigation.

Table 8. 1 Correlations between target proteins in preterm and term placentas and those with and without placental inflammatory responses to demonstrate unique signatures

Cohort	Target Protein										
	hCAP18 IFN-γ	hCAP18 IL-6	hCAP18 MMP-9	hCAP18 SLPI	IFN-γ IL-6	IFN-γ IL-10	IFN-γ MMP-9	IFN-γ SLPI	IL-6 MMP-9	IL-6 SLPI	MMP-9 SLPI
Preterm											
Term											
PIR											
No PIR											

hCAP18; human cathelicidin antimicrobial protein*, IFN-g;* interferon-gamma, *IL-6*; interleukin-6, *IL-10*; interleukin-10; *MMP-9*; matrix maetalloproteinase-9, *PIR;* placental inflammatory response*, SLPI;* secretory leukocyte peptidase inhibitor.

To date, these associations have not been described in full thickness placental tissue samples, nor have correlations specific to term and preterm cohorts been compared to placentas with and without PIR. Elevated proinflammatory cytokines and chemokines in the placenta may result in premature activation of placental p38 MAPK and NFκB signalling pathways and parturition (710), as well as contributing to IUGR, preeclampsia and stillbirth in cases of sterile inflammation following oxidative stress or hypoxia (711). Therefore, expression levels or ratios of targeted proinflammatory proteins may be viable biomarkers of underlying placental pathology and severity in a context wider than PTB.

8.1 Strengths and Limitations of the Study

Strengths of this study included the use of freshly collected human tissue from an unselected cohort. Physiological and molecular characteristics differ between animal and human specimens, thus, using human tissue for research advances a more targeted understanding of disease mechanisms and therapeutics. Furthermore, studying a large whole organ such as the placenta allowed for additional sampling and storage of frozen and FFPE tissue useful for future research following these pilot studies (712). Participant selection and recruitment was based on spontaneity of labour and delivering a live born neonate only rather than obstetric or maternal clinical variables such as IUGR, preeclampsia or GDM, adding strength to the study. However, this also presents a limitation and the possibility of selection bias as described in Chapter 3. PPROM rates were higher than average in the study cohort, potentially due to selection bias and recruitment of women from antenatal wards where they were being expectantly managed following PPROM. Likewise, referral bias must also be considered as a limitation to the study since placentas with potential pathological features such as ragged maternal surfaces are more likely to be referred for histopathological examination. It is therefore challenging to generalise these findings to the total population. Methods used in the studies also have strengths and limitations. All histopathological sampling, analyses, examinations and reporting were performed according to published clinical protocols and following the Amsterdam Criteria (103) to reduce interobserver variability. Although these protocols clearly define parameters for placental examination, interpretation of features such as colour of fetal and maternal surfaces or accelerated villous maturation may be subjective and, as described, there remains an uncertainty in whether complete peripheral membranes and/or umbilical cords were submitted for examination. IHC, IF and ELISA are well-established, qualitative methods and, in the case of IHC and IF, add value to the study in localising proteins of interest. Where possible, monoclonal antibodies were used in the study as these produce less background signal and provide enhanced reproducibility compared to polyclonal antibodies, although polyclonal antibodies have higher specificity than monoclonal antibodies and are capable of recognising multiple epitopes (713). To further minimise background staining and improve reproducibility, negative controls with species-matched non-immune IgG were run with all IF staining samples and all samples were stained in one batch using the same positive and negative controls. IHC was performed at SCH using an automated process to the United Kingdom Accreditation

Service (UKAS) International Organisation for Standardisation (ISO) 15189:2012-accredited standards with positive and negative placental tissue control samples. As a relatively small pilot study, the size of the cohort is an obvious limitation, particularly in studies of macrophage numbers and protein concentrations (*Chapters 5 and 6*). Although patterns in histopathological features can be determined quite clearly, smaller numbers in each preterm cohort, or those where confounding factors were present, limit the validity of statistical tests performed to explore the clinical and histopathological data. The original total cohort size of one hundred subjects was chosen for the wider PRIME project and was in line with other pilot studies of placental histopathology and adverse pregnancy outcome. Given the study objectives, resources and Covid-19 pandemic restriction constraints, the cohort size was considered appropriate.

8.2 Future Work

Unfortunately, this study was greatly affected by the Covid-19 pandemic restrictions and lockdowns. Initial plans included collection of one hundred placentas from fifty women delivering preterm and fifty at term. Although more women than this were recruited, only ninety-eight participants met all eligibility requirements or their placental samples were considered suitable for examination and evaluation, with many of these due to issues primarily related to pandemic restrictions. Nonetheless, these numbers were considered sufficient for pilot studies addressing histopathological evaluation. However, being a tertiary teaching centre with specialist clinics and facilities equipped to manage and treat complex obstetric disorders and the most preterm infants, a challenge in the research was to address the live-birth and/or selection bias which may have affected the validity and generalisability of the results through either sociodemographic characteristics of the cohorts, uptake or timing of interventions to prolong pregnancy or improve outcomes for neonates (tocolytics or corticosteroids, for example), or more active perinatal care for extremely preterm infants than in other centres. To overcome this limitation, several avenues of future work could be explored, including collecting data from a broader and more diverse set of sources. This should include questionnaires at booking appointments or incorporating study visits to allow for interviewing patients to collect longitudinal data to track changes in selection bias over time, identify patterns and trends in the biases and develop strategies to mitigate their effects. Adjustments to the experimental design also need to be implemented to include

more specific controls, such as recruitment of women undergoing elective Caesarean section (non-labouring) to evaluate inflammatory responses which may be considered normal physiological processes of labour. To further strengthen causal inferences between cohorts, gestational age-matched controls should be included in future experimental design.

In respect of live-birth bias, anecdotal evidence provided by Professor Marta Cohen based on presence of viral inclusions in the placenta shows viral infections, specifically CMV and HSV-1/2, to be almost exclusively related to stillbirths in UK cohorts. These observations are supported by studies which show HSV and CMV to be significantly associated with an increased risk of spontaneous abortion and stillbirth, both of which were excluded from this study (494, 679, 680). By incorporating late miscarriage, stillbirth and neonatal death data into future studies, a more comprehensive understanding and broader overview of the entire spectrum of outcomes associated with placental pathologies related to viral infection and PTB can be considered alongside identifying risk factors and potential interventions before PTB occurs. This is particularly important in respect of SARS-CoV-2 which remains an endemic viral infection and continued risk to pregnant women. Recent evidence has linked SARS-CoV-2 infection to preterm birth and suggested that maternal M1 macrophages and neutrophils expressing ACE2 may be able to traffic to the placenta and increase the risk of Covid-19 infection, although the mechanism is not entirely clear (714). Further work is therefore necessary in this area, as is whether vaccination against Covid-19 prevents placental damage observed in some cases of infection or mitigates the risks of adverse pregnancy outcomes. Furthermore, without neonatal or maternal follow up, as was the case in this thesis, it is not possible to accurately reflect the long-term fetal or maternal effects of placental histopathological findings; a key factor in future studies should be to incorporate immediate and long-term neonatal follow-up to advance understanding in pathology, obstetrics and neonatology and seek to refine the clinical significance of placental lesions. To address these clinical and selection heterogeneities, future studies should also seek to recruit women to studies from early pregnancy and continuously collect data on obstetric health. This would ensure that women were still categorised to either the preterm or term cohort after delivery but with a complete maternal and obstetric history without distortion of association due to omission or inclusion of specific characteristics or conditions such that the population no longer reflects the population of interest.

Macrophage studies in this thesis assessed the feasibility of immunofluorescence staining as a technique to determine polarisation state and location in the placenta. Although this approach identified significant associations between gestational age, placental inflammatory response status and macrophage phenotype, the complete expression profiles of both M1 and M2 macrophages remain unknown, and it is not clear whether these populations are able to polarise further to distinct subtypes. Pre-Covid-19 restrictions, it was intended to double-stain macrophages with CD68 and CD80 or CD68 and CD163 to confirm an M1 or M2 phenotype, respectively. In addition, determination of the origin of macrophages, that is whether they were maternal or fetal, was planned through the use of a Y-chromosome marker and a fluorescence in situ hybridisation technique on samples from placentas delivered preterm, term, with PIR and without PIR from pregnancies with a known male fetus. However, it proved challenging to optimise these techniques and once laboratory restrictions were relaxed, it was not deemed cost effective to resume this aspect of the study. As discussed in *Chapter 6*, macrophage polarisation contributing to normal labour processes and/or preterm birth may not be limited to polarisation solely to M1 and M2 phenotypes; indeed, subpopulations of M2 macrophages are widely reported in the literature and, more recently, a polarisation state considered as 'intermediate' or 'non-classical' has been described where macrophages secrete combinations of proinflammatory and anti-inflammatory mediators. Furthermore, these have been shown to be parallel to inflammatory processes indicative of labour and are characterised by gestational age-dependent increases of the transcription factor STAT1 (584, 715, 716). To fully characterise macrophage phenotype in future experiments, immunofluorescence staining should incorporate double staining to include a transcription factor involved in polarisation to each phenotype as well as phenotype-specific cell surface markers, for example hypoxia inducible factor-1 alpha (HIF-1α) and CD80 (M1), STAT3 and CD163 (M2) and STAT1 and CD16 (intermediate). Given that elevated numbers of intermediate macrophages have been reported in preeclamptic and preterm placentas, investigations are necessary to elucidate whether this is also the case in cases of placental malperfusion disorders, particularly when identified in preterm births. The more widespread role of HIF-1α in preterm birth is not yet fully understood. However, as a transcription factor involved in polarisation of macrophages to an M1 phenotype and subsequently a proinflammatory expression profile with an additional critical role in the

response to hypoxia, it is considered to be an important protein to examine for its involvement in physiological and pathological pregnancy. Furthermore, given the significant findings of both inflammatory and malperfusion lesions in preterm placentas in the studies presented in this thesis, the dual role of HIF-1α makes it a promising target for future work. Research studies should focus initially on measurement of HIF-1α levels through immunohistochemical, ELISA or quantitative PCR methodologies in placentas collected across gestational ages to determine differences in those considered pathological, i.e., preterm, versus term gestational ages to assess the significance of HIF-1α expression in normal pregnancy and in placental insufficiency as this is yet to be clearly defined. As discussed in this thesis, danger-associated molecular patterns (DAMPs) and cytokines affect placental function via their expression and effects on initiator and target cells and production of these proteins has been shown to be promoted by HIF-1α. Studies should therefore also seek to understand the source of intracellular DAMPs and cytokine concentrations, particularly where these are elevated in the presence of placental malperfusion disorders. Monitoring of HIF-1α levels and activity in pregnant women may help to identify those at risk of placental insufficiency and/or preterm birth as an emerging biomarker for early detection and intervention. Further work to interrogate whether interventions such as lifestyle modifications, nutritional changes or pharmacological agents can regulate HIF-1α activity and reduce the risk of preterm birth requires further exploration.

Correlation of histopathological data with maternal clinical data revealed an association between PPROM, HCA and APH in a small subset of participants and a relationship between incomplete or ragged peripheral membranes and PPROM. However, a limitation of this particular aspect of the study was uncertainty in whether membranes were truly *incomplete* or *incompletely submitted* to pathology for examination. To investigate the hypothesis of PPROM leading to HCA, weakening of the decidua and subsequent APH, retrospective data extraction from placental examination reports should be undertaken in a larger cohort study. Future studies would also benefit from detailed information on completeness of membranes *at delivery* being included on the pathology request form to inform analyses of membrane integrity and a more-defined approach to research-specific terminology for placental histopathological examination.

8.3 Conclusions

This thesis presents a series of pilot studies to explore novel histopathological characterisations and potential biomarkers of PTB. Histopathologically, these studies revealed defined variations in the type and frequency of inflammatory and non-inflammatory lesions reported in preterm and term placentas and that it is possible to categorise placentas according to WHO-defined gestational age groups and histopathological lesions. Extending these findings further, this thesis developed models capable of predicting histopathological features and stage and grade of inflammatory responses based on gestational age in weeks, each of which may improve management of extremely and very preterm births. Immunohistochemical studies showed that neither CMV nor HSV-1/2 are major contributors to PTB in UK cohorts, nonetheless, these IHC staining findings support the value of histopathological examination of the placenta in cases where maternal infection is suspected or diagnosed and there is a risk of fetal or neonatal transmission or placental injury. Assessment of macrophage phenotype, abundance and location elucidated a potential biomarker for HCA-associated PTB and ELISAs identified similarities in antimicrobial proteins and proinflammatory cytokine profiles between preterm placentas and those with PIR. In conjunction with presenting potential single or combinatory biomarkers of PTB and/or PIR with the capability of providing clearer understanding of the molecular mechanisms underlying inflammation-associated PTB (*Chapters 6* and *7*), these pilot studies have uncovered novel relationships between inflammatory and non-inflammatory placental pathologies and their contribution to PTB (*Chapter 4*), notably at extremely preterm gestations. These distinct histopathological features and signalling pathways in PTB and PIR may elucidate clinical biomarkers. This study has highlighted the importance of the placental pathology report, not only to healthcare professionals, but also as a mechanism by which to provide mothers and families with explanations of the relationship between placental pathology and the adverse outcome. Understanding underlying pathologic changes in preterm placentas is vital to inform clinical obstetric practice. Moreover, it may prevent delays or missed opportunities in postpartum clinical management of the mother and neonate, support effective counselling of PTB recurrence risk and promote discussion of prevention strategies.

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APPENDICES

APPENDIX I

Figure AI.Ia Health Research Authority ethics approval (page 1)

NHS
Health Research Authority
London - Fulham Research Ethics Committee
Barlow House
3rd Floor, 4 Minshull Street
Manchester
M1 3DZ

Telephone: 0207 104 8021

12 December 2018

Professor DOC Anumba
Professor and Honorary Consultant Obstetrician and Gynaecologist
The University of Sheffield
58 Woodholm Road
Sheffield
S11 9HT

Dear Professor Anumba

Study title:	**Elucidating the mechanisms and markers of infection- and inflammation-associated preterm birth - Sheffield studies (NIHR PRIME).**
REC reference:	**18/LO/2044**
Protocol number:	**STH20635**
IRAS project ID:	**256135**

Thank you for your submission. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 21 November 2018

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Covering letter on headed paper		06 December 2018
GP/consultant information sheets or letters [GP letter]	2	30 November 2018
IRAS Checklist XML [Checklist_07122018]		07 December 2018
Participant consent form [Consent - Swab and Placental Studies]	2	30 November 2018
Participant consent form [Consent placental histopathology]	2	30 November 2018
Participant information sheet (PIS) [PIS - Swab and Placental Studies]	2	30 November 2018
Participant information sheet (PIS) [PIS - Placental and Histopathological studies]	2	30 November 2018

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Patient Advert]	1	15 October 2018
Covering letter on headed paper		06 December 2018
GP/consultant information sheets or letters [GP letter]	2	30 November 2018

Figure AI.Ib Health Research Authority ethics approval (page 2)

IRAS Application Form [IRAS_Form_01112018]		01 November 2018
IRAS Application Form XML file [IRAS_Form_01112018]		01 November 2018
IRAS Checklist XML [Checklist_07122018]		07 December 2018
Letter from funder [Funding Letter]		05 March 2018
Participant consent form [Consent - Swab and Placental Studies]	2	30 November 2018
Participant consent form [Consent placental histopathology]	2	30 November 2018
Participant information sheet (PIS) [PIS - Swab and Placental Studies]	2	30 November 2018
Participant information sheet (PIS) [PIS - Placental and Histopathological studies]	2	30 November 2018
Research protocol or project proposal [Protocol]	1	15 October 2018
Summary CV for Chief Investigator (CI) [CI CV]		02 August 2018

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

18/LO/2044 **Please quote this number on all correspondence**

Yours sincerely

Anna Bannister
REC Manager
E-mail: nrescommittee.london-fulham@nhs.net

Copy to: *Mrs Angela Pinder, Sheffield Teaching Hospitals*

Figure AI.II IRAS HRA ethics approval (page 1)

Ymchwil Iechyd a Gofal Cymru
Health and Care Research Wales

NHS
Health Research Authority

Professor DOC Anumba
Professor and Honorary Consultant Obstetrician and Gynaecologist
The University of Sheffield
58 Woodholm Road
Sheffield S11 9HT

Email: hra.approval@nhs.net
Research-permissions@wales.nhs.uk

12 December 2018

Dear Professor Anumba,

HRA and Health and Care Research Wales (HCRW) Approval Letter

Study title:	**Elucidating the mechanisms and markers of infection- and inflammation-associated preterm birth - Sheffield studies (NIHR PRIME).**
IRAS project ID:	**256135**
Protocol number:	**STH20635**
REC reference:	**18/LO/2044**
Sponsor:	**Sheffield Teaching Hospitals NHS Foundation Trust**

I am pleased to confirm that **HRA and Health and Care Research Wales (HCRW) Approval** has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications received. You should not expect to receive anything further relating to this application.

How should I continue to work with participating NHS organisations in England and Wales?
You should now provide a copy of this letter to all participating NHS organisations in England and Wales, as well as any documentation that has been updated as a result of the assessment.

This is a single site study sponsored by the site. The sponsor R&D office will confirm to you when the study can start following issue of HRA and HCRW Approval.

It is important that you involve both the research management function (e.g. R&D office) supporting each organisation and the local research team (where there is one) in setting up your study. Contact details of the research management function for each organisation can be accessed here.

How should I work with participating NHS/HSC organisations in Northern Ireland and Scotland?
HRA and HCRW Approval does not apply to NHS/HSC organisations within the devolved administrations of Northern Ireland and Scotland.

Figure AI.IIIa Patient Information Sheet (PIS) (page 1)

Sheffield Teaching Hospitals NHS
NHS Trust

Elucidating the mechanisms and markers of infection- and inflammation-associated preterm birth - Placental histopathological studies

Participant Information Sheet

Chief Investigator: Professor Dilly Anumba. Honorary Consultant in Obstetrics & Gynaecology/Subspecialist in Fetomaternal Medicine

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1. What is the purpose of the study?
Up to half of all premature births are associated with features of infection or inflammation in the reproductive tract, and sometimes in the fluid around the baby or the babies themselves. The purpose of this study is to investigate what features of infection and inflammation associated with premature birth can be found in the placenta at the time of birth. We will examine and take samples from your placenta, looking for features of infections which are not usually detected in the pregnant woman but which can silently cause preterm birth. By comparing the features of these infections and inflammation in placentas which are delivered prematurely to those of placentas which are delivered at term we will be able to understand how they contribute to infection, and how pre-pregnancy and prenatal care can be improved to reduce the chance of such infections causing premature birth or fetal infection.

2. Why have I been chosen?
You have been chosen because you are pregnant and will be delivering in hospital. We will also examine your placenta after birth to look for any features that account for why you delivered at term or preterm.

3. Do I have to take part?
No. It is up to you to decide whether to take part or not. You are free to withdraw from the study at any time and without giving a reason. Whether you join the study or not will not affect your care at the hospital in any way.

4. What will happen to me if I take part?
If you decide to take part you will be given this information sheet to keep. You will be asked to sign a consent form after we have explained the study to you in detail. We will ask your permission to obtain and examine your placenta after birth. If you deliver at term your placenta will usually be discarded following normal hospital procedure. If you deliver preterm your placenta will usually be sent for standard examination in the hospital's laboratories. Using a variety of laboratory techniques, we will conduct a number of

Figure AI.IIIb Patient Information Sheet (PIS) (page 2)

additional research tests on samples taken from your placenta looking for features that may explain why you delivered either at term or preterm before the placenta is discarded. We will compare findings from the placentas of women who deliver at term to those of women who deliver preterm to find out the differences in infection and inflammation patterns.

5. What will happen to the samples taken?
Samples will be collected from your placenta and processed and stored in the research laboratories of the University of Sheffield for studies by researchers within the research group employing a number of molecular biological techniques, including genetic analysis. We will look to identify features which may explain the timing of your delivery. We will keep any unused samples, and identifiable information about you, for up to 6 years so that we can carry out future follow-on research studies. At the end of this period all the samples will be destroyed.

6. What are the benefits of taking part?
You will not receive any direct benefits from taking part in the study. The studies of the placenta may enable better pre-pregnancy and prenatal care to prevent or treat infections that may be associated with preterm birth.

7. Will this test harm me or my baby in any way?
No. The tests are conducted on your placenta and not on yourself or your baby. They are safe and will not harm you or your baby in any way. You will receive no medication as part of the study.

8. What if I am harmed?
If you are harmed by your participation in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action.

9. What will happen with the results of this study?
We will analyse the data to see if the results can be used to improve care of women before and during pregnancy by preventing or treating infections associated with preterm birth, or by developing drugs which can reduce the inflammation of the placenta associated with preterm birth. We will also publish our results in medical journals and share our findings at relevant conferences, so that the worldwide research community on preterm birth can be informed of our findings and improve care globally. You will not be identified in any report/publication. If you would like a copy of the research report we will record this request on your consent form and send this to you.

10. Will my taking part in the study be kept confidential?
Sheffield Teaching Hospitals NHS Foundation Trust is the sponsor for this study based in United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Sheffield Teaching Hospitals NHS Foundation Trust will keep identifiable information about you for up to 6 years after the study has finished.

Researchers from the University of Sheffield will also be involved in the research and will be involved in analysing your information.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and

Figure AI.IIIc Patient Information Sheet (PIS) (page 3)

accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information at
https://www.sheffieldclinicalresearch.org/

Sh https://www.sheffieldclinicalresearch.org/ d the University of Sheffield will use your name and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from Sheffield Teaching Hospitals NHS Foundation Trust, the University of Sheffield and regulatory organisations may look at your medical and research records to check the accuracy of the research study. The only people in Sheffield Teaching Hospitals NHS Foundation Trust or the University of Sheffield who will have access to information that identifies you will be people who need to contact you to undertake the research or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, or contact details.

When the results are published, no names will be used, and it will not be possible to identify anyone who has taken part.

11. Will anyone else be told about my participation in the study?
No. However, if you wish us to, we will inform your GP that you are helping with this study.

12. What will happen if I do not want to carry on with the study?
You will receive the same quality of clinical care even if you withdraw from the study.

13. Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by the London-Fulham Research Ethics Committee (REC reference:18/LO/2044).

14. Who is organizing and funding the research?
Professor Dilly Anumba (Consultant Obstetrician and Gynaecologist) is organising the research within the Obstetrics, Gynaecology & Neonatology Directorate at Sheffield Teaching Hospitals NHS Foundation Trust. The study is funded from the Department of Health and Social Care of the UK through its National Institute for Health Research (NIHR).

15. What if I wish to complain about the way in which this study has been conducted?
If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

If you have any complaints, queries or concerns you may contact either:
Chief Investigator: Professor Dilly Anumba Consultant Obstetrician and Gynaecologist, Jessop Wing, Tree Root Walk, Sheffield S10 2SF on 0114 226 1075
OR the Patient Services Team (PST), Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF, Tel: 0114 2712400, Email: pst@sth.nhs.uk.

Figure AI.IV Consent form

Sheffield Teaching Hospitals NHS
NHS Trust

STH20635
Patient Identification Number for this study:

CONSENT FORM

Elucidating the mechanisms and markers of infection - and inflammation-associated preterm birth - placental histopathological studies.

Principal Researcher:

Professor Dilly Anumba MD, FRCOG. Consultant in Obstetrics and Fetomaternal Medicine

	Please initial box
1. I confirm that I have read and understand the information sheet Version 2 dated 30/11/2018 for the above study and have had the opportunity to ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3. I agree to take part in the above study.	☐
4. I agree to examination of samples taken from my placenta after birth as part of these studies	☐
5. I agree to genetic tests on the samples and storage of the samples (delete as appropriate)	YES/NO
6. I understand that all the information that is collected will be kept strictly confidential.	☐
7. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the University of Sheffield, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
8. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers	☐
9. I request that my General Practitioner is informed of my participation in the study	☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

APPENDIX II

Figure AII.Ia Request for placental examination SCH Document Number 322.4 (2021) (page 1)

REQUEST FOR PLACENTAL EXAMINATION

Mother's name:…… DOB:……..... NHS No:…… Hospital No:……..... Address:…… *Alternatively, attach patient label*	SCH Lab No: Obstetrician:……..... Hospital:……. Ward/Unit:…….. Name of person completing the form and contact number:……

HAS MOTHER HAD COVID DURING PREGNANCY: Y☐ N☐ IF YES, DATE OF POSITIVE TEST:
SYMPTOMATIC: YES ☐ NO☐
BODY MASS INDEX: MATERNAL ETHNICITY:

CLINICAL INFORMATION:

GESTATIONAL AGE: ……..weeks. **Birth weight**:……g.

Date of delivery: …….. **Mode of delivery**: ☐ SVD ☐ Instrumental ☐ CS.

Outcome: ☐ Livebirth ☐ TOP. Indication:……..

☐ Spontaneous miscarriage (pregnancy loss <24w)

☐ Missed miscarriage (pregnancy loss <24 weeks, clinically silent)

☐ Stillbirth (pregnancy loss >24w)

If a pregnancy loss has occurred has a PM been requested: ☐ Yes ☐ No ☐ Undecided

Sex: ☐ Male ☐ Female ☐ Indeterminate ☐ Not assessed.

INDICATION FOR PLACENTAL EXAMINATION (please tick and give details when necessary)**:**

☐ Multiple pregnancy. Indicate chorionicity / identification of umbilical cords / and indicate if specific pathology is suspected:
……

☐ Pregnancy loss. Specify:……

☐ Fetal abnormalities. Specify:……

☐ Fetal distress. Specify: Apgar score:…..at 1st min; …..at 5th min. Admission to NNU: □Yes □No

☐ Intrauterine growth restriction (IUGR).

☐ Maternal pyrexia ☐ Clinical chorioamnionitis ☐ PROM ☐ Prematurity

☐ Oligohydramnios ☐ Polyhydramnios ☐ Meconium stained liquor

☐ Antepartum haemorrhage (APH) ☐ Abruption ☐ Morbidly adherent placenta

☐ Pre-eclampsia ☐ Gestational Diabetes ☐ Maternal Hypertension ☐ Diabetes Mellitus

☐ Any other indications (please, give details):…….

Please tick this box if the patient/parents/guardian has objected to non-research use of any left over samples ☐

Figure AII.Ib Request for placental examination SCH Document Number 322.4 (2022) (page 1)

REQUEST FOR PLACENTAL EXAMINATION

Mother's name:…… DOB:…… NHS No:…… Hospital No:…… Address:…… *Alternatively, attach patient label*	SCH Lab No: Obstetrician:…… Hospital:…… Ward/Unit:…… Name of person completing the form and contact number:……

BODY MASS INDEX:	MATERNAL ETHNICITY:

CLINICAL INFORMATION:

GESTATIONAL AGE: ……..weeks. **Birth weight**:……g.

Date of delivery: …….. **Mode of delivery**: ☐ SVD ☐ Instrumental ☐ CS.

Outcome: ☐ Livebirth
☐ TOP. Indication:……..
☐ Miscarriage (pregnancy loss 14+0 - 23+6 weeks)
☐ Stillbirth (pregnancy loss >24w)

If a pregnancy loss has occurred has a PM been requested: ☐ Yes ☐ No ☐ Undecided

Sex: ☐ Male ☐ Female ☐ Indeterminate ☐ Not assessed.

INDICATION FOR PLACENTAL EXAMINATION (please tick and give details when necessary)**:**

☐ Pre-term birth (less than 32+0 weeks gestation)

☐ Monochorionic twins with TTTS

☐ Severe fetal distress. Classed as pH<7.05 / BE ≥–12/ scalp lactate >4.8 mmol.

☐ Admission to Level 3 NICU / NNU: ☐Yes ☐No

☐ Severe sepsis with maternal ITU admission and/or fetal sepsis requiring ventilation

☐ Intrauterine growth restriction (IUGR). Classed as <3rd centile or drop in growth velocity >50 percentiles.

☐ Abnormal umbilical artery Dopplers (absent or reversed end diastolic flow)

☐ Fetal hydrops

☐ Early onset (<32 weeks) severe pre-eclampsia requiring iatrogenic delivery

☐ Caesarean peripartum hysterectomy for morbidly adherent placenta

☐ Massive placental abruption with retroplacental clot

☐ Any other indications (please, give details):……

Please tick this box if the patient/parents/guardian has objected to non-research use of any leftover samples ☐

For general enquiries and results:
Telephone: 0114 27 17247/17254
Histopathology Department
Sheffield Children's Hospital, Western Bank

Figure AII.IIa Singleton placenta dissection reporting form (SCH Document Number 326.34) (page 1)

Sheffield Children's Hospital NHS Foundation Trust/PRIME

Singleton Placenta Dissection Reporting Form

Patient name: _____ **Hospital number:** _____ **Unique identifier:**

Date cut up: _____ **Pathologist:** _____ **BMS:** _____

General
The placenta is received ☐ Fresh ☐ Fixed

Placenta weight: _____g (___percentile-normal range 10th-90th) and measures _____ x _____ x _____ cm

Fetoplacental weight ratio: _____ (___percentile-normal range 10th-90th)

Shape of placenta:
- ☐ Discoid
- ☐ Irregular
- ☐ Bilobate
- ☐ Succenturiate

Peripheral membranes:
- ☐ Complete
- ☐ Incomplete
- ☐ Translucent
- ☐ Green
- ☐ Opaque
- ☐ Insertion is marginal
- ☐ Insertion is circumvallate _____% circummarginate _____%

Umbilical cord measures: _____ x _____ cm

There are _____ coils to the _____ (UCI ____coils/cm – normal range 0.07-0.30 coils/cm. Indentations are _____

- ☐ Central insertion
- ☐ Eccentric insertion
- ☐ Marginal insertion
- ☐ Velamentous insertion
- ☐ Number of vessels _____
- ☐ There is a _____ knot

Fetal Surface
- ☐ Translucent
- ☐ Opaque
- ☐ Green
- ☐ Normal chorionic vessels

Maternal Surface
- ☐ Intact
- ☐ Ragged
- ☐ Prominent calcification
- ☐ Normal colour
- ☐ Pale
- ☐ Attached blood clot

Cut surface shows
- ☐ No focal lesions
- ☐ _____ firm, white lesions. The largest measures _____ x _____ cm
- ☐ _____ haemorrhagic lesions. The largest measures _____ x _____ cm
- ☐ Pale areas
- ☐ The lesions comprise altogether _____ % of volume

Block												
Pieces												

SCH Doc. No. 326.4.34 Version No.: 22 Author: C Peres Authorised by: J Ager
Amended for PRIME research project by: K Parris Date 01/05/2020

Figure AII.IIa Singleton placenta dissection reporting form (SCH Document Number 326.34) (page 2)

Field	Definition
C2 C3 D5 D6	Maternal vascular malperfusion of the placental bed (MVM) includes both gross and microscopic findings. Gross findings include placental hypoplasia, infarction and retroplacental haemorrhage.
H5	Placental hypoplasia: weight below 10th centile and/or thin cord (blow 10th centile or 8mm diameter at term). Any infarction seen in a preterm placenta, and, at term, anything more than 5% of nonperipheral infarction must be described. Microscopy: distal villous hypoplasia and/or accelerated villous maturation.
D4 G11	Infarcts – if possible, define as recent or remote. Haemorrhage encased by infarction: infarction haematoma.
D5 H5	Retroplacental haemorrhage or haematoma, blood accumulation beneath and dissecting the decidua and compression of the overlying intervillous space with villous crowding, congestion and/or intravillous haemorrhage with touching villi. There is also a smudged appearance as evidence of early coagulation necrosis of the syncytiotrophoblast nuclei and pale appearance of syncytiotrophoblast nuclei.
C2 D5	Distal villous hypoplasia (DVH): paucity of villi in relation to surrounding stem villi. Thin, relatively elongated appearance with increased syncytial knots seen in the lower two-thirds and involving at least 30% of one full-thickness parenchymal slide. Grading: focal – lesion in one full-thickness slide only, diffuse – present in two or more full-thickness slides sampled. Diffuse distal villous hyperplasia is associated with early-onset FGR.
C3 D6	Accelerated villous maturation (AVM): small or short hypermature villi for gestational age, usually accompanied by an increase in syncytial knots. Diffuse pattern of term-appearing villi with increased syncytial knots and intervillous fibrin, usually alternating with areas of villous paucity. Syncytial knots at term is up to 33% of villi. AVM: mild, moderate or severe forms.
A6 H3	Decidual arteriopathy: in the membrane roll and/or basal plate. Findings: acute atherosis, fibrinoid necrosis with or without foam cells, mural hypertrophy, chronic perivasculitis, absence of spiral artery remodelling, arterial thrombosis and persistence of intramural endovascular trophoblast in the third trimester.
D9	Fetal vascular malperfusion (FVM): likely due to obstruction in fetal blood flow from umbilical cord lesions, hypercoagulability, complications of fetal cardiac dysfunction, such as hypoxia, among others. Findings: thrombosis, segmental avascular villi and villous stromavascular karyorrhexis. Other possible markers: vascular intramural fibrin deposition, stem vessel obliteration/fibromuscular sclerosis and vascular ectasia. Thrombosis is considered a premortem process. Discreet population of avascular villi may be possible in cases of intrauterine death, where they contrast with the more cellular pattern of villous involution. FVM patters: segmental or global. Segmental: thrombotic occlusion or obliteration of chorionic or stem villous vessels. Global: partially obstructed umbilical blood flow with venous ectasia, intramural fibrin deposition in large vessels and/or small foci (<5 villi per focus) of avascular or karyorrhectic villi (partial or intermittent obstruction). Grading: high grade (more than one focus of avascular villi with or without thrombus – cumulative assessment of ≥45 avascular villi over three sections examined or an average of ≥15 villi per section), or two or more occlusive or nonocclusive thrombi in the chorionic plate or major stem villi, or multiple nonocclusive thrombi. Specify whether the thrombosis is arterial or not and its location: at the umbilical, chorionic plate, stem vascular level or any combination thereof.
D9	Avascular villi foci – small: 3 or more foci of 2 to 4 terminal villi; intermediate: 5 to 10 villi; large: more than 10 villi.
E4	Intramural fibrin deposition: state whether isolated or not, recent (fibrin) or remote (calcification).
D9	Villous stromal-vascular karyorrhexis: 3 or more foci of 2 to 4 terminal villi showing karyorrhexisof fetal cells (nucleated erythrocytes, leukocytes, endothelial cells and/or stromal cells) with preservation of surrounding trophoblast.
D9	Stem vessel obliteration: marked thinning of the vessel wall and resultant obliteration of the vascular lumen.
D9	Vascular ectasia: vessels that are four times the luminal diameter of the surrounding corresponding vessel.
C4 D8	Delayed villous maturation (DVM): seen after 36 weeks and rarely before 34 weeks of gestation. Features: monotonous villous population (≥10 villi) with reduced numbers of vasculosyncytial membranes for the period of gestation, as well as a continuous cytotrophoblastic layer and centrally placed capillaries in ≥30% of one full thickness parenchymal slide. Grading: focal (in single) and diffuse (≥2 slides).
D10	Villitis of unknown aetiology: lymphohistiocytic with or without plasma cells. Grading: low (≤10 contiguous villi in any one focus), high (multiple foci on more than one section, at least one of which shows inflammation affecting more than 10 contiguous villi). Low grade: focal/multifocal. High grade: patchy/diffuse (≥30% of all distal villi are involved). "Ungradable: possible low grade/high grade".
D10	Vascular damage. Inflammatory cell damage of the muscular wall: villitis with stem vessel obliteration. Avascular villi seen with villitis: chronic villitis with associated avascular villi.
D10	Villitis may cause impairment of the fetoplacental circulation. Such damage is associated with adverse effects such as neurologic impairment. Although occasional avascular villi with scattered inflammatory cells may indicate "burnt-out villitis", and large areas of contiguous, uniformly hyalinised avascular villi may suggest upstream vascular occlusion, it can be difficult to ascribe the avascular villi to either the inflammatory or the obstructive process. Location: parabasal/paraseptal, randomly in the midparenchyma or subchorionic zone, or a combination thereof.
H2	Other inflammatory lesions: eosinophilic/T-cell vasculitis, chronic intervillositis and chronic deciduitis should be noted.

Staging and Grading of the Maternal and Fetal Inflammatory Responses in Ascending Intrauterine Infection

		Maternal Inflammatory Response	
A2 A3 A4	G4	Stage 1—acute subchorionitis or chorionitis Stage 2—acute chorioamnionitis: polymorphonuclear leukocytes extend into fibrous chorion and/or amnion Stage 3—necrotizing chorioamnionitis: karyorrhexis of polymorphonuclear leukocytes, amniocyte necrosis, and/or amnion basement membrane hypereosinophilia	Grade 1—not severe as defined Grade 2—severe: confluent polymorphonuclear leukocytes or with subchorionic microabscesses
		Fetal Inflammatory Response	
B3 B4 B5	E3	Stage 1—chorionic vasculitis or umbilical phlebitis Stage 2—involvement of the umbilical vein and one or more umbilical arteries Stage 3—necrotizing funisitis	Grade 1—not severe as defined Grade 2—severe: near-confluent intramural polymorphonuclear leukocytes with attenuation of vascular smooth muscle

SCH Doc. No. 326.4.34 Version No.: 22 Author: C Peres Authorised by: J Ager
Amended for PRIME research project by: K Parris Date 01/05/2020

Figure AII.IIc Singleton placenta dissection reporting form SCH Document Number 326.34 (page 3)

Sheffield Children's Hospital NHS Foundation Trust/PRIME

Unique Identifier:

Singleton Placenta Dissection Reporting Form

Delete words in **bold**

Field	A - Peripheral Membranes
A1	Normal
a2	Acute chorionitis (early maternal inflammatory response)
A3	Acute chorioamnionitis (**at the border of rupture/diffuse**) (intermediate maternal inflammatory response)
A4	Acute necrotising chorioamnionitis (advanced maternal inflammatory response)
A5	Chronic deciduitis
A6	Decidual arteriopathy
A7	Reactive amniotic epithelium (meconium-induced)
A8	Haemosiderin-laden macrophages
A9	Laminar necrosis/chorionic cysts
A10	Amnion nodosum
A11	
	C- Chorionic Villous Maturation
C1	Consistent with gestational age
C2	Distal villous hyperplasia (distal villous maldevelopment)
C3	Accelerated maturation (distal villous maldevelopment)
C4	**Focal/Diffuse** delayed villous maldevelopment
C5	Not assessable
C6	
	E- Chorionic/Stem Villous Vessels
E1	Normal
E2	Acute vasculitis (fetal inflammatory response)
E3	**(Mural/Occlusive**) (**recent/remote**) thrombosis
E4	Intramural fibrin deposition
E5	Eosinophilic/T-cell vasculitis
E6	
	G - Intervillous Space
G1	Normal
G2	Small, focal (**recent/remote**) subchorionic thrombosis
G3	**Extensive/Massive, recent/remote** subchorionic thrombosis
G4	Acute subchorionitis (maternal inflammatory response)
G5	Acute intervillositis
G6	Chronic histiocytic intervillositis
G7	Abnormal maternal red blood cells
G8	**Small/Focal** perivillous fibrin deposition
G9	**Extensive/Massive** perivillous fibrin deposition
G10	**Focal/Multifocal**, **recent/remote** intervillous heamatoma
G11	**Focal/Multifocal**, **recent/remote** infarction heamatoma
G12	

Field	B - Umbilical Cord
B1	Normal
B2	Single umbilical artery
B3	Isolated cord phlebitis (early fetal inflammatory response)
B4	Phlebitis and arteritis (intermediate fetal inflammatory response)
B5	Funisitis with concentric inflammation of Wharton's jelly (advanced fetal inflammatory response)
B6	Meconium-induced myonecrosis of the vessel walls
B7	
	D – Chorionic Villi
D1	Normal
D2	**Focal/Diffuse, mild/moderate/severe** stromal oedema
D3	**Focal/Diffuse** chorangiotic changes
D4	**Focal/Multifocal, early/remote** villous infarct
D5	Thin, elongated villi with increased syncytial knots
D6	Small, hypermature villi for age with increased syncytial knots
D7	**(small/large), (isolated/multiple) (infarcted)** chorangioma
D8	**Focal/Diffuse** monotonous villi with centrally placed capillaries and decreased vasculosyncytial membranes
D9	**High/Low** grade, **segmental/global** fetal vascular malperfusion
D10	**High/Low** grade chronic villitis of unknown aetiology
D11	Increased nucleated fetal red blood cells (fetal hypoxic stress)
D12	Abnormal villi suggestive of chromosomal abnormality
D13	Regressive changes secondary to fetal demise
D14	**CMV/Herpes/Toxoplasma** villitis
D15	
	F – Chorionic Plate
F1	Normal
F2	Acute chorioamnionitis (maternal inflammatory response)
F3	Meconium-induced changes
F4	Haemosiderin-laden macrophages
F5	
	H – Basal Plate
H1	Within normal limits
H2	**Focal/Diffuse, mild/moderate/severe** chronic deciduitis
H3	Decidual arteriopathy
H4	Increased numbers of giant trophoblast cells (fetal hypoxic stress)
H5	**Fresh/Remote, marginal/retroplacental** haematoma
H6	

Conclusion:

Comment:

Date Reported: **Reporter:**

SCH Doc. No. 326.4.34 Version No.: 22 Author: C Peres Authorised by: J Ager
Amended for PRIME research project by: K Parris Date 01/05/2020

APPENDIX III

Figure AIII.I Percentiles, means and standard deviations for placental weights by gestational age

Gestational Age (Weeks)	n		Mean	SD	Percentile						
			3	5	10	25	50	75	90	95	97
21	3	143			114	128	143	158	172		
22	19	189	89	99	107	130	166	206	285	499	
23	16	190	41		127	168	188	208	262		
24	16	190	42		128	157	192	222	252		
25	26	197	70	105	128	153	184	216	299	400	
26	22	226	100	107	138	179	200	259	281	570	
27	22	240	77	119	130	166	242	310	332	381	
28	41	223	66	103	128	140	173	214	261	321	361	371
29	37	269	96	124	135	161	214	252	309	352	496	629
30	42	324	88	185	190	208	269	316	374	433	502	570
31	57	314	105	142	152	175	246	313	360	417	479	579
32	69	325	77	161	214	241	275	318	377	436	461	465
33	117	351	83	190	224	252	286	352	413	446	475	504
34	160	381	84	221	260	283	322	382	430	479	527	558
35	260	411	99	232	250	291	344	401	471	544	600	626
36	538	447	110	270	291	320	369	440	508	580	628	679
37	1103	467	107	303	324	349	390	452	531	607	660	692
38	2469	493	103	320	325	365	420	484	560	629	675	706
39	3932	500	103	330	350	379	426	490	564	635	683	713
40	4114	510	100	340	360	390	440	501	572	643	685	715
41	1982	524	100	358	379	403	452	515	583	655	705	738
42	321	532	99	370	388	412	460	525	592	658	700	771

Figure AIII.II Fetal-placental weight ratio percentiles by gestational age

Gestational Age (Weeks)	n	Mean	SD	Percentile						
				3	5	10	25	50	75	90	95	97
21		2.54						2.54				
22	19	2.9	0.8		1.0	1.0	2.0	2.4	3.6	3.9	4.3	
23	16	3.3	0.7				2.4	2.9	3.6	4.5		
24	16	3.4	1				2.0	2.6	4.0	4.6		
25	26	4.0	1.4		1.7	2.3	3.2	3.8	4.6	6.0	7.4	
26	22	4.1	1.2		2.1	2.8	3.4	3.7	4.8	5.2	7.7	
27	22	4.5	1.1		2.6	3	3.3	3.6	4.5	6.0	7.1	
28	41	4.8	1	2.3	3.6	3.9	4.2	4.7	6.5	6.6	6.9	
29	37	5.2	1.4	1.9	2.5	3.7	4.4	5.0	5.7	7.5	8.0	9.2
30	42	5.2	1.1	2.7	3.1	3.6	4.5	5.1	5.8	6.8	6.9	7.6
31	57	5.5	1.1	3.3	4.1	4.4	4.7	5.4	6.2	6.9	7.3	8.2
32	69	5.9	1.2	3.2	4.1	4.4	5.0	5.8	6.8	7.7	7.9	8.4
33	117	6	1.1	4.3	4.5	4.7	5.2	6.0	6.6	7.7	8.2	8.7
34	160	6.2	1.0	4.4	4.7	5.0	5.5	6.1	6.7	7.5	7.9	8.2
35	260	6.4	1.2	4.5	4.7	5.0	5.6	6.3	7.2	8.0	8.6	9.1
36	538	6.6	1.1	4.8	4.9	5.3	5.8	6.4	7.3	8.1	8.4	8.8
37	1103	6.8	1.1	4.9	5.1	5.4	6.0	6.7	7.4	8.2	8.8	9.1
38	2469	6.9	1.1	5.1	5.2	5.6	6.1	6.8	7.5	8.3	8.9	9.2
39	3932	7.1	1.1	5.2	5.4	5.7	6.3	7.0	7.7	8.5	9.1	9.4
40	4114	7.2	1.1	5.3	5.5	5.8	6.4	7.1	7.9	8.6	9.1	9.5
41	1982	7.2	1.1	5.4	5.6	5.9	6.5	7.1	7.8	8.6	9.1	9.4
42	321	7.1	1.1	5.3	5.5	5.9	6.4	7.1	7.8	8.5	8.9	9.1

Figure AIII.III Fetal-placental weight ratio percentiles by gestational age

Kruskal-Wallis test DoL				
P value	0.1705			
Kruskal-Wallis statistic	5.017			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
Extremely Preterm vs. Very Preterm	29.75	No	ns	0.2298
Extremely Preterm vs. Moderately Preterm	15.57	No	ns	0.7185
Extremely Preterm vs. Term	11.36	No	ns	>0.9999
Very Preterm vs. Moderately Preterm	-14.18	No	ns	>0.9999
Very Preterm vs. Term	-18.39	No	ns	0.6468
Moderately Preterm vs. Term	-4.214	No	ns	>0.9999

Figure AIII.IV Fetal-placental weight ratio percentiles by gestational age

Kruskal-Wallis test DROM				
P value	0.0054			
Kruskal-Wallis statistic	12.68			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
Extremely Preterm vs. Very Preterm	-12.17	No	ns	>0.9999
Extremely Preterm vs. Moderately Preterm	0.8889	No	ns	>0.9999
Extremely Preterm vs. Term	11.74	No	ns	0.6437
Very Preterm vs. Moderately Preterm	13.06	No	ns	0.9795
Very Preterm vs. Term	23.91	Yes	*	0.0388
Moderately Preterm vs. Term	10.85	No	ns	0.1115

Table AIII.V Birthweight centile conversions for all participants' neonates

GA (Weeks)	Birthweight (g)	Sex	Birthweight Centile	GA (Weeks)	Birthweight (g)	Sex	Birthweight Centile
23.9	642	Female	55	37.4	2710	Female	20
24.0	654	Male	36	37.4	3230	Female	73
24.1	655	NR	37	37.4	3570	Male	>90
24.6	737	Female	64	37.4	NR	NR	
25.1	509	Female	<2.5	37.6	2710	Male	13
25.8	455	Female	<2.5	37.8	2290	Female	<2.5
26.4	978	Male	49	37.9	2690	Female	12
27.3	1082	Male	43	37.9	3820	Male	>90
27.3	770	Female	<2.5	38.1	3150	Male	41
27.6	1148	NR	9	38.1	3610	Male	83
27.9	1162	Female	58	38.3	3170	Female	47
28.6	1240	Male	32	38.6	2550	Male	<2.5
29.0	1220	Male	15	38.6	3900	Male	>90
29.4	963	Female	<2.5	38.8	3750	Male	84
29.5	1390	Male	29	38.9	3490	Male	59
29.6	1170	Male	<2.5	38.9	NR	NR	
29.8	1420	Female	33	39.0	NR	NR	
31.4	1730	Male	33	39.1	3680	Male	73
31.5	1965	Female	73	39.4	4380	Male	97
32.8	1731	Female	11	39.6	3450	Male	40
33.0	1455	Female	<2.5	39.8	3330	Female	34
33.1	1765	Male	6	39.9	3210	Female	21
33.1	1721	Female	7	39.9	2680	Male	<2.5
33.4	2340	Male	66	39.9	NR	NR	
33.7	1810	Male	4	40.0	3290	Female	25
33.7	2418	Male	68	40.0	3580	Male	44
33.8	2200	Female	48	40.1	2880	Male	5
34.0	2308	Male	45	40.1	3000	Male	7
34.0	2540	Female	83	40.3	3090	Male	10
34.4	2461	Male	53	40.3	NR	NR	
34.4	2680	Female	83	40.4	3560	Female	51
34.4	1780	Male	<2.5	40.4	3960	Male	80
34.8	1960	Male	3	40.4	4100	Male	89
35.3	2380	Male	22	40.6	NR	NR	
35.4	2326	Female	20	40.8	3360	Female	32
35.4	2600	Male	44	40.8	3690	Female	66
35.4	1900	Female	<2.5	40.8	NR	NR	
35.4	1966	Male	<2.5	40.9	3410	Female	37
36.1	2270	Female	8	40.9	3540	Female	49
36.1	2920	Female	71	41.0	3600	Female	56
36.1	3010	Male	72	41.1	3030	Male	8
36.1	2330	Female	<10	41.1	3420	Female	38
36.3	2810	Male	47	41.1	3640	Male	50
36.3	2980	Female	74	41.1	NR	NR	
36.4	2260	Female	6	41.3	NR	NR	
36.4	2910	Male	54	41.6	3710	Female	69
36.4	3030	Male	67	41.8	NR	NR	
37.3	2870	Male	30	42.0	3690	Male	55
37.3	3080	Male	52	42.3	4020	Male	84

NR; not recorded

Figure AIII.VI Birthweight percentiles by gestational age

Kruskal-Wallis test Birthweight Centiles				
P value	0.1367			
Kruskal-Wallis statistic	5.533			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
Extremely Preterm vs. Very Preterm	5.063	No	ns	>0.9999
Extremely Preterm vs. Moderately Preterm	-2.911	No	ns	>0.9999
Extremely Preterm vs. Term	-12.95	No	ns	0.8094
Very Preterm vs. Moderately Preterm	-7.973	No	ns	>0.9999
Very Preterm vs. Term	-18.01	No	ns	0.4064
Moderately Preterm vs. Term	-10.04	No	ns	0.6506

Figure AIII.VII Differences in umbilical cord diameter assessed by gestational age

Kruskal-Wallis Test umbilical cord diameter				
P value	0.0006			
Dunn's multiple comparisons test	**Mean diff.**	**Significant?**	**Summary**	**Adjusted P Value**
Extremely Preterm vs. Very Preterm	-6.267	No	ns	>0.9999
Very Preterm vs. Moderately Preterm	-11.63	No	ns	>0.9999
Moderately Preterm vs. Term	-14.84	No	ns	0.1413

APPENDIX IV

Supplementary Data

Figure AIV.I Reported MIR and FIR stages and grades for total cohort (*n*=98) by gestational age (weeks)

GA (Weeks)	MIR Stage	MIR Grade	FIR Stage	FIR Grade	GA (Weeks)	MIR Stage	MIR Grade	FIR Stage	FIR Grade
23.9	3	2	3	2	**37.4**	1	1	0	0
24.0	0	0	0	0	**37.4**	0	0	0	0
24.1	3	2	3	2	**37.4**	0	0	0	0
24.6	3	2	3	2	**37.4**	0	0	0	0
25.1	2	1	3	1	**37.6**	1	1	0	0
25.8	1	1	0	0	**37.8**	0	0	0	0
26.4	3	1	3	2	**37.9**	0	0	0	0
27.3	0	0	0	0	**37.9**	1	1	0	0
27.3	1	1	0	0	**38.1**	0	0	0	0
27.6	2	1	2	2	**38.1**	0	0	0	0
27.9	2	2	3	2	**38.3**	0	0	0	0

28.6	0	0	0	0	**38.6**	3	2	0	0
29.0	2	2	3	2	**38.6**	1	1	0	0
29.4	0	0	0	0	**38.8**	0	0	0	0
29.5	2	1	2	1	**38.9**	1	1	0	0
29.6	1	1	0	0	**38.9**	0	0	0	0
29.8	2	1	2	1	**39.0**	0	0	0	0
31.4	2	2	3	1	**39.1**	2	1	1	2
31.5	1	1	1	1	**39.4**	2	2	2	1
32.8	0	0	0	0	**39.6**	1	1	0	0
33.0	0	0	0	0	**39.8**	1	1	0	0
33.1	3	2	3	2	**39.9**	1	1	0	0
33.1	0	0	0	0	**39.9**	0	0	0	0
33.4	3	2	3	2	**39.9**	1	1	0	0
33.7	0	0	0	0	**40.0**	0	0	0	0
33.7	2	1	2	1	**40.0**	1	1	1	1
33.8	0	0	0	0	**40.1**	0	0	0	0
34.0	1	1	0	0	**40.1**	0	0	0	0
34.0	0	0	0	0	**40.3**	2	1	2	1
34.4	0	0	0	0	**40.3**	1	1	0	0
34.4	0	0	0	0	**40.4**	3	1	3	2
34.4	0	0	0	0	**40.4**	0	0	0	0
34.8	0	0	0	0	**40.4**	3	2	0	0
35.3	0	0	0	0	**40.6**	0	0	0	0
35.4	2	1	1	1	**40.8**	0	0	0	0
35.4	2	1	2	1	**40.8**	1	1	0	0
35.4	0	0	0	0	**40.8**	1	1	0	0
35.4	0	0	0	0	**40.9**	3	2	2	2
36.1	2	1	1	1	**40.9**	1	1	1	1
36.1	0	0	0	0	**41.0**	0	0	0	0
36.1	0	0	0	0	**41.1**	0	0	0	0
36.1	0	0	0	0	**41.1**	2	2	1	1
36.3	2	2	2	2	**41.1**	3	2	1	1
36.3	0	0	0	0	**41.1**	0	0	0	0
36.4	0	0	0	0	**41.3**	1	1	0	0
36.4	0	0	0	0	**41.6**	0	0	0	0
36.4	0	0	0	0	**41.8**	1	1	0	0
37.3	0	0	0	0	**42.0**	2	1	3	2
37.3	0	0	0	0	**42.3**	2	2	2	2

FIR; fetal inflammatory response, *GA;* gestational age, *MIR*; maternal inflammatory response.

Figure AIV.II Chi square test for trends -from chi confounding data sheet: all cases

Comparison	***P* Value**	**χ^2 Value**	**df**
MIR Present/Absent	0.2149 ns	1.538	1
MIR Stage 0/Stage 1	0.1957 ns	1.674	1
MIR Stage 1/Stage 2	0.0105	6.548	1
MIR Stage 2/Stage 3	0.9603 ns	0.0025	1
MIR Grade 0/Grade 1	0.5440 ns	0.3681	1
MIR Grade 1/Grade 2	0.2416 ns	1.371	1
FIR Present/Absent	0.0016	9.950	1
FIR Stage 0/Stage 1	0.9948 ns	4.173	1
FIR Stage 1/Stage 2	0.4459 ns	0.5810	1
FIR Stage 2/Stage 3	0.0309 ns	4.656	1

FIR Grade 0/Grade 1	0.0519 ns	3.780	1
FIR Grade 1/Grade 2	0.3971 ns	0.7179	1

df; degrees of freedom, *FIR*; fetal inflammatory response, *MIR*; maternal inflammatory response

Figure AIV.III Simple linear regression lines data for predictive values

GA (Weeks)	MIR Stage			MIR Grade			FIR Stage			FIR Grade		
	Mean	+ Error	- Error	Mean	+ Error	- Error	Mean	+ Error	- Error	Mean	+ Error	- Error
23.9	1.44	0.56	0.56	0.92	0.40	0.40	1.68	0.54	0.54	0.99	0.38	0.38
24.1	1.43	0.55	0.55	0.91	0.39	0.39	1.67	0.53	0.53	0.99	0.38	0.38
24.3	1.43	0.54	0.54	0.91	0.39	0.39	1.65	0.52	0.52	0.98	0.37	0.37
24.5	1.42	0.54	0.54	0.91	0.38	0.38	1.63	0.52	0.52	0.97	0.37	0.37
24.6	1.41	0.53	0.53	0.90	0.38	0.38	1.62	0.51	0.51	0.96	0.36	0.36
24.8	1.40	0.52	0.52	0.90	0.37	0.37	1.60	0.50	0.50	0.95	0.36	0.36
25.0	1.39	0.52	0.52	0.89	0.36	0.36	1.59	0.49	0.49	0.94	0.35	0.35
25.2	1.39	0.51	0.51	0.89	0.36	0.36	1.57	0.49	0.49	0.94	0.35	0.35
25.4	1.38	0.50	0.50	0.89	0.35	0.35	1.56	0.48	0.48	0.93	0.34	0.34
25.6	1.37	0.49	0.49	0.88	0.35	0.35	1.54	0.47	0.47	0.92	0.34	0.34
25.7	1.36	0.49	0.49	0.88	0.34	0.34	1.52	0.47	0.47	0.91	0.33	0.33
25.9	1.35	0.48	0.48	0.87	0.34	0.34	1.51	0.46	0.46	0.90	0.33	0.33
26.1	1.34	0.47	0.47	0.87	0.33	0.33	1.49	0.45	0.45	0.90	0.32	0.32
26.3	1.34	0.47	0.47	0.87	0.33	0.33	1.48	0.45	0.45	0.89	0.32	0.32
26.5	1.33	0.46	0.46	0.86	0.32	0.32	1.46	0.44	0.44	0.88	0.31	0.31
26.7	1.32	0.45	0.45	0.86	0.32	0.32	1.45	0.43	0.43	0.87	0.31	0.31
26.8	1.31	0.44	0.44	0.85	0.31	0.31	1.43	0.43	0.43	0.86	0.30	0.30
27.0	1.30	0.44	0.44	0.85	0.31	0.31	1.41	0.42	0.42	0.85	0.30	0.30
27.2	1.30	0.43	0.43	0.85	0.30	0.30	1.40	0.41	0.41	0.85	0.30	0.30
27.4	1.29	0.42	0.42	0.84	0.30	0.30	1.38	0.41	0.41	0.84	0.29	0.29
27.6	1.28	0.42	0.42	0.84	0.29	0.29	1.37	0.40	0.40	0.83	0.29	0.29
27.8	1.27	0.41	0.41	0.84	0.29	0.29	1.35	0.39	0.39	0.82	0.28	0.28
27.9	1.26	0.40	0.40	0.83	0.29	0.29	1.34	0.39	0.39	0.81	0.28	0.28
28.1	1.25	0.40	0.40	0.83	0.28	0.28	1.32	0.38	0.38	0.81	0.27	0.27
28.3	1.25	0.39	0.39	0.82	0.28	0.28	1.30	0.37	0.37	0.80	0.27	0.27
28.5	1.24	0.38	0.38	0.82	0.27	0.27	1.29	0.37	0.37	0.79	0.26	0.26
28.7	1.23	0.38	0.38	0.82	0.27	0.27	1.27	0.36	0.36	0.78	0.26	0.26
28.9	1.22	0.37	0.37	0.81	0.26	0.26	1.26	0.35	0.35	0.77	0.25	0.25
29.1	1.21	0.36	0.36	0.81	0.26	0.26	1.24	0.35	0.35	0.76	0.25	0.25
29.2	1.21	0.36	0.36	0.80	0.25	0.25	1.22	0.34	0.34	0.76	0.24	0.24
29.4	1.20	0.35	0.35	0.80	0.25	0.25	1.21	0.34	0.34	0.75	0.24	0.24
29.6	1.19	0.34	0.34	0.80	0.24	0.24	1.19	0.33	0.33	0.74	0.24	0.24
29.8	1.18	0.34	0.34	0.79	0.24	0.24	1.18	0.32	0.32	0.73	0.23	0.23
30.0	1.17	0.33	0.33	0.79	0.23	0.23	1.16	0.32	0.32	0.72	0.23	0.23
30.2	1.16	0.33	0.33	0.78	0.23	0.23	1.15	0.31	0.31	0.72	0.22	0.22
30.3	1.16	0.32	0.32	0.78	0.23	0.23	1.13	0.31	0.31	0.71	0.22	0.22
30.5	1.15	0.31	0.31	0.78	0.22	0.22	1.11	0.30	0.30	0.70	0.22	0.22
30.7	1.14	0.31	0.31	0.77	0.22	0.22	1.10	0.30	0.30	0.69	0.21	0.21
30.9	1.13	0.30	0.30	0.77	0.21	0.21	1.08	0.29	0.29	0.68	0.21	0.21
31.1	1.12	0.30	0.30	0.77	0.21	0.21	1.07	0.28	0.28	0.67	0.20	0.20
31.3	1.12	0.29	0.29	0.76	0.21	0.21	1.05	0.28	0.28	0.67	0.20	0.20
31.4	1.11	0.29	0.29	0.76	0.20	0.20	1.04	0.27	0.27	0.66	0.20	0.20
31.6	1.10	0.28	0.28	0.75	0.20	0.20	1.02	0.27	0.27	0.65	0.19	0.19
31.8	1.09	0.27	0.27	0.75	0.19	0.19	1.00	0.26	0.26	0.64	0.19	0.19
32.0	1.08	0.27	0.27	0.75	0.19	0.19	0.99	0.26	0.26	0.63	0.19	0.19
32.2	1.07	0.26	0.26	0.74	0.19	0.19	0.97	0.25	0.25	0.63	0.18	0.18
32.4	1.07	0.26	0.26	0.74	0.18	0.18	0.96	0.25	0.25	0.62	0.18	0.18
32.5	1.06	0.26	0.26	0.73	0.18	0.18	0.94	0.25	0.25	0.61	0.18	0.18
32.7	1.05	0.25	0.25	0.73	0.18	0.18	0.93	0.24	0.24	0.60	0.17	0.17
32.9	1.04	0.25	0.25	0.73	0.17	0.17	0.91	0.24	0.24	0.59	0.17	0.17
33.1	1.03	0.24	0.24	0.72	0.17	0.17	0.89	0.23	0.23	0.58	0.17	0.17
33.3	1.03	0.24	0.24	0.72	0.17	0.17	0.88	0.23	0.23	0.58	0.16	0.16
33.5	1.02	0.24	0.24	0.72	0.17	0.17	0.86	0.23	0.23	0.57	0.16	0.16
33.7	1.01	0.23	0.23	0.71	0.16	0.16	0.85	0.22	0.22	0.56	0.16	0.16
33.8	1.00	0.23	0.23	0.71	0.16	0.16	0.83	0.22	0.22	0.55	0.16	0.16
34.0	0.99	0.23	0.23	0.70	0.16	0.16	0.82	0.22	0.22	0.54	0.15	0.15
34.2	0.98	0.22	0.22	0.70	0.16	0.16	0.80	0.21	0.21	0.54	0.15	0.15
34.4	0.98	0.22	0.22	0.70	0.16	0.16	0.78	0.21	0.21	0.53	0.15	0.15
34.6	0.97	0.22	0.22	0.69	0.15	0.15	0.77	0.21	0.21	0.52	0.15	0.15

34.8	0.96	0.22	0.22	0.69	0.15	0.15	0.75	0.21	0.21	0.51	0.15	0.15
34.9	0.95	0.21	0.21	0.68	0.15	0.15	0.74	0.21	0.21	0.50	0.15	0.15
35.1	0.94	0.21	0.21	0.68	0.15	0.15	0.72	0.20	0.20	0.49	0.15	0.15
35.3	0.94	0.21	0.21	0.68	0.15	0.15	0.71	0.20	0.20	0.49	0.15	0.15
35.5	0.93	0.21	0.21	0.67	0.15	0.15	0.69	0.20	0.20	0.48	0.14	0.14
35.7	0.92	0.21	0.21	0.67	0.15	0.15	0.67	0.20	0.20	0.47	0.14	0.14
35.9	0.91	0.21	0.21	0.66	0.15	0.15	0.66	0.20	0.20	0.46	0.14	0.14
36.0	0.90	0.21	0.21	0.66	0.15	0.15	0.64	0.20	0.20	0.45	0.14	0.14
36.2	0.89	0.21	0.21	0.66	0.15	0.15	0.63	0.20	0.20	0.45	0.14	0.14
36.4	0.89	0.21	0.21	0.65	0.15	0.15	0.61	0.20	0.20	0.44	0.15	0.15
36.6	0.88	0.21	0.21	0.65	0.15	0.15	0.60	0.20	0.20	0.43	0.15	0.15
36.8	0.87	0.21	0.21	0.65	0.15	0.15	0.58	0.20	0.20	0.42	0.15	0.15
37.0	0.86	0.22	0.22	0.64	0.15	0.15	0.56	0.21	0.21	0.41	0.15	0.15
37.1	0.85	0.22	0.22	0.64	0.15	0.15	0.55	0.21	0.21	0.40	0.15	0.15
37.3	0.85	0.22	0.22	0.63	0.15	0.15	0.53	0.21	0.21	0.40	0.15	0.15
37.5	0.84	0.22	0.22	0.63	0.16	0.16	0.52	0.21	0.21	0.39	0.15	0.15
37.7	0.83	0.22	0.22	0.63	0.16	0.16	0.50	0.21	0.21	0.38	0.15	0.15
37.9	0.82	0.23	0.23	0.62	0.16	0.16	0.48	0.22	0.22	0.37	0.16	0.16
38.1	0.81	0.23	0.23	0.62	0.16	0.16	0.47	0.22	0.22	0.36	0.16	0.16
38.3	0.80	0.23	0.23	0.61	0.16	0.16	0.45	0.22	0.22	0.36	0.16	0.16
38.4	0.80	0.24	0.24	0.61	0.17	0.17	0.44	0.23	0.23	0.35	0.16	0.16
38.6	0.79	0.24	0.24	0.61	0.17	0.17	0.42	0.23	0.23	0.34	0.16	0.16
38.8	0.78	0.24	0.24	0.60	0.17	0.17	0.41	0.23	0.23	0.33	0.17	0.17
39.0	0.77	0.25	0.25	0.60	0.18	0.18	0.39	0.24	0.24	0.32	0.17	0.17
39.2	0.76	0.25	0.25	0.59	0.18	0.18	0.37	0.24	0.24	0.31	0.17	0.17
39.4	0.76	0.26	0.26	0.59	0.18	0.18	0.36	0.25	0.25	0.31	0.18	0.18
39.5	0.75	0.26	0.26	0.59	0.19	0.19	0.34	0.25	0.25	0.30	0.18	0.18
39.7	0.74	0.27	0.27	0.58	0.19	0.19	0.33	0.26	0.26	0.29	0.18	0.18
39.9	0.73	0.27	0.27	0.58	0.19	0.19	0.31	0.26	0.26	0.28	0.19	0.19
40.1	0.72	0.28	0.28	0.58	0.20	0.20	0.30	0.27	0.27	0.27	0.19	0.19
40.3	0.71	0.28	0.28	0.57	0.20	0.20	0.28	0.27	0.27	0.27	0.19	0.19
40.5	0.71	0.29	0.29	0.57	0.20	0.20	0.26	0.28	0.28	0.26	0.20	0.20
40.6	0.70	0.29	0.29	0.56	0.21	0.21	0.25	0.28	0.28	0.25	0.20	0.20
40.8	0.69	0.30	0.30	0.56	0.21	0.21	0.23	0.29	0.29	0.24	0.20	0.20
41.0	0.68	0.30	0.30	0.56	0.22	0.22	0.22	0.29	0.29	0.23	0.21	0.21
41.2	0.67	0.31	0.31	0.55	0.22	0.22	0.20	0.30	0.30	0.22	0.21	0.21
41.4	0.67	0.32	0.32	0.55	0.22	0.22	0.19	0.30	0.30	0.22	0.22	0.22
41.6	0.66	0.32	0.32	0.54	0.23	0.23	0.17	0.31	0.31	0.21	0.22	0.22
41.7	0.65	0.33	0.33	0.54	0.23	0.23	0.15	0.31	0.31	0.20	0.22	0.22
41.9	0.64	0.33	0.33	0.54	0.24	0.24	0.14	0.32	0.32	0.19	0.23	0.23
42.1	0.63	0.34	0.34	0.53	0.24	0.24	0.12	0.33	0.33	0.18	0.23	0.23
42.3	0.62	0.35	0.35	0.53	0.25	0.25	0.11	0.33	0.33	0.18	0.24	0.24

Figure AIV.IV Probability value of categorisation to each histologic group by gestational age (weeks)

GA (Weeks)	Predicted probability: A/PIR	Predicted probability: VM	Predicted probability: PIR/VM	Predicted probability: NP	GA (Weeks)	Predicted probability: A/PIR	Predicted probability: VM	Predicted probability: PIR/VM	Predicted probability: NP
23.9	0.1550	0.4272	0.5825	0.0562	**37.4**	0.3808	0.1619	0.0792	0.3307
24.0	0.1561	0.4247	0.5775	0.0570	**37.4**	0.3808	0.1619	0.0792	0.3307
24.1	0.1573	0.4223	0.5725	0.0579	**37.4**	0.3808	0.1619	0.0792	0.3307
24.6	0.1634	0.4101	0.5470	0.0623	**37.4**	0.3808	0.1619	0.0792	0.3307
25.1	0.1696	0.3981	0.5214	0.0670	**37.6**	0.3851	0.1592	0.0762	0.3377
25.8	0.1786	0.3814	0.4853	0.0742	**37.8**	0.3893	0.1566	0.0734	0.3447
26.4	0.1866	0.3674	0.4544	0.0810	**37.9**	0.3914	0.1552	0.0720	0.3483
27.3	0.1992	0.3467	0.4089	0.0921	**37.9**	0.3914	0.1552	0.0720	0.3483
27.3	0.1992	0.3467	0.4089	0.0921	**38.1**	0.3957	0.1526	0.0693	0.3554
27.6	0.2035	0.3399	0.3940	0.0961	**38.1**	0.3957	0.1526	0.0693	0.3554
27.9	0.2079	0.3332	0.3793	0.1003	**38.3**	0.4000	0.1501	0.0666	0.3627

28.6	0.2184	0.3179	0.3459	0.1106	**38.6**	0.4065	0.1463	0.0629	0.3736
29.0	0.2246	0.3092	0.3275	0.1169	**38.6**	0.4065	0.1463	0.0629	0.3736
29.4	0.2309	0.3008	0.3096	0.1236	**38.8**	0.4108	0.1438	0.0605	0.3810
29.5	0.2341	0.2966	0.3008	0.1270	**38.9**	0.4130	0.1426	0.0593	0.3847
29.6	0.2373	0.2924	0.2922	0.1305	**38.9**	0.4130	0.1426	0.0593	0.3847
29.8	0.2390	0.2904	0.2880	0.1323	**39.0**	0.4152	0.1414	0.0582	0.3884
31.4	0.2643	0.2604	0.2288	0.1617	**39.1**	0.4173	0.1401	0.0571	0.3921
31.5	0.2660	0.2585	0.2252	0.1638	**39.4**	0.4239	0.1366	0.0538	0.4034
32.8	0.2894	0.2344	0.1818	0.1937	**39.6**	0.4283	0.1342	0.0518	0.4110
33.0	0.2931	0.2308	0.1758	0.1987	**39.8**	0.4327	0.1319	0.0498	0.4186
33.1	0.2949	0.2290	0.1728	0.2012	**39.9**	0.4349	0.1308	0.0488	0.4224
33.1	0.2949	0.2290	0.1728	0.2012	**39.9**	0.4349	0.1308	0.0488	0.4224
33.4	0.3006	0.2238	0.1641	0.2088	**39.9**	0.4349	0.1308	0.0488	0.4224
33.7	0.3062	0.2186	0.1558	0.2167	**40.0**	0.4371	0.1296	0.0479	0.4262
33.7	0.3062	0.2186	0.1558	0.2167	**40.0**	0.4371	0.1296	0.0479	0.4262
33.8	0.3081	0.2169	0.1531	0.2194	**40.1**	0.4393	0.1285	0.0469	0.4301
34.0	0.3120	0.2135	0.1478	0.2248	**40.1**	0.4393	0.1285	0.0469	0.4301
34.0	0.3120	0.2135	0.1478	0.2248	**40.3**	0.4437	0.1263	0.0451	0.4378
34.4	0.3197	0.2069	0.1377	0.2359	**40.3**	0.4437	0.1263	0.0451	0.4378
34.4	0.3197	0.2069	0.1377	0.2359	**40.4**	0.4459	0.1252	0.0442	0.4416
34.4	0.3197	0.2069	0.1377	0.2359	**40.4**	0.4459	0.1252	0.0442	0.4416
34.8	0.3276	0.2004	0.1282	0.2474	**40.4**	0.4459	0.1252	0.0442	0.4416
35.3	0.3375	0.1925	0.1171	0.2623	**40.6**	0.4504	0.1230	0.0425	0.4494
35.4	0.3395	0.1909	0.1150	0.2653	**40.8**	0.4548	0.1209	0.0409	0.4571
35.4	0.3395	0.1909	0.1150	0.2653	**40.8**	0.4548	0.1209	0.0409	0.4571
35.4	0.3395	0.1909	0.1150	0.2653	**40.8**	0.4548	0.1209	0.0409	0.4571
35.4	0.3395	0.1909	0.1150	0.2653	**40.9**	0.4570	0.1198	0.0401	0.4610
36.1	0.3537	0.1804	0.1011	0.2873	**40.9**	0.4570	0.1198	0.0401	0.4610
36.1	0.3537	0.1804	0.1011	0.2873	**41.0**	0.4593	0.1188	0.0393	0.4649
36.1	0.3537	0.1804	0.1011	0.2873	**41.1**	0.4615	0.1177	0.0385	0.4688
36.1	0.3537	0.1804	0.1011	0.2873	**41.1**	0.4615	0.1177	0.0385	0.4688
36.3	0.3579	0.1774	0.0974	0.2937	**41.1**	0.4615	0.1177	0.0385	0.4688
36.3	0.3579	0.1774	0.0974	0.2937	**41.1**	0.4615	0.1177	0.0385	0.4688
36.4	0.3599	0.1760	0.0956	0.2970	**41.3**	0.4659	0.1157	0.0370	0.4766
36.4	0.3599	0.1760	0.0956	0.2970	**41.6**	0.4726	0.1126	0.0349	0.4884
36.4	0.3599	0.1760	0.0956	0.2970	**41.8**	0.4771	0.1106	0.0335	0.4962
37.3	0.1550	0.4272	0.5825	0.0562	**42.0**	0.4816	0.1087	0.0322	0.5041
37.3	0.1561	0.4247	0.5775	0.0570	**42.3**	0.4883	0.1058	0.0303	0.5158

FIR; fetal inflammatory response, *GA;* gestational age, *MIR*; maternal inflammatory response

Figure AIV.V Total protein concentrations for all samples used in Chapter 7 analysed using QuBit® Protein Assay Kit (Life Technologies, California, USA) and QuBit® Fluorometer (Life Technologies, California, USA

Group	Unique Identifier	TOTAL PROTEN	
		µg/ml	pg/ml
PT/I	SU0006	12300.0000	12300000000.0000
PT/I	SU0029	13080.0000	13080000000.0000
PT/I	SU0041	14640.0000	14640000000.0000
PT/I	SU0049	11760.0000	11760000000.0000
		0.0000	0.0000
T/I	SU0043	12495.0000	12495000000.0000

T/I	SU0054	16050.0000	16050000000.0000
T/I	SU0059	16800.0000	16800000000.0000
T/I	SU0070	9135.0000	9135000000.0000
		0.0000	0.0000
PT/NI	SU0030	16800.0000	16800000000.0000
PT/NI	SU0034	13710.0000	13710000000.0000
PT/NI	SU0045	14400.0000	14400000000.0000
PT/NI	SU0072	11430.0000	11430000000.0000
		0.0000	0.0000
T/NI	SU0022	14535.0000	14535000000.0000
T/NI	SU0035	11580.0000	11580000000.0000
T/NI	SU0052	15450.0000	15450000000.0000
T/NI	SU0069	16500.0000	16500000000.0000

PT/I; preterm/inflamed*, PT/NI;* preterm/non-inflamed*, T/I*; term/inflamed*, T/NI;* term/non-inflamed

AIV.Va ELISA raw data: standards concentrations (pg/µl) for runs 1-3, sample 1-3 concentrations, values after multiplication by dilution factor and means per group (pg/ml)

https://docs.google.com/spreadsheets/d/1uvbFv2AZyCgcWTn3axJSwFrdAIwchRu4/edit?usp=share_link&ouid=104219403155318123493&rtpof=true&sd=true

Figure AIV.VII Kruskal-Wallis and Dunn's post-hoc tests of MIR and FIR grades against all target proteins

Kruskal-Wallis test hCAP18				
P value	0.2748			
Kruskal-Wallis statistic	6.337			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
hCAP18 MIR Grade 0 vs. MIR Grade 1	-6.3	No	ns	>0.9999
hCAP18 MIR Grade 0 vs. MIR Grade 2	-13.5	No	ns	0.499
hCAP18 MIR Grade 0 vs. FIR Grade 0	-1.944	No	ns	>0.9999
hCAP18 MIR Grade 0 vs. FIR Grade 1	-8	No	ns	>0.9999
hCAP18 MIR Grade 0 vs. FIR Grade 2	-7.5	No	ns	>0.9999
MIR Grade 1 vs. MIR Grade 2	-7.2	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 0	4.356	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 1	-1.7	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 2	-1.2	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 0	11.56	No	ns	0.9637
MIR Grade 2 vs. FIR Grade 1	5.5	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 2	6	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 1	-6.056	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 2	-5.556	No	ns	>0.9999
FIR Grade 1 vs. FIR Grade 2	0.5	No	ns	>0.9999
Kruskal-Wallis test IFN-γ				
P value	0.8921			
Kruskal-Wallis statistic	1.674			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
IFN-γ MIR Grade 0 vs. MIR Grade 1	-0.9	No	ns	>0.9999
IFN-γ MIR Grade 0 vs. MIR Grade 2	-6.5	No	ns	>0.9999
IFN-γ MIR Grade 0 vs. FIR Grade 0	-1.389	No	ns	>0.9999
IFN-γ MIR Grade 0 vs. FIR Grade 1	-4	No	ns	>0.9999
IFN-γ MIR Grade 0 vs. FIR Grade 2	1.5	No	ns	>0.9999
MIR Grade 1 vs. MIR Grade 2	-5.6	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 0	-0.4889	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 1	-3.1	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 2	2.4	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 0	5.111	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 1	2.5	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 2	8	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 1	-2.611	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 2	2.889	No	ns	>0.9999
FIR Grade 1 vs. FIR Grade 2	5.5	No	ns	>0.9999

Kruskal-Wallis test IL-6				
P value	0.0566			
Kruskal-Wallis statistic	10.75			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
IL-6 MIR Grade 0 vs. MIR Grade 1	-7.35	No	ns	>0.9999
IL-6 MIR Grade 0 vs. MIR Grade 2	-13.08	No	ns	0.5865
IL-6 MIR Grade 0 vs. FIR Grade 0	-1.972	No	ns	>0.9999
IL-6 MIR Grade 0 vs. FIR Grade 1	-7.75	No	ns	>0.9999
IL-6 MIR Grade 0 vs. FIR Grade 2	-9.083	No	ns	>0.9999
MIR Grade 1 vs. MIR Grade 2	-5.733	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 0	5.378	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 1	-0.4	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 2	-1.733	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 0	11.11	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 1	5.333	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 2	4	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 1	-5.778	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 2	-7.111	No	ns	>0.9999
FIR Grade 1 vs. FIR Grade 2	-1.333	No	ns	>0.9999
Kruskal-Wallis test IL-8				
P value	0.2471			
Kruskal-Wallis statistic	6.661			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
IL-8 MIR Grade 0 vs. MIR Grade 1	-2.1	No	ns	>0.9999
IL-8 MIR Grade 0 vs. MIR Grade 2	-15.17	No	ns	0.2516
IL-8 MIR Grade 0 vs. FIR Grade 0	-0.05556	No	ns	>0.9999
IL-8 MIR Grade 0 vs. FIR Grade 1	-3.5	No	ns	>0.9999
IL-8 MIR Grade 0 vs. FIR Grade 2	-13.83	No	ns	0.4373
MIR Grade 1 vs. MIR Grade 2	-13.07	No	ns	0.8418
MIR Grade 1 vs. FIR Grade 0	2.044	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 1	-1.4	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 2	-11.73	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 0	15.11	No	ns	0.2329
MIR Grade 2 vs. FIR Grade 1	11.67	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 2	1.333	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 1	-3.444	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 2	-13.78	No	ns	0.4104
FIR Grade 1 vs. FIR Grade 2	-10.33	No	ns	>0.9999
Kruskal-Wallis test IL-10				
P value	0.697			
Kruskal-Wallis statistic	3.019			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**

IL-10 MIR Grade 0 vs. MIR Grade 1	-3.6	No	ns	>0.9999
IL-10 MIR Grade 0 vs. MIR Grade 2	-10	No	ns	>0.9999
IL-10 MIR Grade 0 vs. FIR Grade 0	-1.556	No	ns	>0.9999
IL-10 MIR Grade 0 vs. FIR Grade 1	-4.5	No	ns	>0.9999
IL-10 MIR Grade 0 vs. FIR Grade 2	-5.333	No	ns	>0.9999
MIR Grade 1 vs. MIR Grade 2	-6.4	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 0	2.044	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 1	-0.9	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 2	-1.733	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 0	8.444	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 1	5.5	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 2	4.667	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 1	-2.944	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 2	-3.778	No	ns	>0.9999
FIR Grade 1 vs. FIR Grade 2	-0.8333	No	ns	>0.9999
Kruskal-Wallis test MMP9				
P value	0.9152			
Kruskal-Wallis statistic	1.481			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
MMP9 MIR Grade 0 vs. MIR Grade 1	-1.8	No	ns	>0.9999
MMP9 MIR Grade 0 vs. MIR Grade 2	-7.667	No	ns	>0.9999
MMP9 MIR Grade 0 vs. FIR Grade 0	-1.889	No	ns	>0.9999
MMP9 MIR Grade 0 vs. FIR Grade 1	-2.5	No	ns	>0.9999
MMP9 MIR Grade 0 vs. FIR Grade 2	-1.667	No	ns	>0.9999
MIR Grade 1 vs. MIR Grade 2	-5.867	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 0	-0.08889	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 1	-0.7	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 2	0.1333	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 0	5.778	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 1	5.167	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 2	6	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 1	-0.6111	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 2	0.2222	No	ns	>0.9999
FIR Grade 1 vs. FIR Grade 2	0.8333	No	ns	>0.9999
Kruskal-Wallis test SLPI				
P value	0.0519			
Kruskal-Wallis statistic	10.97			
Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted P Value**
SLPI MIR Grade 0 vs. MIR Grade 1	-7.05	No	ns	>0.9999
SLPI MIR Grade 0 vs. MIR Grade 2	-16.25	No	ns	0.1559
SLPI MIR Grade 0 vs. FIR Grade 0	-1.139	No	ns	>0.9999
SLPI MIR Grade 0 vs. FIR Grade 1	-9.25	No	ns	>0.9999
SLPI MIR Grade 0 vs. FIR Grade 2	-12.25	No	ns	0.8009
MIR Grade 1 vs. MIR Grade 2	-9.2	No	ns	>0.9999

MIR Grade 1 vs. FIR Grade 0	5.911	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 1	-2.2	No	ns	>0.9999
MIR Grade 1 vs. FIR Grade 2	-5.2	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 0	15.11	No	ns	0.2329
MIR Grade 2 vs. FIR Grade 1	7	No	ns	>0.9999
MIR Grade 2 vs. FIR Grade 2	4	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 1	-8.111	No	ns	>0.9999
FIR Grade 0 vs. FIR Grade 2	-11.11	No	ns	>0.9999
FIR Grade 1 vs. FIR Grade 2	-3	No	ns	>0.9999

APPENDIX V

Recipes for Buffers

Appendix AV.I Buffers for Immunofluorescent Staining

Appendix AV.Ia Citrate Buffer for Antigen Retrieval

To prepare 1 L, add 1.92 g of anhydrous Citric acid (10mM) (Cat. No. C0759-5006, Sigma, St. Louis, MO, USA) to 1000 ml of distilled water and mix to dissolve. Adjust the pH to 6.0 with 1N NaOH/HCl (Cat. No. 10326140/10467640, Fisher Scientific, Loughborough, UK). Add 0.5 ml Tween-20 (Cat. No. P1379, Sigma, St. Louis, MO, USA) and mix well.

Appendix AV.Ib Wash Buffer – 0.5% Tween-1X PBS

To prepare 1 L, add 5 ml Tween-20 (Cat. No. P1379, Sigma, St. Louis, MO, USA) to 995 ml 1X PBS (pH 7.4) (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, MA, USA) and mix well.

Appendix AV.Ic Permeabilisation Buffer – 0.2% Triton X-100-1X PBS

To prepare 500 ml, add 1 ml Triton X-100 (Cat. No. T8787, Sigma, St. Louis, MO, USA) to 499 ml 1X PBS (pH 7.4) (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, MA, USA) and mix well.

Appendix AV.II Buffers for Protein Extraction

Appendix AV.IIa Lysis Buffer – 0.1% IGEPAL® CA-630-1X Protease Inhibitor Cocktail-1X PBS (pH 7.4)

To prepare 10 ml 10X Protease Inhibitor Cocktail stock solution, dissolve one SIGMA*FAST*® Protease Inhibitor Cocktail tablet (Cat. No. 58830, Sigma, St. Louis, MO, USA) in 10 ml 1X PBS (pH 7.4) (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, MA, USA).
To prepare 10 ml Lysis Buffer, add 1 ml 10X Protease Inhibitor Cocktail stock solution and 10µl IGEPAL® CA-630 (Cat. No. I3021, Sigma, St. Louis, MO, USA) to 8.99 1X PBS (pH 7.4) (Cat. No. 10010056, Thermo Fisher Scientific, Waltham, MA, USA) and mix well.