Immunology of Choriodecidua and Onset of Labour

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This thesis is dedicated to my little world…

Appa, Amma

Sarangan

Shakti and Shivanya

STATEMENT OF ORIGINALITY

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise by reference in the text, and that this work has not been submitted for any other degree or professional qualification.

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ABSTRACT

Introduction:

Labour is a well-orchestrated process associated with inflammation; its onset is a poorly understood retrospective diagnosis. Immunology of pregnancy and labour is an area of wide interest; however, knowledge is lacking in what occurs at the maternal-fetal interface, the decidua. The aim of this thesis was to better understand the role of decidual immune cells in preterm birth and term labour while associating metabolites with immune cells that may be pivotal in labour.

Methodology:

Multiple cytokine assays were used to quantify cytokines/chemokines in choriodecidua/placenta. Flow cytometry was used to identify immune cells in decidua, placenta, maternal and cord blood. Mass spectrometry was used to quantify urinary metabolites.

Results:

Study one showed that choriodecidua was the most inflammatory gestational tissue analysed. Cytokines/chemokines indicated that NK cells, monocytes, T cells and neutrophils may play a significant role in preterm birth. Increased IL-8 in choriodecidua was associated with worse neonatal outcomes.

Study two revealed a shift from predominantly $CD56^{bright}$ NK cells to a predominant subset of $CD56^{+}CD16^{+}$ NK cells with onset of term labour in decidua. Decidual NK^{bright} cells and T_{regs} work together to maintain uterine quiescence at term, which is lost with labour. CD4+ Tregs and CD8+ Tregs were significantly higher in decidua than placenta/blood at term. Decidual monocytes, T cells and neutrophils did not alter with the onset of labour.

Study three did not identify a signature urinary metabolic profile that is associated with the onset of labour. It highlighted that factors such as diet and lifestyle may cause inter-personal variability.

Conclusions:

This thesis highlights that the tolerant immune cells and their interaction with cytokines/chemokines maintain an immunological equilibrium in the decidua. This is altered with the onset of labour. Urinary metabolites inconsistently altered with the onset of labour, there was no signature urinary metabolic profile associated with labour.

LIST OF CONFERENCE PRESENTATIONS/PUBLICATIONS

1. Isolation of single cells from human uterus in the third trimester of pregnancy: myometrium, decidua, amnion and chorion Alexander TH Cocker, Emily M Whetlock, Brendan Browne, Pei F Lai, Jonathan KH Li, **Sivatharjini P Sivarajasingam**, Nesrina Imami, Mark R Johnson, Victoria Male, Sept 2022, Oxford Open Immunology – IN PRESS

2. Cocker A, **Sivarajasingam S**, Sassine A, Raj I, Dermont S, Khan A, Imami N, Johnson M, 2018, Regulatory T-cell compartment in HIV-1+ pregnancies loses gestation adaptations and function, European Congress for Immunology (ECI) 2018, Amsterdam, 2-5 September 2018

3. **Sivarajasingam S**, Cocker ATH, Sassine AJ, Imami N, Johnson M, Disrupted Immunological Equilibrium of Decidual Natural Killer Cells and Regulatory T Cells with Onset of Labour, Society of Reproductive Investigation, San Diego, March 2018

4. Cocker ATH, **Sivarajasingam SP**, Dermont S, Khan W, Imami N, Johnson MR, Gestational Immunological Adaptations and Anti-Viral Responses are disrupted in HIV-1+ Pregnancies, Society of Reproductive Investigation, San Diego, March 2018

5. Sassine AJ, **Sivarajasingam SP**, Cocker ATH, Wang Y, Imami N, Crawford MA, Johnson MR, Erythrocyte oleic acid is correlated with increasing natural killer cells in maternal blood, Society of Reproductive Investigation, San Diego, March 2018

6. Cocker ATH, **Sivarajasingam SP**, Dermont S, Khan W, Imami N, Johnson MR, Amplified CMV-specific responses are reflective of excessive immune exhaustion in HIV-1+ pregnancies, British Society of Immunology, Brighton Dec 2017

7. **Sivarajasingam SP**, Cocker ATH, Sassine AJ, Imami N, Johnson MR, Regulatory T cells maintain comparable frequency in decidua and placenta albeit higher than in maternal blood as gestation advances, Annual preterm birth conference, Leeds Sept 2017

8. **Sivarajasingam SP**, Cocker ATH, Sassine AJ, Johnson MR, Decidual NK cells initiate the inflammatory trigger of human labour, Annual preterm birth conference, Leeds Sept 2017

9. **Sivarajasingam SP**, Das A, Herbert BR, Singh N, Imami N, Johnson MR, The immunology of the choriodecidua, placenta and amnion in pre-term deliveries secondary to chorioamnionitis, Society of Reproductive Investigation, Orlando, March 2017

10. **Sivarajasingam S**, Cocker ATH, Imami N, Johnson MR, Increased anergic $CD56^{-}CD16^{+}$ Decidual and Cytokine producing $CD56^{bright}CD16^{-}$ placental NK cell frequencies observed in term, non-labouring women, British Society of Immunology/NVVI Annual Congress, Liverpool, Dec 2016

11. **Sivarajasingam SP**, Imami N, Johnson MR, Cytokine and myometrial signalling in human labour J Endocrinol. 2016 Dec;231(3):R101-R119. Epub 2016 Sep 19 *Journal of Endocrinology, Sept 2016*

12. Fainardi V, Nagakumar P, **Sivarajasingam S**, Johnson MR, Saglani S, Lloyd CM, IL-4 and IL-13 secreting type 2 innate lymphoid cells are present in human cord blood, International Congress of Paediatric Pulmonology, Naples Italy, June 2016

13. **Sivarajasingam S**, Das S, Herbert BR, Singh N, Imami N, Johnson MR, Inflammation in Choriodecidua and Amnion in Preterm Birth Subtypes, European Spontaneous Preterm Birth Conference, Gothenburg, Sweden May 2016

ABBREVIATIONS

ACA Acetoacetate

AEA N-Arachidonoylethanolamine

BAFF B cell activation factor

CA Chorioamnionitis

CD Choriodecidua

DB Decidua basalis

DC Dendritic cells

DSC Decidual stromal cells

FAO Fatty acid oxidation

FAS Fatty acid synthesis

FCS Fetal calf serum

FWB FACS wash buffer

IDO Indoleamine-pyrrole 2,3-dioxygenase

IL Interleukin

IQR Interquartile range

IVH Intraventricular haemorrhage

LPS Lipopolysaccharide

MMP Matrix metalloproteinase

NEC Necrotising enterocolitis

NK Natural killer cells

NND Neonatal death

PA Placental abruption

PB Peripheral blood

PBMC Peripheral blood mononuclear cells

PBS Phosphate buffered saline

PCA Principal component analysis

PDA Patent ductus arteriosus

PPP Pentose phosphate pathway

PROM premature rupture of membranes

PTB Preterm birth

PTL Preterm labour

PTNL Preterm non labour

RDS Respiratory distress syndrome

RCF Relative centrifugal force

RPM Revolutions per minute

TCA Tricarboxylic acid

TECK Thymus expressed chemokine

TNF Tumour necrosis factor

TEL Term early labour

TNL Term non labour

Treg T regulatory cells

TwL Twins labouring

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1. Introduction

1.1 Preterm Birth

Preterm birth is defined as the birth of a baby before 37 weeks of gestation which can be sub-divided into three further categories: extremely preterm (less than 28 weeks), very preterm (28-32 weeks) and moderate to late preterm (32-37 weeks). Currently every year 13.4 million babies are born prematurely and unfortunately this number is continuing to rise which is around 10% of all births (WHO, 2023). One million of these children die due to complications of preterm birth, and those that survive are at increased risk of neurodevelopmental impairments, respiratory and gastrointestinal complications (1). There is an inverse relationship between gestational age at delivery and the risk of neonatal morbidity, thus the extremely premature babies that survive tend to have the most severe prematurity complications such as cerebral palsy (2). Unfortunately, the aetiology of preterm birth is still poorly understood and is probably best described as a syndrome initiated by one or more mechanism (1) and in many cases, frustratingly this mechanism remains undefined. The cost of preterm birth to the society is considerable and this has been estimated in the UK to be £3.4 billion (Reducing preterm birth: Guidelines for Commissioners and Providers, 2019) and up to $26 billion in USA (3). With this all taken into consideration prediction and prevention is the focus of most research, as markers that can direct prophylactic therapies are needed. Unfortunately, due to the complexity of this condition, differences observed in populations and agents, there is no single reliable predictive model yet, and it is highly likely that with significantly more research it will be a combination of tests that will enable better clinical predictivity of preterm birth (4).

1.2 Epidemiology of Preterm Birth

There is significant variation in preterm birth (PTB) rates worldwide (Figure 1.1) and although it affected 10.6% of live births globally in 2014, 80% of these were in Asian and Sub-Saharan Africa (5). This reinforces the notion that PTB rates are higher in countries with low to middle income and the leading cause for this may be infective processes. There has been some

evidence though that PTB rates are also increasing in higher income countries; in European countries this may be swayed by increasing multiple pregnancy preterm deliveries (6) however in America there is a persistent increase in PTB. It is notably, the only high-income country to rank in the top ten list for greatest overall number of preterm births per year (7).

Here in the UK, PTB is the single biggest cause of neonatal mortality and morbidity affecting 8% of all live births in England and Wales in 2015, and there has been no decline in the preterm birth rate in the UK over the last 10 years (444).

	Estimated preterm birth rate* (%, UI)	UNDP estimated number of livebirths	Proportion of global livebirths (%)	Estimated number of preterm births (n, UI)	Proportion of global preterm births (%)
Asia	10·4% (8·7–11·9)	75 441 991	53·9%	7 847 643 (6 579 297–8 987 184)	52·9%
Europe	8·7% (6·3–13·3)	7 927 034	5·7%	690 931 (497 738–1 051 737)	4·7%
Latin America and the Caribbean	9·8% (8·6–11·3)	10 814 139	7·7%	1 062 800 (931 611–1 220 105)	7·2%
North America	11·2% (9·5–13·2)	4 394 185	3·1%	491 297 (416 479–578 367)	3·3%
North Africa	13·4% (6·3–30·9)	5 771 560	4·1%	773 687 (365 845–1 782 375)	5·2%
Oceania	10·0% (7·9–12·7)	643 749	0·5%	64 227 (50 706–81 961)	0·4%
Sub-Saharan Africa	12·0% (8·6–16·7)	34 953 292	25·0%	4 182 440 (2 994 834–5 838 104)	28·2%
Global	10·6% (9·0–12·0)	139 945 950	100·0%	14 835 606 (12 654 938–16 728 926)	100·0%

Regions are based on the United Nations Standard Country or Area Codes for Statistical Use (M49) major regional groups. UI=uncertainty interval. UNDP=United Nations Development Programme. *Preterm births per 100 livebirths.

***Table 2*: Estimated preterm birth rates and numbers of preterm births in 2014**

Figure 1. 1 Estimate preterm birth rate and numbers of preterm births in 2014. (Chawanpaiboon et al., 2019) Image reproduced with permission of the rights holder Elsevier, © Elsevier.

85% of preterm births occur in the late preterm period (5) and although these babies have a far reduced mortality rate compared to the very premature babies, it is now well recognised they have significant morbidity compared to term babies. Morbidities that the neonates are at higher risk of include respiratory distress, feeding difficulties, hypothermia (Figure 1.2) and later in life they are at increased risk of long-term morbidities such as neuro-developmental delay, behavioural problems and chronic medical illnesses for example diabetes (8).

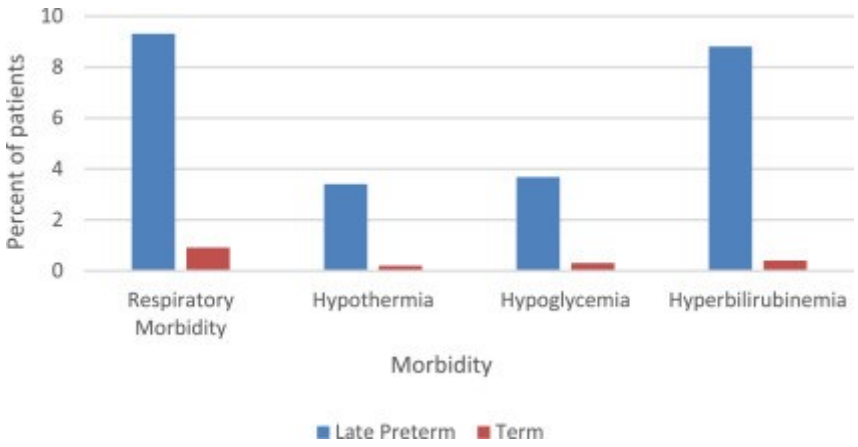


Figure 1. 2 Clinical morbidities noted during birth hospitalisation from pooled data for late preterm and term patients (Huff et al, 2019). Image reproduced with permission from Springer Nature.

1.3 Causes of preterm birth

PTB can be subdivided into spontaneous and iatrogenic, with spontaneous preterm birth (SPTB) contributing to 70% of preterm deliveries (9). The remainder are medically indicated deliveries due to fetal or maternal concerns such as pre-eclampsia, fetal growth restriction and multiple pregnancies (1).

SPTB is caused by a plethora of pathologies (Figure 1.3) and is probably best described as a syndrome with multiple mechanisms in addition to social, genetic and environmental factors.

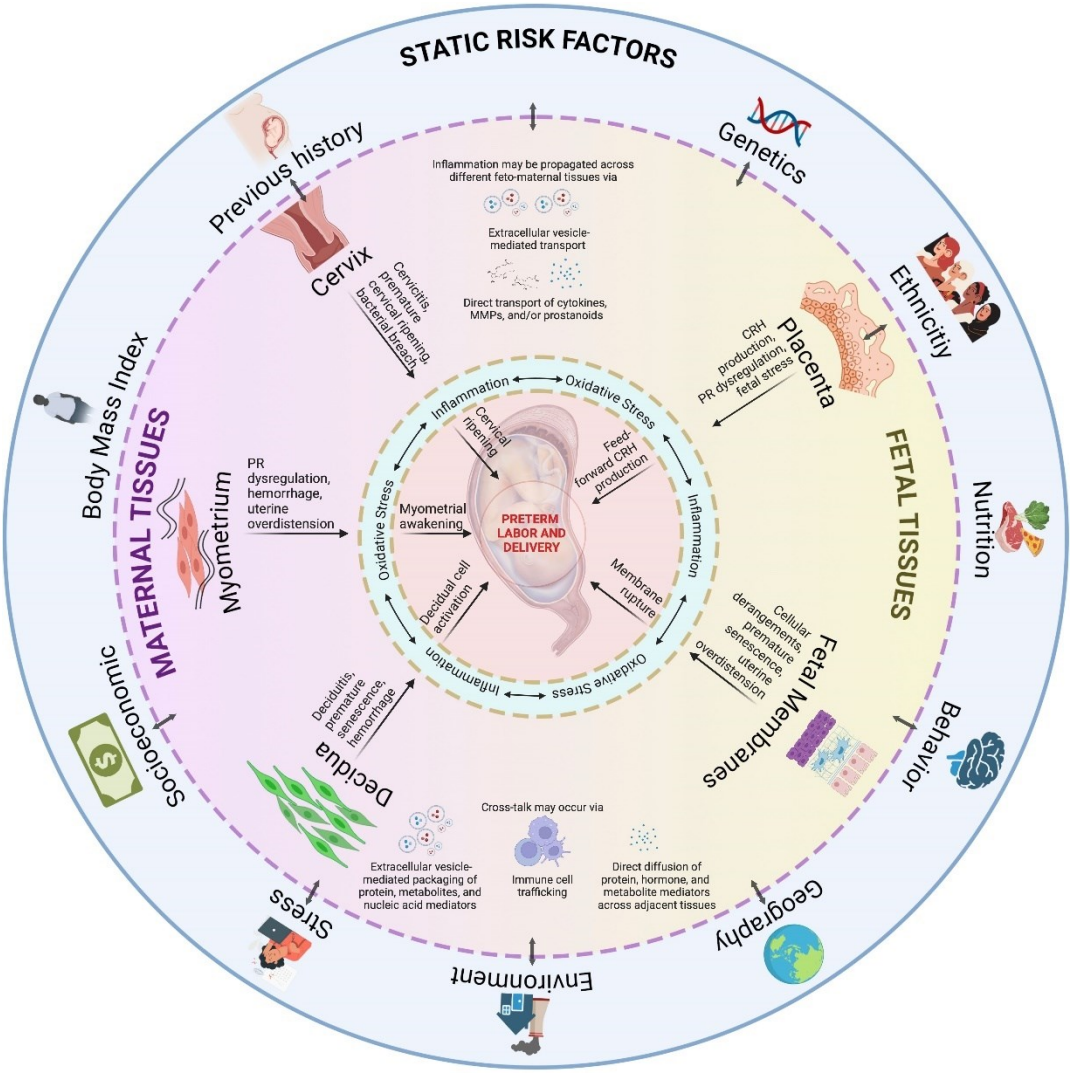


Figure 1. 3 An integrated model for preterm birth which highlight that PTB is not a disease of single system acting in silo but contributed by systems working together to produce an outcome (Vidal et al, 2022). Image reproduced with permission from creative commons attribution license.

Of the mechanisms mentioned in the above figure, those that are most relevant to this thesis are the role of the decidua and breakdown of maternal-fetal tolerance in the trigger of labour, whether it be physiologically normal at term or abnormally at a preterm gestation.

Inflammation has been implicated in the mechanisms responsible for term and preterm parturition. In fact, infection which is a heightened inflammatory process is the only evidenced causal link confirmed to trigger PTB (10). Infections can be intrauterine, extra-uterine, systemic, clinical, or even sub-clinical especially when intrauterine. The micro-

organisms that cause the intrauterine infections are most commonly ascending infections from the vagina and cervix, but can also enter via haematogenous dissemination from the placenta, retrograde through fallopian tubes from abdominal cavity or via amniocentesis (1). Once the ascending infection breaks the antimicrobial strategies in the cervix, it can gain access to the fetal membranes causing chorioamnionitis and an inflammatory insult (11).

1.4 Gestational tissues

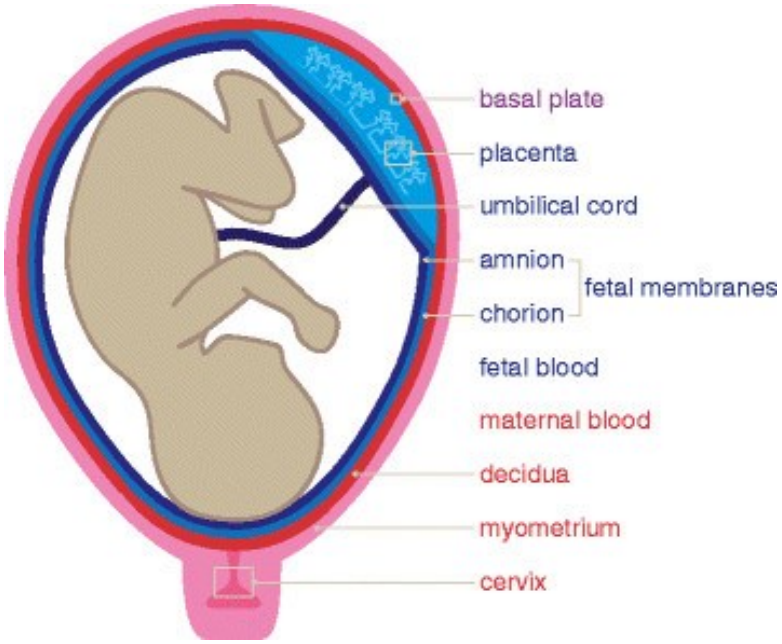


Figure 1. 4 The tissues of pregnancy (Eidem, Ackerman, McGary, Abbot, & Rokas, 2015) Image reproduced with permission from Springer Nature.

1.4.1 Decidua

The decidua is formed during the first trimester from the endometrium of the uterus and can be sub-divided once the placenta is formed into decidua basalis which surrounds the placenta, whilst the decidua parietalis is the decidual tissue in contact with fetal membranes, specifically the chorion (Cunningham *et al*, 2014 textbook). Once embryonic implantation is complete, the endometrium is called the decidua and decidualisation is essential for

successful embryonic growth (12). Insufficiency in decidualisation may cause infertility, recurrent miscarriages, and placental pathologies such as pre-eclampsia (13) (14).

The decidua is probably the least studied gestational tissue even though it is located directly at the maternal-fetal interface, hence ideally placed to have a key role in the events leading to the onset of labour (15). Most research has been focused on defining the molecular events underlying myometrial contractions, fetal membrane rupture and cervical ripening (15) but since 1988 when Casey *et al* proposed that the decidua may play a role in the onset of labour, there has been some interest in this understated gestational tissue (16).

Being the maternal-fetal interface, the decidua is an area of immunological interest. For a pregnancy to be maintained healthily and to term, the maternal immune system must adapt in order to tolerate allogenic cells whilst maintaining maternal immune competence (17). In human and interestingly rodent pregnancies, the placentae are haemochorial which is the most invasive type of placenta (18). This means the embryo derived trophoblast cells invade the maternal uterine tissue (19). This leads to the trophoblast cells being in close contact with the maternal immune cells, which make up around 40-50% of cells in the decidua throughout pregnancy (18). Considering fetal and placental cells are semi-allogenic to the mother, a "normal" immune response would lead to rejection, like that of allogenic tissue transplantation (20), however we know that this does not occur. This phenomenon was noted and first described as early as in the 1950s by Sir Peter Medawar as "the immunological paradox of pregnancy" (21). Historically this was believed to be possible due to a physical blood-placental barrier, but this theory is clearly disproved by the deep invasive placentation with constant transplacental exchange of cells, further confirmed by cell-free fetal DNA found in maternal blood used as a screening tool for fetal trisomies (22). As a result, it was accepted that this paradox was only possible by regulating immune cells involved in pregnancy and specifically, at the decidual level in more recent years.

1:4:2 Immune Cells in the Decidua

The immune cells can be divided into innate and adaptive immune cells (Table 1.1).

Table 1. 1 Immune cells: effector mechanisms and their role in the pregnant uterus (The bars in the column "present in the uterus" aim to evidence that some populations are more abundant than others; however, no quantitative data are provided) (Solano, 2019). Image reproduced with permission of the

rights holder Elsevier, © Elsevier. Abbreviations MHC Major histocompatibility complex, Treg T regulatory cell, KIR2DL4 Killer cell immunoglobulin-like receptor 2LD4, HLA-C/E/G Human leukocyte antigen C/E/G, APC antigen-presenting cell, LILRB2 Leukocyte immunoglobulin-like receptor subfamily B member 2, TCR T cell receptor, ILC Innate lymphoid cell

lineage	immune responses	immune cell population		main effector mechanisms	interacts with MHC molecules in trophoblast cells?	present in uterus	role in human decidua	references
myeloid	innate	granulocytes	neutrophils	phagocytosis, extracelullar traps, cytokines			immune defense, angiogenesis, Treg induction	6
			basophils, eosynophils	cytotoxicity, cytokine secretion		-	-	
			Mast cells	cytotoxicity, cytokine secretion	KIR2DL4 binds HLA-G		trophoblast invasion, spiral arteries modification	4;7
		mononuclear phagocytes	Monocytes	phagocytosis, cytokine secretion		-	-	4
			Macrophages	phagocytosis, APC, cytokine secretion	LILRB1 (subp) binds HLA-G		homeostasis, tissue remodeling, spiral arteries modification, immune survillance, Treg induction	4;38;90
			dendritic cells	APC, cytokine secretion	LILRB2 (subp) binds HLA-G		tolerance, Treg induction	4
lymphoid		Innate lymphoid cells (ILC)	Natural killer cells	cytokine secretion, cytotoxicity	KIRs bind HLA-C, NKG2 bind HLA-E, LILRB1 HLA-G (subp)		cytokines, angiogenic mediators, and growth factor secretion: promote trophoblast invasion	3;48
			ILC1-3	cytokine secretion			immune defense, tissue remodelling	20
	adaptive	T cells	Natural killer T cells	cytokine secretion, cytotoxicity	TCR binds MR1		immune defense, regulation?	17
			MAIT		TCR binds CD1d		immune defense	19
			gamma delta T cells		CD94/NKG2A binds HLA-E		immune defense, regulatory cytokines	18
			CD_4+ T con	cytokine secretion, $CD8^+$ T and B cell activation	LIRB1 (subp) binds HLA-G		cytokine secretion: supports vascular changes	13
			CD_4+ T reg	immune regulation			multimodal immune regulation and angiogenesis	15
			CD8+ T cells	cytokine secretion, cytotoxicity	TCR binds HLA-C		immune defense, immune regulation	13
		B cells		antibody production, cytokine secretion			immune regulation	14

The decidua is often seen to be the maternal-fetal interface and this interface consists of decidual immune cells, decidual stromal cells and trophoblast cells (23). Several immune cell subsets make up the decidual immune cell population, these include natural killer cells, mononuclear phagocytes (monocytes, macrophages, dendritic cells) and T cells (24).

1:5 Natural Killer Cells

Natural killer (NK) cells are probably the most studied innate immune cell group within the decidua, in particular the innate lymphoid cells subset. This is likely to be as during the first trimester, when the decidua is being formed approximately 70-80% of early pregnancy decidual lymphocytes are made up by NK cells (25). NK cells were historically deemed to be a homogenous population of innate lymphocytes with limited phenotypic and functional diversity, however more recent research has proven that these cells can be subdivided into distinct populations with distinct diversities (26).

NK cells are commonly divided into two main subsets $CD56^{bright}$ and $CD56^{dim}$ NK cells (27). $CD56^{bright}$ cells are typically cytokine producing cells with poor cytotoxic ability whereas $CD56^{dim}$ cells are known for their cytotoxic ability (28). The difference in the two types of cells are best summarised below in Table 1.2 and Figure 1.5 (26).

Table 1. 2 NK cell subsets subdivided on expression of CD56, its known function and expression of other receptors.

NK cells subset	Function	Expression
$CD56^{bright}$	- Cytokine producing - Poor cytotoxicity	- CD127 - CD117
$CD56^{dim}$	- Cytotoxic	- CD16 - KIRs (killer cell immunoglobulin-like receptor) - CD57

More recently NK cells have been accepted as being more diversified than previously thought, which is partially due to genetic means i.e. via combining the KIR gene content (29). In addition, distinct tissue-resident NK cell subpopulations have been identified and these have largely contributed to NK cell diversity due to differing local tissue environments (30) (31).

1.5.1 Peripheral NK cells

NK cells are mainly derived in the bone marrow and secondary lymphoid tissue such as the spleen and lymph nodes (32), however there is emerging evidence that NK cells can be derived in peripheral tissues such as uterine and liver tissue (30, 31). There was always some confusion regarding whether NK cells circulated from blood to tissues, but it is now understood that tissue specific NK cells express CD69 which seem to enable retainment of these NK cells in the tissue (33). Contrastingly, NK cells in blood lack expression of CD69 and interestingly uterine NK cells hold a unique phenotypic/functional profile (34) which seem to be a mix of both more and less differentiated NK cells that are defined CD69+, CD103+ and/or $CD49a^{+}$, as shown in Figure 1.5 (26).

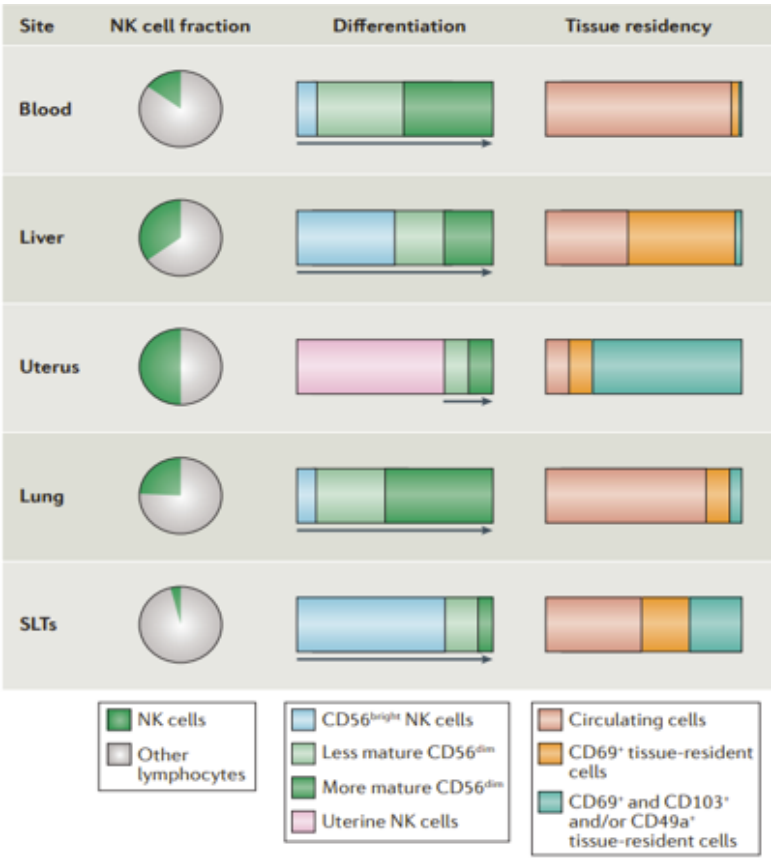


Figure 1. 5 Characteristics of human NK cells in circulation and peripheral tissues (Björkström et al., 2016) Adapted from Image reproduced with permission from Springer Nature.

What do we know about uterine NK cells?

It is known that uterine NK cells differ from peripheral NK cells (35), however it remains undefined where uterine NK cells originate from. There is a possibility they may be recruited from the periphery and/or may differentiate locally; this may be helped by decidual stromal cells which uterine NK cells are attracted to (36). The potential crosstalk between NK cells and the cells they interact with is highlighted in Figure 1.6.

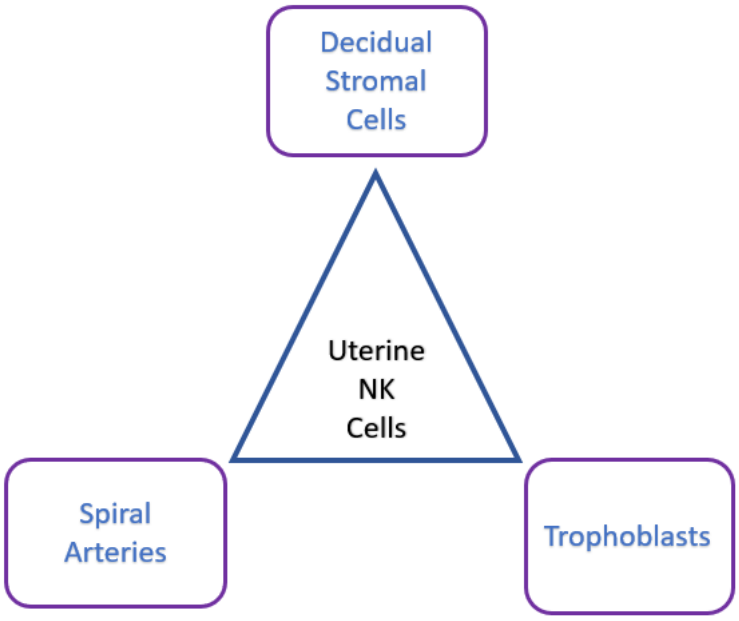


Figure 1. 6 Uterine NK cells and their interactors – Uterine NK cells interact with decidual stromal cells, spiral arteries and trophoblasts in the first trimester and this interaction is vital for healthy implantation and placentation.

Decidual stromal cells are non-hematopoietic cells that are capable of immunomodulation as well as having an anti-inflammatory effect (37, 38). Stromal cells can be isolated from varying tissues including the placenta (38), cord blood (39) and of course bone marrow. Decidual stromal cells have been shown to inhibit the expression of major activating NK receptors and sharply suppress NK cell proliferation, cytotoxicity, and cytokine production (40). Decidual stromal cells also promotes the differentiation of $CD56^{bright}$ $CD16^{-}$ NK with high levels of inhibitory receptors, immunotolerant, and angiogenic cytokines by secreting IL-24 (23).

Uterine NK cells are also widely known to interact with trophoblasts, and they can interact with HLA-E, HLA-G and HLA-C via a range of receptors including KIRs and leukocyte immunoglobulin receptors (41). Uterine NK cells, unlike peripheral NK cells have a biased interaction with HLA-C and HLA-G which is key to limit cytotoxicity to trophoblast cells and maintain immunotolerance (24). Earlier studies confirmed that first trimester trophoblast cells recruit $CD56^{bright}CD16^{-}$ NK cells via expression and secretion of CXCL12 and stromal cell-derived factor 1 (42). More recent studies have shown that certain combinations of maternal

KIRs and fetal HLA-C genetic variants are associated with pregnancy disorders such as pre-eclampsia, in which trophoblast invasion is deficient (43).

Remodelling of spiral arteries in the decidua basalis and underlying superficial myometrium is essential to ensure minimal vessel resistance and high blood flow of nutrients and oxygen to the placenta and so to the growing fetus (44). Resistance and poor flow can lead to pregnancy complications such as fetal growth restriction and pre-eclampsia. It has been shown in a mouse model, that uterine NK cells enhance vasculature construction in the early decidua basalis (45). In human models, uterine NK cells initiate the remodelling of and differentiation of spiral artery vascular smooth muscle (46), and impaired decidual NK cell regulation of vascular remodelling in early human pregnancies has led to high uterine artery resistance (47).

There have been differing studies on the role of uterine NK cells in pregnancy however, it is widely accepted that NK cells are poorly cytotoxic to both classic NK targets as well as trophoblasts (48). They can on the other hand activate cytotoxicity in response to viruses in the decidua such as cytomegalovirus (49).

The progression of uterine NK cells to decidual NK cells is presumed to happen following conception under the stimulation of IL-15 which is a key cytokine in the differentiation of hematopoietic progenitor to NK cells (50). Most studies informing us about decidual NK cells and in particular their function have been based on early pregnancy and delivery samples for obvious ethical reasons. Decidual NK cells are potent cytokine producers which include IL-8, IP-10, IL-10, G-CSF, GM-CSF, M-CSF, TNF-α which are all necessary for a successful healthy pregnancy (51) (52). As pregnancy progresses decidual NK cells have less granules in their cytoplasm which may be due to a functional shift that is necessary for the onset of labour (53). Most interestingly one study identified that there is a significant increase in $CD56^{dim}CD16^{+}$ NK cells in the decidua, with no change in $CD56^{bright}CD16^{-}$ NK cells when comparing spontaneous vaginal delivery to elective Caesarean section samples in uncomplicated human pregnancies (54). This suggests that NK cells play a role in the inflammatory induction of labour. Overall, $CD56^{bright}CD16^{-}$ NK cells are essential in maintaining a healthy pregnancy and changes in decidual NK cells subsets/status and function can be associated with several pregnancy complications such as spontaneous recurrent

miscarriage where $CD56^{+}$ decidual NK cells were increased and also the phenotype of NK shifted from $CD56^{bright}CD16^{-}$ to $CD56^{dim}CD16^{+}$ (55).

1.6 Monocytes

Monocytes develop from pre-cursors in the bone marrow and comprise about 5-10% of the circulating blood leukocytes (55). There are three types of monocytes that are present in humans; classical, non-classical and intermediate monocytes which can be differentiated by their expression of CD14 and CD16. Classical monocytes ($CD14^{high}CD16^{-}$) make up the largest proportion of monocytes in peripheral blood, around 80%, and are efficient phagocytes. Non-classical monocytes ($CD14^{low}CD16^{high)}$ are weak phagocytes but efficient producers of pro-inflammatory cytokines whilst the intermediate monocytes ($CD14^{high}CD16^{intermediate}$) as denoted by the name, are transitional having both phagocytic and inflammatory capabilities (56).

In healthy pregnancy, there are fewer classical monocytes and an increased number of intermediate monocytes (56) in circulation. After 1-2 days in circulation, monocytes migrate to the uterus driven by oestrogen and progesterone (55) and become resident macrophages (57). Decidual macrophages make up around 20-25% of total decidual leukocytes and tend to be classified as all tissue macrophages into M1 and M2 (55). M1 macrophages are microbicidal and inflammatory, while M2 macrophages are immunomodulatory, inducing tolerance and resolution of inflammation (58). Their immune-suppressive activity may be due to their high levels of IL-10 and indoleamine 2-3 deoxygenase (IDO) production, along with the production of prostaglandin-E_2 (PGE_2), which blocks the activation of cytotoxic leukocytes (59). Recently evidence has further subdivided the macrophages at the maternal-fetal interface into three subsets based on CCR2 and CD11c, very interestingly it identified that the macrophage subset that was distributed in the decidua - $CCR2^{-}CD11c^{LO}$ macrophages, had an anti-oxidative and anti-inflammatory effect (60). This again works toward maintaining the immune-tolerant microenvironment.

Macrophages play a role in blastocyst implantation by creating a pro-inflammatory environment (61), and prepare the spiral arteries for further remodelling by trophoblasts. In

addition to this, macrophages play a key role in spiral artery modelling. They engulf apoptotic cells that are a by-product of spiral artery modelling and trophoblast invasion; by doing so macrophages prevent potential inflammatory sequelae within the decidua (62).

Decidual macrophages appear to be more like M2 macrophages as they express M2 markers such as CD206, CD163 and DC-SIGN (63, 64), however they do not behave exactly like M2 macrophages as they are not induced by Th2 cytokines, such as IL-4, but by M-CSF and IL-10 (65) which are abundant in the decidua (66) (67). There is also evidence suggesting there are two subsets of decidual macrophages, one which is more inflammatory and the other more regulatory, the latter has higher numbers within the decidua (68).

Macrophages continue to be present in the decidua at term, albeit they become the smallest cohort of the decidual leukocytes (53). The macrophages that are in the decidua from the second trimester through to term are of the M2 subset which likely prevents placental and fetal rejection ((69) (70). A recent study has shown that vascular endothelial growth factor (VEGF) may enhance macrophage migration and induce M1 macrophages to shift to an M2 phenotype, with the possibility that VEGF inhibition may lead to a M1 bias resulting in pregnancy complications such as pre-eclampsia (71). The precise role that macrophages play towards the end of the pregnancy is still unclear, but it is likely that they maintain an immunotolerant role whilst mopping up apoptotic cells (61). It is interesting however that macrophages are increased in both human and rodent decidua preceding the onset of labour, term and preterm, which suggests they play a role in the inflammatory process that characterises human labour and are possibly recruited in to do so (72). In addition, Gonzalez *et al* showed that depletion of macrophages in pregnant mice protected these animals from lipopolysaccharide (LPS)-induced preterm birth, indicative of the significant role macrophages may play in preterm labour (73).

Dendritic cells (DCs) are the last of the subset of mononuclear phagocytes, although similar to macrophages, they are more potent antigen presenting cells than macrophages (74). Human DCs are typically divided into myeloid and plasmacytoid, the myeloid DCs ($CD14^{-}CD11c^{+}$) reside in the spleen and lymph nodes and regulate pro-inflammatory responses. Plasmacytoid DCs ($CD123^{+}CD11c^{-}$) are found in non-lymphoid peripheral tissues and generate Th2 responses (55). During normal pregnancy, peripheral myeloid DCs are

extremely tolerogenic however it has been noted that this tolerance is reduced in third trimester compared to second trimester, suggesting diminishing tolerance leading up to the onset of labour (75).

Decidual DCs are still not fully understood as there is no single specific marker for decidual DCs; identification of decidual DCs have been via lineage negative and HLA-DR+ as a combination marker (76). These cells further expressed CD11c which indicates they are myeloid in origin. Kammerer *et al* went further and characterised immature DCs by DC-SIGN and confirmed the maturation of $CD14^+DC\text{-}SIGN^+$ decidual cells into $CD83^+$ mature DCs. Once conception occurs, the decidual DCs can be divided into a major population of $CD14^+DC\text{-}SIGN^+$ and a smaller population of the CD83+ mature DCs (63).

DCs, especially the DC-SIGN+ ones have been reported to induce T regulatory cells which is likely to improve maternal tolerance to fetal antigens, and the lack of such induction of T regulatory cells can underly pregnancy complications such as pre-eclampsia (77). $DC\text{-}SIGN^+$ cells are also known to release IL-15 during decidualisation (78, 79) which can recruit decidual NK cells to the endometrium (80) and upregulates CD56 expression (81), which we know is essential for a successful healthy pregnancy.

With regards to the onset of labour and PTB, there is little to no work on the role of decidual dendritic cells in the onset of labour. This is partly due to the difficulty of decidual mononuclear cell isolation and the rarity of decidual DCs. In a mouse model, there was some suggestion of decidual DCs activation when LPS was injected into T and B cell-deficient mice ($Rag1^{-/-}$) to induce preterm birth (82). Further work is necessary to understand decidual DCs and their role in human pregnancy and labour.

1.7 Granulocytes

Granulocytes are made up of neutrophils, basophils, eosinophils and mast cells (17). Neutrophil and mast cells are probably the most relevant to the decidua and onset of labour.

1.7.1 Neutrophils

Neutrophils are the most abundant white cells in the human circulation and are commonly considered as the first line innate immune defence against infection (83). They play a crucial role in immune defence against bacteria and fungus as well as participate in developing an

inflammatory reaction (84). They are characterised by a multi-lobed nucleus, granular cytoplasm and short lifespan which is because they classically die while undertaking antimicrobial function (83), however decidual neutrophils have a longer lifespan due to exposure to cytokines such as GM-CSF, IFN-y and TNF-α (85) *(86)*.

In blood the most abundant granulocytes are neutrophils, and this is reflected in the decidua too, where their frequency increases steeply from the 1st to the 2nd trimester of pregnancy (87) (88). Neutrophils are known to rapidly increase in damaged tissues, as they are usually the first cells to be recruited at inflammatory sites, where they can capture and kill microbes (89). Decidual neutrophils exhibit an activated phenotype (88) and are probably in waiting to counteract local infections such as those in amniotic fluid (90).

With regards to decidual neutrophils, it is known that they are of maternal origin and are present in the decidua from the first trimester (91, 92). Although neutrophils are historically considered to be inflammatory, they can also adopt the ability to regulate and maintain tolerance. They can secrete anti-inflammatory mediators like arginase-1 and IDO and promote homeostatic functions (93) (88, 94). Equally to counter these observations, improper activation of neutrophils can lead to tissue damage during an autoimmune or exaggerated inflammatory reaction (84)((95). Little is still known about the neutrophil population that exist in the decidua, particularly in the third trimester and whether they can convert to the more proinflammatory phenotypes as a result of exposure to inflammatory mediators or to physiological triggers resulting in PTB or term labour respectively. Interestingly it has been shown that a lower frequency of neutrophils was detected in pathological decidual samples and when isolated that they are of an activated phenotype with increased resistance to apoptosis (92).

It is known that neutrophils can interact with decidual innate lymphoid cell 3; it is possible that this interaction leads to neutrophil recruitment to the decidua with activation of the immunoregulatory factors (HB-EGF and IL1Ra) (96, 97). Additionally, NK cells have been shown to communicate with neutrophils resulting in a range of outcomes including neutrophil activation, expression of activation markers, production of cytokines and angiogenic factors (86, 98, 99) and even inducing neutrophil apoptosis (100).

1.7.2 Mast cells

Mast cells, another subset of granulocytes is well known to play a pivotal role in Ig-E dependent allergic diseases such as asthma. They release several vasoactive mediators, diverse proteases, chemokines and cytokines (101). It has been reported that mast cells exist in the human uterus, however the role that they play in human pregnancy is not well understood. Mouse model studies have indicated that mast cells are involved in implantation, placenta formation and uterine contraction (102, 103). *In vitro* studies have revealed that mast cell lines promote migration (104) and tube formation (105) of trophoblast cells via secretion of proteases called chymases or by KIR2DL4-dependent interactions, implying a role for mast cells in trophoblast invasion and spiral artery modelling. Mast cells may play a small role in labour as they undergo phenotypical changes, however this role is certainly not pivotal (106). This was further supported by Needham *et al* who confirmed that mast cells do not play a significant role in spontaneous preterm labour (107).

1.8 Adaptive immune system

The adaptive immune system differentiates itself from the innate immune system due to its antigen specificity and immunological memory (108). T cells make up a large part of the adaptive immune system, playing a significant role in feto-maternal immune tolerance throughout pregnancy (109). Gomez-Lopez *et al* have shown that circulating T cells are drawn into the maternal-fetal interface prior to labour and during term labour (110, 111).

T cells make up 10-20% of decidual leukocytes in the 1st trimester of human pregnancy (112) and continue to expand steadily until onset of labour and delivery (113). T cells, that express the remarkably diverse αβ T cell receptors are the most abundant subpopulation which includes CD8+ T cells, CD4+ T cells and T regulatory (Treg) cells (114). Approximately 30–45% of decidual T cells are CD4^{+} T cells, and 45–75% are CD8^{+} T cells, and of the CD4+ T cells(115), 5% are CD25^{hi} FOXP3^{+} Treg cells (116).

1.8.1 CD8+ T cells

CD8+ effector T cells are classically the cytotoxic decidual T cells. They have the potential to recognise fetal antigens and destroy trophoblast cells both directly (via HLA-C on extravillous trophoblasts) and indirectly (via maternal antigen presenting cells) (117). To be devoid of this it has been demonstrated that CD8+ T cells in human decidua express higher levels of immune checkpoint receptors Tim-3 and PD-1 than CD8+ T cells in peripheral blood. These receptors recognise galectin-9 and PD-L1 respectively on extra villous trophoblasts which send out inhibitory signal, enabling trophoblast antigen-specific tolerance (118). Another study also showed that there was an increased percentage of virus-specific CD8+ T cells in the decidua, which implies that there may be a deliberate accumulation of memory T cells at the maternal-fetal interface to protect the fetus from infection (119).

Although CD8+ T cells are commonly cytotoxic, there has been a subpopulation of HLA-DR+ CD8+ T cells, that has a regulatory role in the context of tumours (120) which have also been found present in the decidua ma(121). Much more work needs to be undertaken to begin to understand this subpopulation's contribution to immune tolerance within the decidua.

1.8.2 CD4+ T cells

CD4+ T cells can be divided into three effector subsets: Th1, Th2 and Th17 (122). Th1 cells are mainly involved in encouraging the eradication of virus-infected cells and intracellular pathogens within peripheral tissues (57). They are identified by the transcription factor T-bet and their signature cytokine is IFN-γ, along with TNF-α which are anti-viral and pro-inflammatory (123). Th1 cells are consequently seen as immune cells that cause poor pregnancy outcomes with potential threats to the fetus (123). Anti-inflammatory Th2 cells, identified by transcription factor GATA3, secrete IL-4, IL-5, IL-6, IL-10, and IL-13 and a bias towards Th2 cells could provide a pregnancy protective, less embryotoxic state. This Th2 bias is promoted further by the placenta which produces progesterone and IL-4 which are known to promote Th2 responses (124). Additionally, Nancy and Erlebacher *et al* showed that Th2 cytokines could quell Th1 cell differentiation and function (116). Th17 cells, identified by transcription factor RORγt, are characterised by unique signalling pathways and are involved in host defence against bacteria, fungi and viruses (123). They secrete IL-17 along which is a pro-inflammatory cytokine, and it has been shown that decidual stromal cells promote

proliferation and invasion of human trophoblast cells by recruiting Th17 cells to the decidua (125). Th17 cells have been shown to accumulate in the decidua (126) and are higher in number in pregnancies complicated by chorioamnionitis than not (127). This suggests that adaptive immune cells may play a role especially at the maternal-fetal interface in the complex pathophysiology of preterm labour. It is also known that the percentage of Th17 cells increase in peripheral blood as gestation advances, which may indicate that they have a role in preparing for the onset of term labour (128).

Both CD8 and CD4 effector T cells within the decidua have been shown to demonstrate a unique Th1 pattern of high level of IFN-γ expression along with reduced levels of cytolytic mediators perforin and granzymes (129). This was confirmed by Powell *et al* who extended these findings further by demonstrating that decidual effector cells had increased expression of IL-4 compared to those in peripheral blood (130), and IL-4 is a signature cytokine for Th2 differentiation (131).

1.8.3 Regulatory T cells

Regulatory T cells (Tregs) are a unique subset of CD4+ T cells that express high levels of the IL-2 receptor alpha chain CD25 (CD4+CD25^{high}CD127^{dim}). Their function is controlled by X chromosome-encoded transcription factor Foxp3 (55). CD4+ Tregs can originate in the thymus, commonly known as natural Tregs or in the periphery (induced Tregs) (55). Induced Tregs are derived from naïve CD4+ T cells in response to antigen stimulation, or exposure to TGF-β (132) and IL-2 (57, 133). In human pregnancy, Tregs grow from 5% in early pregnancy to 20% of all decidual CD4+ T cells in the third trimester (116, 130). Originally, this was thought to be due to induced Treg expansion in the decidua (77), however a more recent study has suggested that over 90% of decidual Tregs are natural Tregs (134). It is possible that induced Tregs are necessary in early pregnancy and natural Tregs may be important in late pregnancy to maintain a healthy pregnancy, further work is required to confirm this theory (117).

Tregs are potent immunosuppressors; they limit inflammation and maintain immune homeostasis by suppressing the activity of other immune cells. They prevent autoimmune

diseases by downregulating the immune response, whilst facilitating efficient elimination of pathogenic organisms and minimising its tissue damage (55, 135).

Tregs play a significant role in the success of a healthy pregnancy, beginning in early pregnancy where it has been shown in a mice model that a reduction in Tregs globally (placenta, decidua and peripheral blood) led to increased embryo resorption, increased pro-inflammatory cytokines and abnormal uterine artery function. This indicates that Tregs play a crucial role in normal placental and subsequently fetal development (136). Tregs have also been shown to induce angiogenesis via release of IL-10 (125) and IL-17 (137) which promotes vessel development and trophoblast invasion.

Focusing on decidual Tregs, it is known that Tregs increase in the decidua compared to peripheral blood (138, 139). Studies have also shown that pregnancies complicated by recurrent miscarriages have reduced proportions of decidual Tregs (140). Tregs also seem to selectively inhibit the mixed lymphocytes reaction to umbilical mononuclear cells, suggesting selective migration of fetal antigen-specific Tregs to the decidua (139).

The role of decidual Tregs regarding the onset of labour, preterm or term is not fully understood. Shah *et al* have demonstrated that in peripheral blood, fetal cord blood and myometrium, there is altered Treg function with the onset of labour in humans (141). A very recent study has shed some light on the role of decidual Tregs; it has identified that Tregs are depleted in the decidua of a subset of women who had idiopathic PTB. Interestingly, it also demonstrated that this depletion was not seen in physiological term labour (142).

1.9 Physiological Labour

Clinically labour is defined by a variety of signs and symptoms and is commonly subdivided into three stages - first, second and third. The first stage is defined as the onset of regular uterine activity associated with effacement and dilatation of the cervix, and descent of the presenting part. The second stage is defined from full dilatation of the cervix to delivery of the baby and the final stage is defined from delivery of baby to delivery of placenta (446). Olson *et al* described eloquently that parturition consists of five physiological events: membrane rupture, cervical dilatation, myometrial contractility, placental separation and

uterine involution (143). Although we understand the stages of human labour well, understanding what triggers labour and its progress, is still unknown. Several factors are considered to play a significant role including hormones, immune cells, cytokines/chemokines, mechanical factors, fetal factors and pathology such as infection, bleeding and excessive uterine stretch (144).

Labour is associated with inflammation (15, 145, 146) yet there is no signature inflammatory picture seen in the maternal peripheral blood (10). Subsequently, our group looked at inflammation in the myometrium and confirmed that this is likely to be a consequence, rather than the cause of onset of labour (147). Inflammation has also been demonstrated in the fetal membranes and decidua (148); gaining a better understanding of the inflammatory changes and immune cell communications in the decidua during uncomplicated term labour may give us more of an understanding of preterm birth caused by breakdown of maternal-fetal tolerance (Figure 1.7).

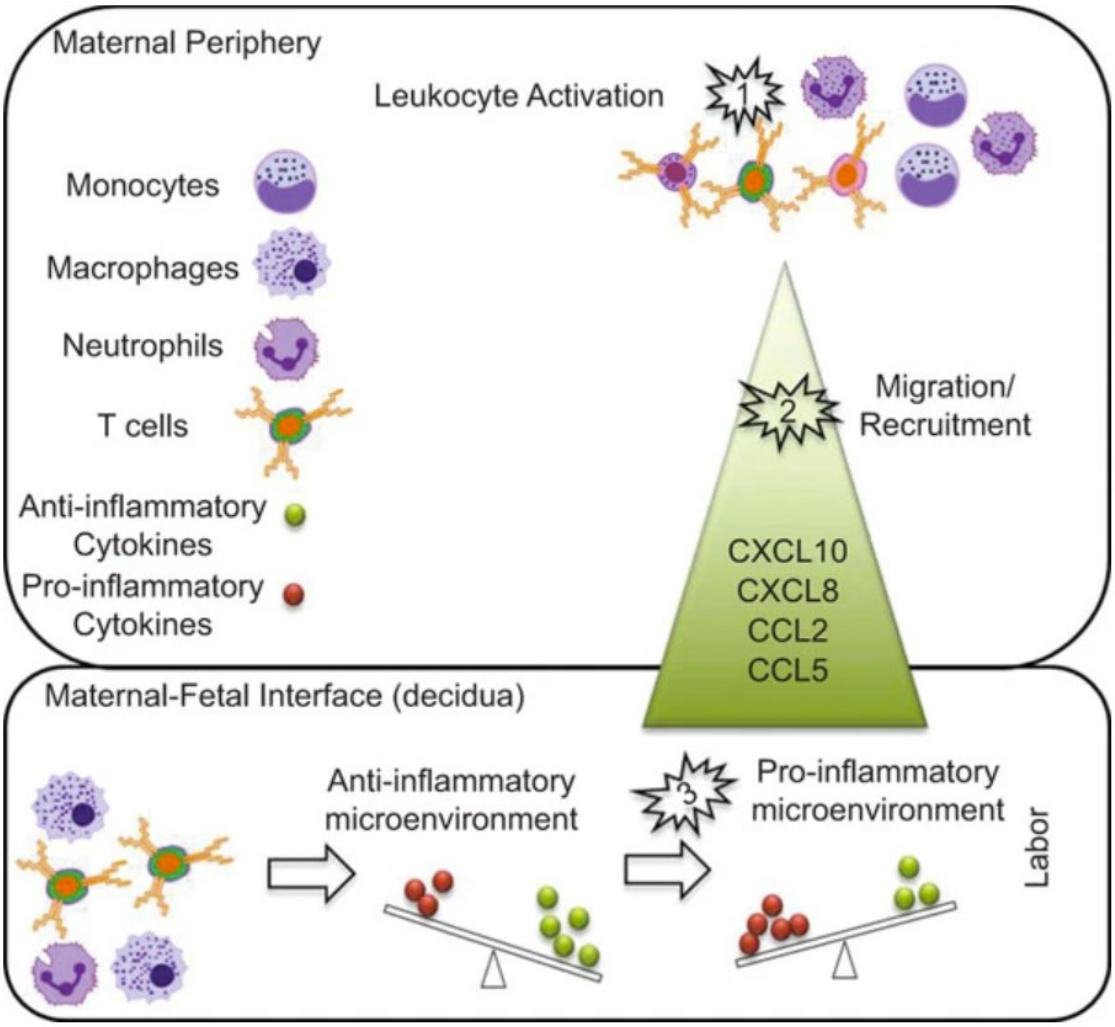


Figure 1. 7 A possible pathway to term or preterm labour (Gomez-Lopez, StLouis, Lehr, Sanchez-Rodriguez, & Arenas-Hernandez, 2014) Image reproduced with permission from creative commons attribution license.

It is clear from several studies that both the innate and the adaptive immune system play a role, and in fact work together to maintain an immune balance. The main cells of interest seem to be the NK cells, macrophages and T cells, in particular T regulatory cells and their proportions within the decidua as pregnancy advances is well demonstrated in Figure 1.8. The change in proportions especially with adaptive immune cells via T cells peaking towards the end of pregnancy may be suggestive of their local role in the onset of labour.

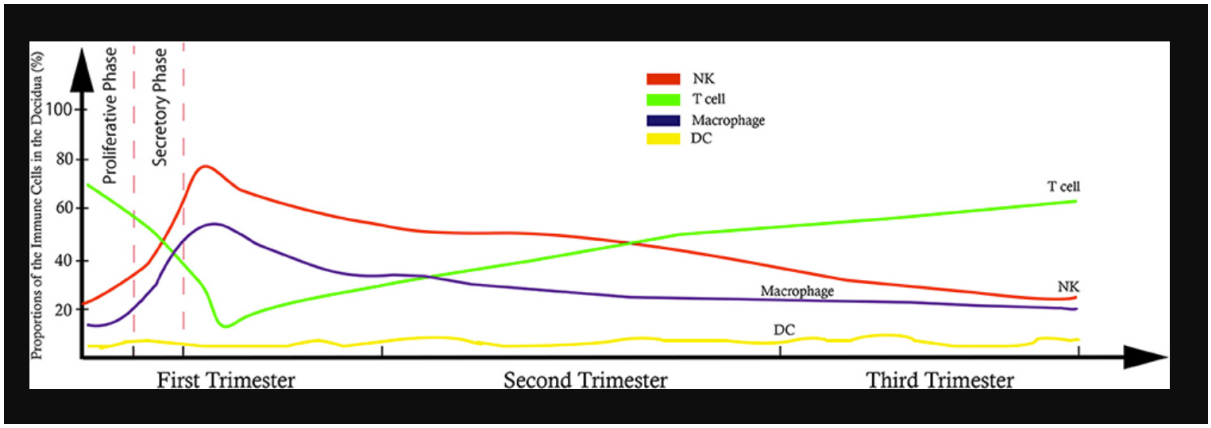


Figure 1. 8 Proportion of immune cells in the decidua (F. Yang et al., 2019). Image reproduced with permission from creative commons attribution license (Frontiers copyright policy).

1.10 Metabolic Adaptations in Pregnancy

Just as the maternal immune system adapts to procure a successful pregnancy and healthy baby, metabolic adaptations are also essential to support the growth and development of the fetus. To put it simply pregnancy changes can be divided into anabolic and catabolic phase (Figure 1.9). The anabolic phase is restricted to the first and second trimester, whilst the catabolic phase to the third trimester (149). Much of the changes, especially in third trimester, have been attributed to hormonal influences.

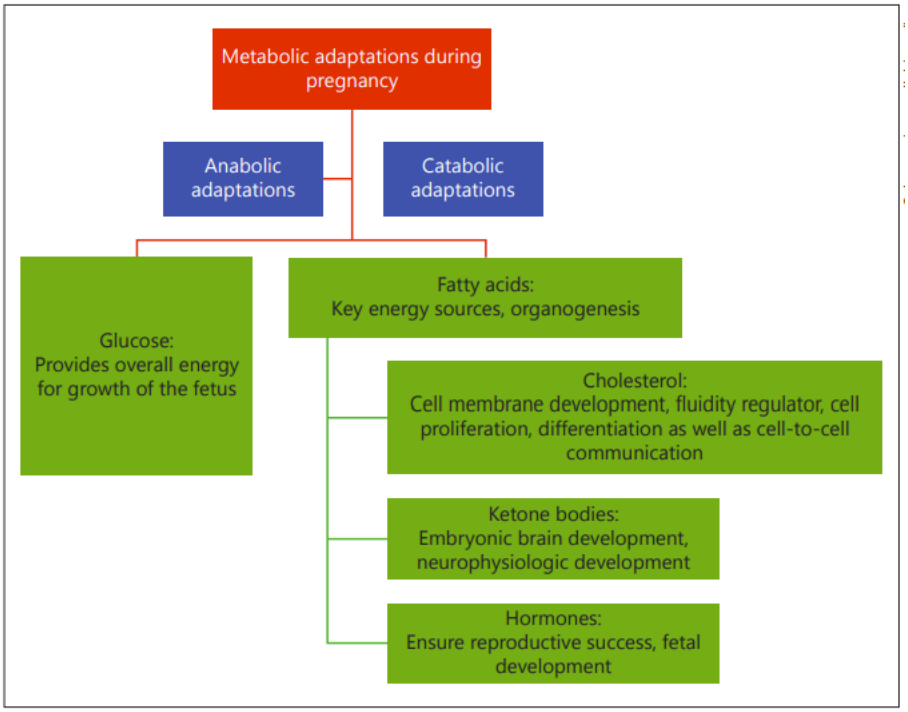


Figure 1. 9 Overview of metabolic adaptations during pregnancy (Zeng et al., 2017). Image reproduced with permission from Karger publishers, Copyright ©2017 Karger Publishers, Basel, Switzerland

Figure 1.9 summarises the overall metabolic adaptations of pregnancy, however what is particularly of interest to this thesis are the metabolic pathways that are important to healthy decidualisation in humans. Six metabolic pathways have been described (Table 1.2).

Table 1. 2 The six metabolic pathways

Metabolic Pathway	Metabolic effect
Glycolysis	• Converts glucose into pyruvate • Generates energy to form ATP and NADH
Tricarboxylic acid (TCA) cycle	• Allows the release of stored energy • Provides the precursors of certain amino acids
Pentose phosphate pathway (PPP)	• Generates NADPH • Diversion of intermediates from the glycolytic pathway towards the synthesis of nucleotide, amino acid precursors, and fatty acids
Fatty acid oxidation (FAO)	• Conversion of fatty acids into products that the cell can further use to generate energy
Fatty acid synthesis	• Generation of lipids necessary for cellular growth and proliferation from precursor cells
Amino acid metabolism	• Anabolic cellular signalling pathways synthesis of proteins and amino acids

Glycolysis is the dominant metabolic pathway in rapidly proliferating and activated cells, which has been shown to be the case in decidual stromal cells ((150-152). Studies have also shown that when glucose metabolism is disturbed during decidualisation, poor pregnancy outcomes are associated(150). Pentose phosphate pathway (PPP) is the other metabolic pathway which has been demonstrated to be involved in decidualisation and inhibition of PPP using dehydroepiandrosterone in human and mouse endometrial stromal cells, prevented decidualisation and implantation (153).

Bringing together both immunology and metabolic pathways is a field of immunometabolism and what is highly interesting is that work unrelated to pregnancy has indicated that distinct immune cell functions are linked to different metabolic pathways (154).

Glycolysis, the dominant metabolic pathway has been reported to be increased in activated immune cells such as NK cells (155), DCs (156), and T cell subsets (157). Glycolysis is also involved in the initial generation of Tregs (158-160). Glycolysis produces enough energy

required for certain immune functions such as phagocytosis, cytokine production and antigen presentation (161).

TCA is present in most quiescent or non-proliferative cells. They play a role in most T-cell subsets especially CD8+ T cells (162). PPP on the other hand is essential for the growth and proliferation of cells and are widely present in macrophages, neutrophils and DCs. A by-product of PPP is nicotinamide adenine dinucleotide phosphate (NADPH), which is used by macrophages and neutrophils to both clear pathogens during an infection but also limit tissue damage by inducing antioxidants (161).

Fatty acid oxidation (FAO) is the fourth metabolic pathway and is the pathway of choice for tolerogenic and non-inflammatory cells. FAO helps to promote generation of Tregs and inhibits effector T cells, which on whole increases immune tolerance (163). Tregs demonstrate increased FAO compared to other T cells, such Th1, Th2 and Th17. FAO has also been linked to the generation of CD8+ T cells (164).

Fatty acid synthesis is the fifth metabolic pathway. It regulates both innate and adaptive immune cells involved in inflammatory response (161). Fatty acid synthesis is increased in antigen presenting cells when subjected to inflammatory stimuli (165). It is also known that saturated fatty acids may promote the synthesis of pro-inflammatory cytokines, so the type of fatty acid synthesis may control cytokine release from T cells (118).

The last metabolic pathway is amino acid metabolism. Amino acids are the building blocks of proteins in our cells and tissues, and after water are the second most abundant compound in mammals (166). There are 20 known essential amino acids (167) of which the most studied regarding its metabolism and role in immune response are glutamine, arginine and tryptophan (161). Tryptophan metabolism necessitates IDO which is expressed by for example activated DCs. IFN-γ also increases IDO expression in cells, therefore promoting the catabolic metabolism of Tryptophan too (168). It has also been widely recognised that tryptophan derived catabolites inhibit T-cell proliferation (169, 170) and may even inhibit NK cells proliferation (171).

Glutamine is known to be consumed by all immune cells at a rate similar to or greater than glucose during infection and or high catabolism (172). Neutrophils consume glutamine at the

highest rates (173) where it is converted to glutamate, aspartate (via Krebs cycle activity), and lactate (166).

The metabolic pathways summarised above and detailed in Figure 1.10, relating to immune cells represent just a simple summary, these pathways are not mutually exclusive nor restricted to an individual immune cell subset. It is well established that immune cells can adapt their functional role, therefore their metabolic properties too.

Inflammation associated with labour is linked with certain immune cell subsets, in particular monocytes, NK cells, T cells, dendritic cells and neutrophils (Figure 1.7). Activation of these cells require energy, which is mostly from glycolysis (161) and it has been shown that when there is disturbed glucose metabolism during decidualisation, there are adverse reproductive outcomes such idiopathic subfertility (161), recurrent miscarriages (174) and pre-eclampsia (175). Furthermore, the growing field of immunometabolism has shown us that metabolic reprogramming of immune cells is essential for both inflammatory as well as anti-inflammatory response (176). This emphasises the importance of appropriate metabolic adaptations to maintain an immunological quiescence. It also supports an emerging concept whereby you repolarise immune cells towards a less inflamed phenotype by manipulating its metabolism using small molecules (176).

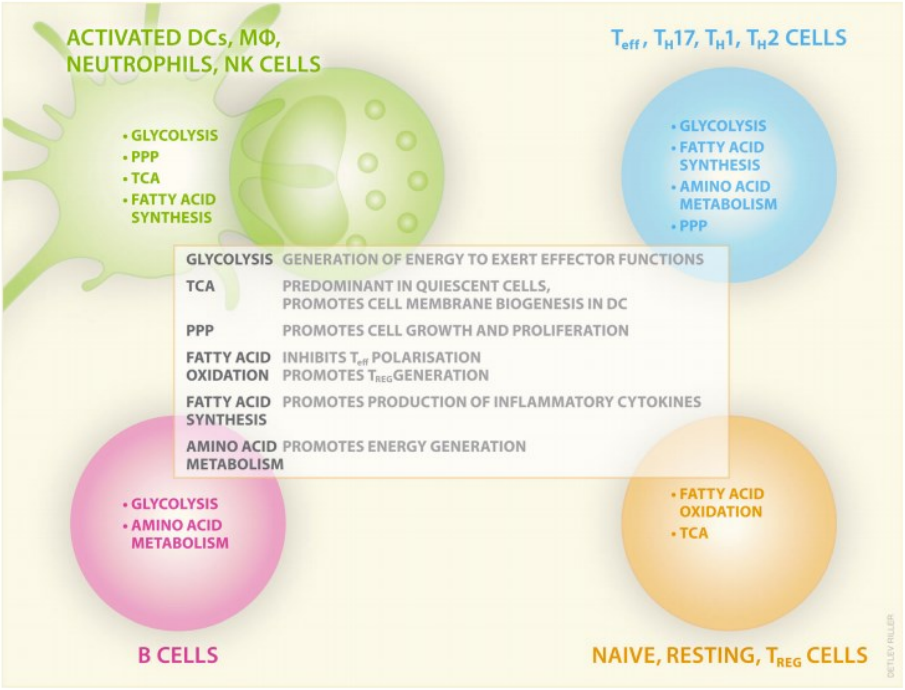


Figure 1. 10 Overview of metabolic pathways described in distinct immune cells subsets, such as activated DCs, macrophages, neutrophils, NK cells, effector T cell subsets, naïve, and resting and regulatory T cells, as well as B cells (Thiele et al., 2018). Image reproduced with permission from Springer nature.

1.11 Urinary metabolites and Pregnancy

Recognising the onset of labour remains one of the greatest challenges in obstetrics and it still forms the basis of a vast amount of scientific research. As mentioned above metabolites and immune cells can be related and the study of metabolites via metabolomic studies may give us an insight into the pathogenesis of physiological conditions such as parturition. Metabolomic studies have been conducted on various tissues and fluid (177, 178), but one that is a common choice of study is urine. Urine has been proven to be a useful tool for the diagnosis of physiologic and pathologic human conditions by providing the underlying metabolic changes (179-181). It is also ideal as a possible clinical investigation in development as it is non-invasive, safe to both mother and fetus and easy to collect. It can be superior to blood which can only provide a snapshot whereas a continuous collection of urine over a period would give a more integrated picture (182). Further, the human metabolome database describes at least 4730 metabolites identified in urine (442).

Metabolomics is a relatively young branch of "omics" science, however over the last few years it has evolved considerably. A major challenge however still exists for the identification of metabolites and validation of metabolites in human populations. It is vital to identify biological meaning to the metabolites in order to understand and piece together the mechanisms of disease (183).

Studies undertaken during second trimester have shown some differences in urinary metabolites in patients who deliver pre-term, however due to small sample numbers, it was insufficient to create a predictive model. The only metabolite that was noted to be significantly decreased in second trimester was citric acid (184). Citric acid is formed via the TCA cycle which is known to a play role in quiescent cells such as Tregs (Figure 1.10).

Urinary metabolites have been studied in pregnancy to a certain extent in various trimesters. Caboni *et al* looked at urinary metabolites at term gestation and in labour at term, identifying potentially 18 discriminate metabolites. These included alanine, glycine, glucose, lactic acid

and creatine which were down-regulated whilst 3-hydroxybutiyric acid, acetoacetic acid and acetone were up-regulated (185). These metabolites on the whole suggest that the pathway that is most relevant are ketone bodies, and ketosis is plausible in labour, where there are increased energy requirements. Glycine was the only discriminant metabolite that was reduced both on GC/MS and NMR analysis with the onset of term labour, which is interesting as this amino acid is recognised to be an anti-inflammatory and immunomodulatory agent (186). There is also evidence that glycine inhibits production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (187), so lack of glycine may support the inflammatory switch that is required for the onset of labour.

Meloni *et al* compared urinary metabolites of patients with preterm rupture of membranes (PROM) at term to patients with intact membrane with and without labour (188). This study highlighted that discriminatory metabolites were noted only really with the change in labour status, and interestingly PROM *per se* did not affect the urinary metabolites much. In general, several metabolites decreased in the urine including succinic acid, alanine, glutamine, citric acid, 2,4-dihydroxybutyric acid, creatinine to name a few, however only three urinary metabolites were significantly raised with labour: phosphate, lactose and uric acid (188). The same group noted when comparing preterm PROM with term PROM that lactic acid, erythritol and ethanolamine levels were higher in the preterm PROM patients. This may be indicative of bacterial infections as these three metabolites are derived from bacterial processes (189).

1.12 Summary and Clinical relevance

Labour is associated with inflammation, yet understanding its onset remains a challenge in clinical medicine and explains the lack of effective therapeutic interventions. The role of immune cells especially at the maternal-fetal interface, the decidua, may be key to understanding what tips the immune-tolerance maintained in pregnancy into an inflammatory cascade resulting in parturition. Understanding these processes at term may give us more insight into preterm birth due to the breakdown of maternal-fetal immunotolerance. This understanding may help us realise the Government's ambitious target to reduce the PTB rate from 8% to 6% in the UK. This understanding will hopefully build a foundation on which we can develop predictive biomarkers and effective therapeutic interventions, both are essential if we are to reduce the PTB rate and its consequences.

1.13 Hypotheses and aims

1.13.1 Hypotheses

This thesis investigates the role of the immune system in term and preterm labour by testing the following hypotheses:

I. Choriodecidual inflammatory changes participate in the initiation of labour and may be a cause of labour, so are a potential target of treatment to prevent PTB.

II. Higher concentrations of cytotoxic NK cells and lower concentrations of Treg cells are observed in the decidua in pregnancies complicated by preterm delivery compared to both non-labour controls and term labour controls.

III. Urinary metabolites will change closer to the onset of labour at term in low-risk pregnancies mirroring immune cells changes in the decidua.

1.13.2 Objectives

I. To understand the inflammatory changes seen in both choriodecidua and placenta in preterm birth due to different causes.

II. Identify which immune cell populations and proportions of these cells are present in four compartments – decidua, placenta, maternal and cord peripheral blood mononuclear cells and how they vary with different pregnancy outcomes.

III. To identify which urinary metabolites increase or decrease as pregnancy advances and the onset of labour is reached, and to which metabolic pathways these metabolites relate to.

2. Material and Methods

2.1 Study subjects

The framework of project methods for the three main studies of this PhD thesis are detailed in Figure 2.1 below.

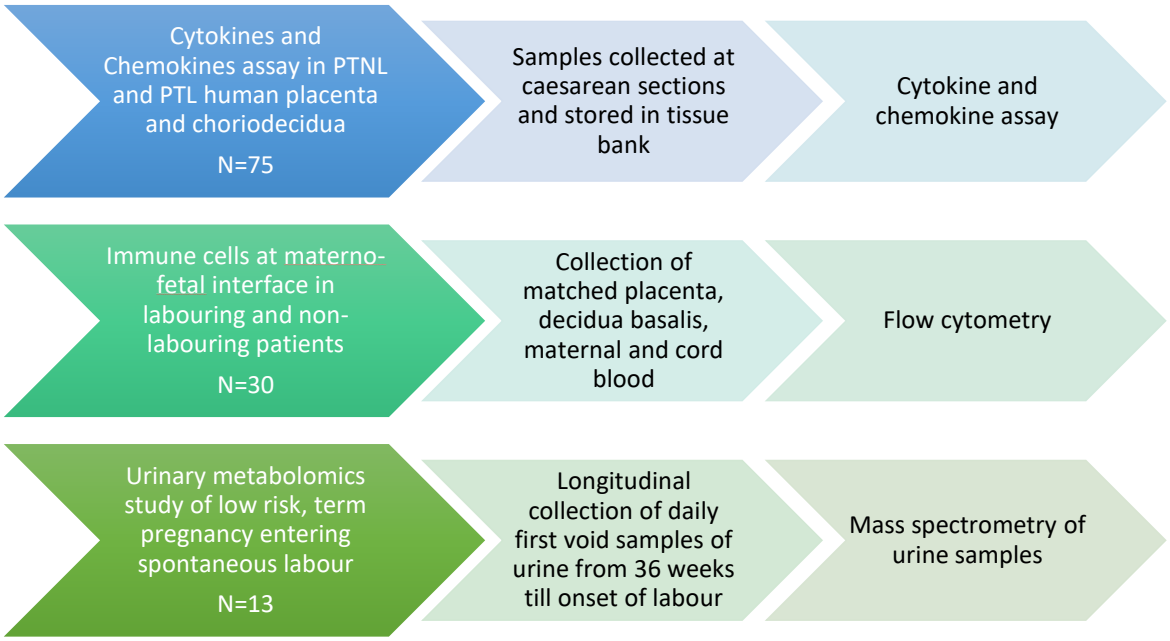


Figure 2. 1 Thesis methods framework for the various studies

All samples collected for this thesis were from patients booked and delivered at Chelsea and Westminster Hospital. They all were counselled and appropriately recruited with written informed consent taken. In the longitudinal studies, patients were continually, verbally consented during each collection of urine samples, which was usually on a weekly basis. In view of the complex changes to a woman's immune system during pregnancy, the decision was made to use term or preterm non-labouring patients as controls to compare the changes in immune cells associated with the onset of labour and various preterm labouring subtypes, respectively. Prior to working independently in the laboratory, specific training and observation by both postdoctoral researchers and supervisor Dr Nesrina Imami was completed. Following completion and assessment, authorisation was granted to perform experimental work in a containment level II laboratory. Table 2.1 summarises the pertinent details of each study, including the inclusion and exclusion criteria, which experimental

procedures were undertaken, and which collaborators were involved in the respective studies.

Table 2. 1 Summary of study inclusion and exclusion criteria, experimental procedures and whether conducted by PhD student or collaborators.

Study	Description	Methods conducted by PhD student	Conducted by others
Chapter 3 – Experimental Study 1 Cytokine and Chemokines in Preterm labour subtypes	Cytokines and Chemokines assay in PTNL and PTL human placenta and choriodecidua N=75 preterm Exclusion and inclusion criteria described in detail in Chapter 3 based on each PTL subtype	Thawing and preparation of both placenta and choriodecidua samples from tissue bank Protein extraction Protein assay Multiple cytokine and chemokine assays Collection of neonatal outcomes of babies born in the chorioamnionitis PTL group and correlation to cytokine/chemokines	Consenting, collection and storage of placentae and choriodecidua at caesarean sections (Ms Natasha Singh and research team) Dr Enitan Ogunidipe (Consultant Neonatologist) for helping with collation of neonatal clinical details

Chapter 4 – Experimental study 2 Immune cells in term and preterm gestation, with the onset of labour	Immune cells at maternal-fetal interface in labouring and non-labouring patients, at both term and preterm gestations N= 30 Inclusion criteria: singleton pregnancy, caesarean deliveries Exclusion criteria: spontaneous rupture of membranes, induction of labour, syntocinon augmentation Early labour was defined as cervical dilatation ≤3cm	Consenting, collection and storage of placentae choriodecidua, maternal and cord blood at caesarean sections Development and optimisation of standard operating procedure for collection and preparation of decidual leucocytes and separation Development and optimisation of flow cytometry panel including titration of antibodies Staining of samples and acquiring samples on flow cytometer	Consenting and separation of some maternal/cord blood samples assisted by Dr Alexander Cocker and Dr AnnieBelle Sassine Set up and optimisation of the BD LSR II as per Perfetto protocol by Dr Alexander Cocker

Chapter 5 – Experimental study 3 Urinary metabolomics and onset of labour	Longitudinal study of daily first void urine samples from 36 weeks till onset of labour (N=13) Inclusion criteria: singleton pregnancy, low risk pregnancy (i.e., no significant medical condition) Exclusion criteria: maternal age >45 years, gestational diabetes mellitus, diabetes, pre-eclampsia and BMI>35, induction of labour	Consenting low-risk patients for longitudinal study Provision of freezer if required for storage of samples Weekly collection of urine samples from patients' home on dry ice and storage at -70^{0}C Thawing, preparation and aliquoting of urine samples (N=400) as per protocol for mass spectrometry Transfer of samples on dry ice to MRC-NIHR National Phenome Centre, Imperial College London	Mass spectrometry undertaken by Dr Frances Jackson, Postdoctoral researcher, MRC-NIHR National Phenome Centre, Imperial College London Data analysis undertaken by Dr Jia Li, Reader, Faculty of Medicine, Imperial College London
All studies	Statistical analysis using GraphPad and STATA	Data entry and cleaning Conducting statistical analysis	Dr AnnieBelle Sassine helped with statistical analysis

2.2 Materials

2.2.1 Reagents

Commercially available reagents used in this thesis are listed below in Table 2.2.

Table 2. 2 Commercially available reagents

Materials/Reagents	Supplier	Catalogue number
Accutase® solution	Sigma-Aldrich	A6964

Cell strainer 70µm	BD Biosciences	352350
EDTA 0.5M	Sigma-Aldrich	200-494-4
FACS tubes	BD Biosciences	352063
Fetal calf serum	Sigma-Aldrich	MFCD00243108
GentleMACS dissociator	Militenyi Biotec	130-093-235
GentleMACS M tubes	Militenyi Biotec	130-096-335
Histopaque	Sigma-Aldrich	H8889-500ml
KOVA glastic slide	Hycor Biomedical Inc	HBI-87157
Lithium heparin vacutainer	BD Biosciences	362753
Pasteur pipette	Appleton Woods Ltd	KC243
Petri dishes 60mm	Corning	391-1917
2% PFA (FACS Fix)	BD Cytofix™	554665
Phosphate buffered saline (Ca+, Mg+ free)	Sigma-Aldrich	MFCD00131855
Trypan blue solution	Sigma-Aldrich	MFCD00003969

2.2.2 Antibodies

Tables 2.3 and 2.4 below show primary and secondary antibody panels used throughout this project.

Table 2. 3 Primary antibodies.

Antibody	Supplier	Catalogue number
Mouse Anti-Human CD56 BV786	BD Biosciences	564058
Mouse Anti-Human CD4 BV711	BD Biosciences	563028
Mouse Anti-Human CD8 BV605	BD Biosciences	564116

Mouse Anti-Human CD127 BV421 (HIL-7R-M21)	BD Biosciences	562436
Mouse Anti-Human CD14 FITC Clone TUK4	Militenyi Biotec	130-080-701
Mouse Anti-Human CD15 PE	BD Biosciences	555402
Mouse Anti-Human CD16 APC	BD Biosciences	561304
Mouse Anti-Human HLA-DR PerCP	BD Biosciences	347402
Mouse Anti-Human CD3 APC-H7	BD Biosciences	560176
Mouse Anti-Human CD25 Pe-Cy7 Clone BC-96	BioLegend	302611
Brilliant Stain Buffer	BD Biosciences	563794
Human TruStain FcX	BioLegend	422301
Fixable Viability Stain 510	BD Biosciences	564406

Table 2.4 Secondary antibodies.

Secondary Antibodies	Supplier	Catalogue Number
BV786 IgG2b κ CD56 Isotype Control	BD Biosciences	564090
BV421 Mouse IgG1 CD127 Isotype Control	BD Biosciences	562438
PE Mouse IgM κ CD15 Isotype Control	BD Biosciences	555584
APC Mouse IgG1 κ CD16 Isotype Control	BD Biosciences	554681

2.3 Media and other Solutions

Standard media and solutions were used; all prepared and made up according to manufacturers' instructions and/or standard operating procedures.

2.3.1 FACS Wash Buffer

Five-hundred ml phosphate buffered saline (PBS) supplemented with 5ml fetal calf serum (FCS) and 2ml 0.5M EDTA (all Sigma-Aldrich).

2.3.2 Cell Lysis Buffer

Nine ml dH2O was supplemented with 1ml 10x cell lysis buffer, 1 tablet protease inhibitor, 100µL phosphatase inhibitor and 40µL phenylmethylsulfonyl fluoride (PMSF).

2.3.3 NMR Urine Phosphate Buffer

Potassium dihydrogen phosphate (KH2PO4), 3-trimethylsilyl propionic acid-d4 acid sodium salt (TSP), supplemented with sodium azide (NaN3), and potassium hydroxide solution (KOH 45% w/w), Sigma-Aldrich, Gillingham, UK) (prepared by Dr Frances Jackson, postdoctoral fellow, Imperial College London).

2.4 Kits

DC Protein Assay Kit - BioRad

A simple colorimetric assay for measuring total protein concentration which is based on the Bradford dye-binding method (Bradford 1976).

2.5 Primary cells

2.5.1 Peripheral Blood Mononuclear Cells Collection and Handling

Chapter four discusses the immune cells present and their proportions in decidua, placenta, PBMC and cord blood obtained from patients with preterm and term delivery, with or without the onset of labour. 30 number of blood samples were collected from the patients in the operating theatre by the anaesthetist at the same time as cannulation. Maternal blood was collected into two 10ml lithium heparin vacutainers (BD, Berkshire, UK), and was kept at room

temperature and processed within 4 hours. All blood samples were overlayered on to a Ficoll-Hypaque gradient for density gradient centrifugation (190). Whole blood was gently layered onto Histopaque (Sigma-Aldrich) at 1:1 ratio in a sterile 50 mL tube and centrifuged at 2000 revolutions per minute (RPM) (800 relative centrifugal force (RCF)) for 20 minutes at room temperature, with no braking at deceleration, to separate the blood components into layers (Figure 2.2). The mononuclear layer was recovered and washed once in 40ml PBS and centrifuged at 1800 RPM (700 RCF) for 10 minutes. The supernatant was discarded, and the cells were re-suspended in 50ml of PBS for counting.

2.5.2 Cord Blood Leucocytes Collection and Handling

Chapter four discusses the immune cells present and its proportions in decidua, placenta, PBMC and cord blood obtained from patients with preterm and term delivery, with or without the onset of labour. 28 number of cord blood samples were collected from the cord immediately after delivery into one 10ml lithium heparin vacutainers (BD). They were kept at room temperature and processed within 4 hours. All cord blood samples were overlayered on to a Ficoll-Hypaque gradient for density gradient centrifugation. Whole blood was gently layered onto Histopaque (Sigma-Aldrich) at 1:1 ratio in a sterile 50 mL tube and centrifuged at 2000 rpm (800 RCF) for 20 minutes at room temperature, with no braking at deceleration, to separate the blood components into layers (Figure 2.2).

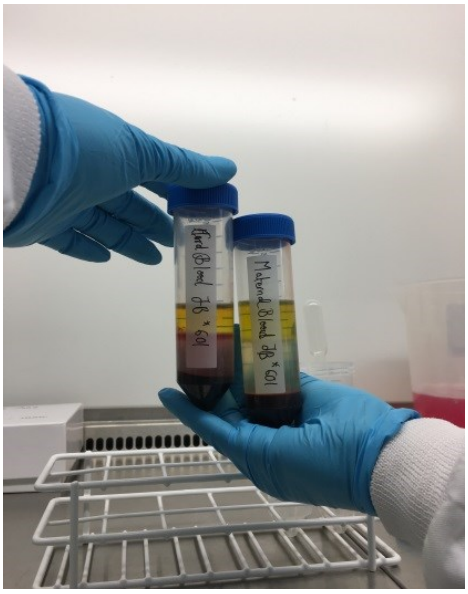


Figure 2. 2 Maternal and cord blood processed using density gradient centrifugation.

The mononuclear layer was recovered and washed once in 40ml PBS and centrifuged at 1800 rpm for 10 minutes. The supernatant was discarded, and the cells were re-suspended in 50ml of PBS for counting.

2.5.3 Cell counting

Twenty µl of cell solution was added to 20µl of Trypan blue dye (Sigma-Aldrich) and 10µl pipetted into a KOVA Glasstic® slide (Hycor Biomedical Inc, Edinburgh, UK). Using a microscope, cells were counted in 2 sets of 9 square blocks and the average number calculated. The average was multiplied by 2 to account for the Trypan blue dilution, by the volume of cell solution (V), and by the KOVA chamber factor (F = 10,000) to reach the total cell count. Thus, formula used to determine total number of cells in sample was as follows:
Av x 2 x K x V = T

2.6 Human Tissue

2.6.1 Ethic statement

Written informed consent was obtained from all patients. All tissues were collected under protocols approved by the Chelsea and Westminster Foundation NHS Trust Tissue Bank and the National Research Ethics Committee in accordance with the Human Tissue Act 2004. Approval for this project was granted by the Chelsea and Westminster Healthcare Tissue Bank, under their HTA research licence and ethics this conveyed through this process by the Multi Research Ethics Committee (MREC).

2.6.2 Decidual and Placental tissue Collection, Handling and Storage

Chapter three discusses the cytokine and chemokine levels present in human placental, choriodecidual and amnion tissues from women who had preterm deliveries due to different causes. All procedures involving human tissues were conducted in compliance with the London Chelsea Ethics Committee and the Human Tissue Authority licence. Samples of

choriodecidua, placenta and amnion were obtained from 183 patients by both members of the medical team and research staff under the supervision of Ms Natasha Singh (Consultant Obstetrician) on labour ward at Chelsea and Westminster Hospital after informed written consent by the patient. All tissues were stored at -80^{o}C immediately after collection and separation of each tissue as part of a tissue bank. There were 75 preterm samples in this tissue bank which were subdivided into groups based on clinical indication for delivery. For singleton pregnancies the preterm samples were divided into preterm non-labour (PTNL), chorioamnionitis (CA), placental abruption and polyhydramnios. For the twin preterm deliveries, they were either classified into twin non-labour or twins in labour. Details of these preterm subtypes can be found in chapter 3, table 3.1. Sample size was based on previous work undertaken within our group, specifically on myometrium which proved to be statistically sufficient.

Chapter four discusses the immune cells present and their proportions in decidua, placenta, PBMC and cord blood obtained from patients with preterm and term delivery, with or without the onset of labour. Extensive literature review was undertaken to help identify similar studies to help with formal power size calculations. There was only two similar studies that were relevant (174, 191) (192) and from that limited information a sample size of 6-10 per group was set, with an aim to recruit above 15. All procedures involving human tissues were conducted in compliance with the London Chelsea Ethics Committee and the Human Tissue Authority licence. Decidua basalis and placental tissue were collected from women undergoing both elective and emergency caesarean sections at Chelsea and Westminster Hospital (London, United Kingdom) following written informed consent by the patient. Exclusion criteria for recruitment included multiple pregnancy, induction of labour, augmentation of labour and vaginal bleeding. The tissue was collected immediately after delivery of the placenta by me and Dr AnnieBelle Sassine (PhD student) in labour ward, Chelsea and Westminster Hospital and processed within two hours of delivery. Decidual basalis (DB) tissue was collected centrally from the maternal aspect of the placenta (Figure 2.3), and matched placental tissue was collected directly beneath the DB biopsy site.

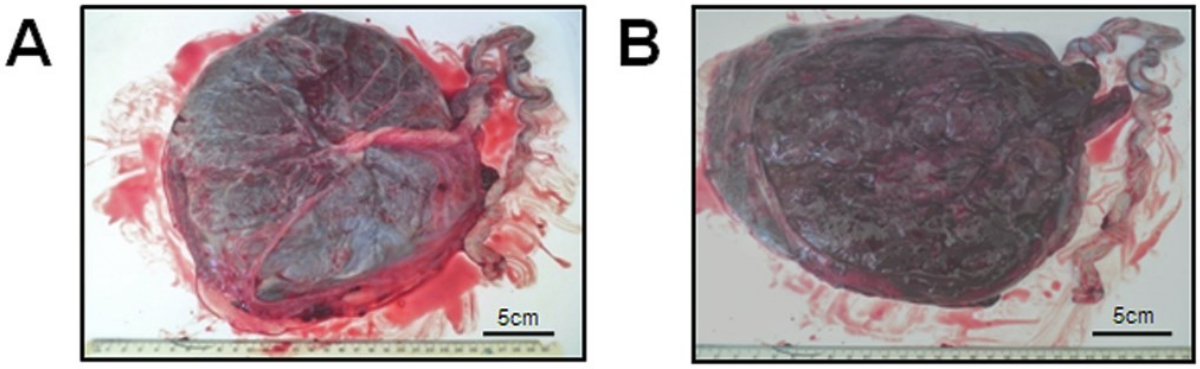


Figure 2. 3 The placenta – A. Fetal side of a term placenta, B. Maternal side of a term placenta. Image published with permission of the rights holder ((193). under the terms of the Creative Commons Attribution Licence. © Warrander et al 2021.

The DB was lifted of the maternal placental surface, undermined, and removed in small pieces using tissue forceps and scissors (Figure 2.4)

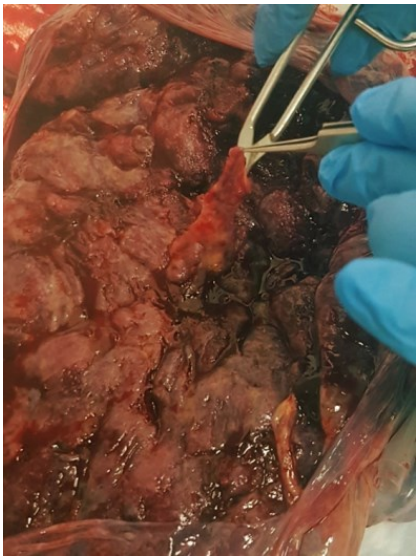


Figure 2. 4 Decidua basalis being lifted off the maternal aspect of the placenta using forceps and scissors.

Placental tissue approximately 1x1x1cm size was cut away directly below the decidual biopsy site. Excess blood was removed from the tissues using sterile gauze and then the tissue was placed in 20ml PBS in a sterile FACS tube. Once samples were back in the laboratory villous tissue and blood vessels were further removed from the DB to maximise purity of the maternal-fetal interface (Figure 2.5).

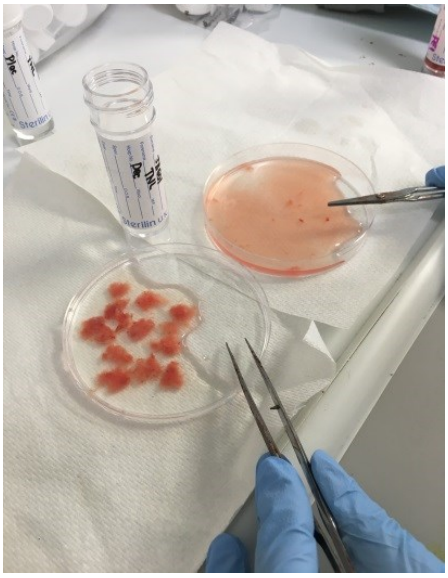


Figure 2. 5 Optimisation of decidua basalis by removal of villous tissue and blood vessels using tissue forceps. To remove any remaining surface blood DB is further washed in PBS. Both DB and placenta were repeatedly washed in PBS on petri dishes to further remove blood. The tissues were then processed within two hours for flow cytometric analysis.

2.7 Human Tissue Digestion

2.7.1 Decidual and Placental Tissue Digestion

After the tissue has been thoroughly washed and processed as shown in Figure 2.3 the optimised DB and placenta are placed in 50ml of PBS in a FACS tube. This is then centrifuged at 1200rpm (300 RCF) for 5 minutes at room temperature in order to form tissue pellets. The supernatant located above the tissue pellets are carefully aspirated without disturbing the tissue pellet. The next step was focused on disaggregating the tissue which is a two-part process. To initiate mechanical disaggregation the tissue pellets were placed in a cell detachment solution which is a cocktail containing proteolytic and collagenolytic enzymes (Accutase®, Sigma-Aldrich) which was pre-warmed to 37^0C at a ratio of 1:2 of cell pellet volume: Accutase®. This combination of tissue pellet and cell detachment solution was transferred to a GentleMACS M tube and placed in the GentleMACS tissue dissociator (Militenyi Biotec) for a one-minute mechanical disaggregation on the spleen setting (Figure 2.6).

Figure 2. 6 The GentleMACSTM Dissociator is a benchtop instrument for the semi-automated dissociation of tissues into single-cell suspensions or through homogenates. Two samples could be mechanically disaggregated in parallel which was ideal for my work on both placenta and decidua basalis.

Once the mechanical disaggregation is complete, the second part of enzymatic disaggregation of the tissue is continued. This is done by placing the GentleMACS M tube containing the tissue and the Accutase® at 37°C for 45 minutes with gentle agitation every 10 minutes. The digested tissue was then passed through a 70µm cell strainer and suspended in 20ml FACS wash buffer (FWB). This was then gently layered onto 20ml Histopaque (Sigma-Aldrich) at 1:1 ratio in a sterile 50 ml FACS tube and centrifuged at 2000 rpm (800 RCF) for 20 minutes at room temperature, with no braking at deceleration, to separate the tissue components into layers. Leucocytes are found in the interface between the density gradient media and the FWB. This mononuclear layer was carefully aspirated using a plastic Pasteur pipette and washed once more in 40ml FWB and centrifuged at 1800rpm for 10 minutes at room temperature. The supernatant was discarded, and the cells were re-suspended in 5ml of FWB for counting.

2.8 Cytokine/Chemokine Assay

Decidual and placental tissue that was originally stored at -70^{0}C were thawed overnight. Protein lysates were produced by lysing the tissue with BioRad Cell Lysis Buffer supplemented

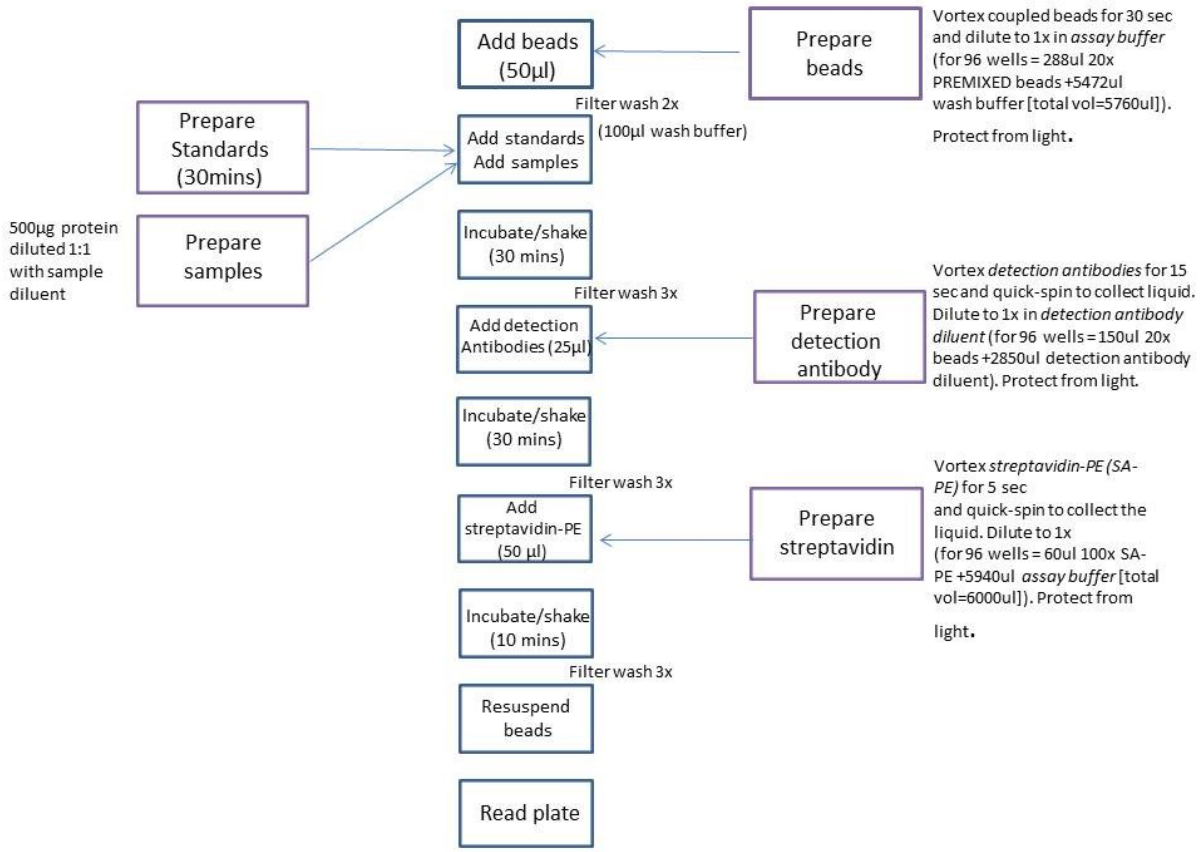


Figure 2. 7 The Bio-plex workflow followed to undertake the cytokine/chemokine assays as per manufacturer's instructions.

with 2mM PMSF and BioRad QG solution according to the manufacturer's instructions. Detailed workflow is shown in Figure 2.7 below.

The tissue was homogenised via four, 20 second cycles at 5000rpm using a Precellys homogeniser. Tissue media was separated from cell debris through centrifugation at 13,000 x g (20 minutes at 4˚C). The protein concentration was then determined by a DC™ Protein Assay (BioRad, Hemel Hempstead, UK). A Human Bio-Plex© Pro™ cytokine assay assessing 19 cytokines/chemokines (Table 2.5) and Human Bio-Plex© Pro™ Human Inflammation panel 37-plex (Table 2.6) panel was performed as per manufacturer's instructions (Figure 2.7). Due to buffer incompatibility, an additional separate Bio-Plex® CCL5 single-plex assay was performed for both placenta and choriodecidual tissue. 500µg of protein at 1:1 ratio with Bio-Plex sample diluents was assayed per well. Samples were run in singlicate in order to allow all samples to be run on the same plate, hence exclude inter-plate variation in the analyses. Appropriate standards and controls were provided with the assays, and both were completed

in accordance with the manufacturer's instructions. Although in total 3 assays were undertaken, appropriate controls were undertaken as mentioned above which should regulate for assay/plate variation. Assays were read using a Bio-Plex© 200 reader and Bio-Plex Manager© v7.1 software. Tissue weight was used to normalise the concentrations of chemokine and cytokine results.

Table 2. 4 Human Bio-Plex© ProTM cytokine assay assessing 19 cytokines/chemokines.

Cytokines/Chemokines			
IL-1B	IL-10	MCP-1/CCL2	*TECK/CCL25*
IL-2	IL-16	RANTES/CCL5	GROa/CXCL1
IL-4	IFN-γ	MCP-3/CCL7	GROb/CXCL2
IL-6	TNF-α	TARC/CCL17	GCP-2/CXCL6
IL-8	CCL1	CCL20	CX3CL1

Table 2. 5 Human Bio-Plex© ProTM Human Inflammation panel 37-plex.

Inflammation Panel			
TNF-R1	GP130	IL-12 p70	IL-34
TNF-R2	IFNα2	IL-19	IL-35
TSLP	IFNβ	IL-20	LIGHT
TWEAK	IFNγ	IL-22	MMP1
APRIL	IL-6Ra	IL-26	MMP2
BAFF	IL-8	IL-27	MMP3
CD30	IL-10	IL-28A	Osteocalcin
CD163	IL-11	IL-29	Osteopotontin
Chitinase	IL-12 p40	IL-32	Pentraxin 3
IL-2	IL-32	LIGHT (TNFSF14)	

2.9 Flow Cytometry

Fluorescently conjugated antibodies were used to identify a range of cell subsets, and to characterise cell properties through observation of associated cell surface markers. NK cells and their surface marker expression were observed in addition to exploring T cells. T cell populations including CD8+, CD4+ and regulatory T cells have been identified. Furthermore, other cells such as classical, intermediate, and non-classical monocytes were investigated. Neutrophils have also been studied. $1x10^6$ – $2x10^6$ cells were used per panel considering known frequencies of cell populations.

2.9.1 Titration of Antibodies

- Initially panels were set up using manufacturer's recommended volumes to prevent delay in sample analysis. Titrations of flow cytometry antibodies were performed to optimise volumes for individual panels and between samples. Titrations were performed using $1x10^6$ PBMCs titrating with antibody dilutions from 1:10 with 7 serial dilutions to 1:640.

2.9.2 Surface Staining

Cells were counted using Trypan blue solution under a microscope as described above. Two million cells of each sample (decidua, placenta, PBMC and cord) were stained as per antibody panel (Table 2.7) in a total volume of 100µl. All antibodies were titrated as described above to ensure optimum staining levels. After incubation, cells were washed once with PBS using centrifugation. Cells were fixed with 2% paraformaldehyde and placed at 4^0C in the dark until acquisition within the following 24 hours.

Table 2. 6 Flow Cytometry Staining Panel

Marker/Clone	Fluorophore	Volume per sample
Viability dye	Aqua	1µl
CD3	APC-H7	2.5µl
CD4	BV711	2.5µl
CD8	BV605	2.5µl

CD14	FITC	20µl
CD15	PE	10µl
CD16	APC	2.5µl
CD25	PE- Cy7	5µl
CD56	BV786	2µl
CD127	BV421	5µl
HLA DR	PerCP	2.5µl

2.9.3 Flow Cytometry Acquisition and Data Analysis

All samples were acquired on a BD LSR II Flow Cytometer equipped with 50mW 405nm violet, 50mW 488nm blue, and 20mW 633nm red lasers. The set up and optimisation of the BD LSR II (Becton Dickinson, Oxford, UK) followed the protocol set out by Perfetto and colleagues in their Nature Protocols paper (194) and this was performed regularly by Dr Alexander Cocker. This set up reduced fluorescent spill-over between detectors, improved the distinction of positive and negative population fluorescence, and daily calibration ensured that samples acquired were longitudinally comparable for the duration of the study. In brief, the cytometer detectors were assessed to find their optimal range for acquisition using 8-peak Rainbow Calibration Particles (BD), and detector-specific mean fluorescence intensity (MFI) target values were generated for daily calibration using FL1 mid-range Rainbow Fluorescent Particles (BD) so that the detection of sample fluorescence was normalised. Data acquisition was carried out using FACSDiva v6.0 (Becton Dickinson), and data was analysed using FlowJo v10 (TreeStar, Ashland, OR, USA).

2.10 Urinary Metabolomics

2.10.1 Urine collection and storage

Morning first void urine samples were collected daily in dry universal bottles by recruited patients from 36+0 weeks gestation till onset of labour. Samples were initially stored at -20^{0}C at the patients' homes for the first 7 days either in their personal home refrigerators or in refrigerators provided to the patients by our research team at their request. Once a week the samples were collected and transported on dry ice by me to Chelsea and Westminster Hospital to be stored at -80^{0}C till analysis.

2.10.2 Sample preparation

Prior to mass spectrometry, all 400 samples had to be aliquoted. 400 samples were processed in 4 batches of 100 samples in order to manage time effectively. 100 samples were thawed overnight for 13 hours at room temperature. Each sample was initially centrifuged at room temperature, at 1000rpm for 10 minutes. 540µl of urine supernatant from each sample was aliquoted to a 1.5ml Eppendorf, to which 60µl of human urine buffer was added. Following vortexing, the sample was sat to mix well for 5 minutes and centrifuged at room temperature, at 1300rpm for 10 minutes. Aliquoted prepped samples were stored at -80^{0}C and transferred to the MRC-NIHR National Phenome Centre, Imperial College London, on dry ice. 575µl of prepped urine supernatant was transferred into a 5mm NMR tube, carefully avoiding bubbles. A pooled sample from all urine extracts were created for quality control (QC) purposes. The process is summarised in Figure 2.8.

2.10.3 Acquisition of 1 H NMR Spectral Profiles of Urine and Blank Samples

The acquisition was undertaken by Dr Frances Jackson who has described the process as below.

^{1}H NMR spectra were acquired on a Bruker DRX-600 spectrometer (Bruker Biospin, Karlsruhe, Germany) operating at 600.29 MHz for proton observation using a standard one-dimensional water pre-saturation pulse sequence [relaxation delay-90°-t_1-90°-t_m-90°-acquire free

induction decay (FID)]. The relaxation delay was 4 seconds, with application of a 90° radio frequency pulse, t_1, referring to the interpulse delay, which was set to 3 µl, while t_m is the mixing time of 100 ms. The probe was matched and tuned automatically to the proton transmitter resonance frequency before acquisition for each sample and samples were run at a temperature of 27°C (300K). Processing of ^{1}H NMR spectra was carried out using TOPSPIN 3.1 software package (Bruker Biospin, Rheinstetten, Germany). The FIDs were transformed into a spectrum by Fourier transformation. The 400 spectra were manually phased, baseline corrected and calibrated to the TSP signal at δ 0.0 for the urine samples. This was undertaken by Dr Frances Jackson, a postdoctoral research fellow.

2.10.4 Assessment of Dilution Factor and Spectral Normalization on Human Urine Profiles

This was undertaken by Dr Frances Jackson who describes the process as below.

The spectral data were imported into Matlab software (version 2014a, the Mathworks Inc, MA, USA) and were transformed into 32K data points. Resonance of the water (δ4.7 -5.05) was also removed from each spectra. Each 1H NMR spectrum was aligned using an in-house algorithm (195) and normalised using Probalistic Quotient Normalisation (PQN) (196) in order to remove variation in metabolite linked to osmolarity and dilution (197).

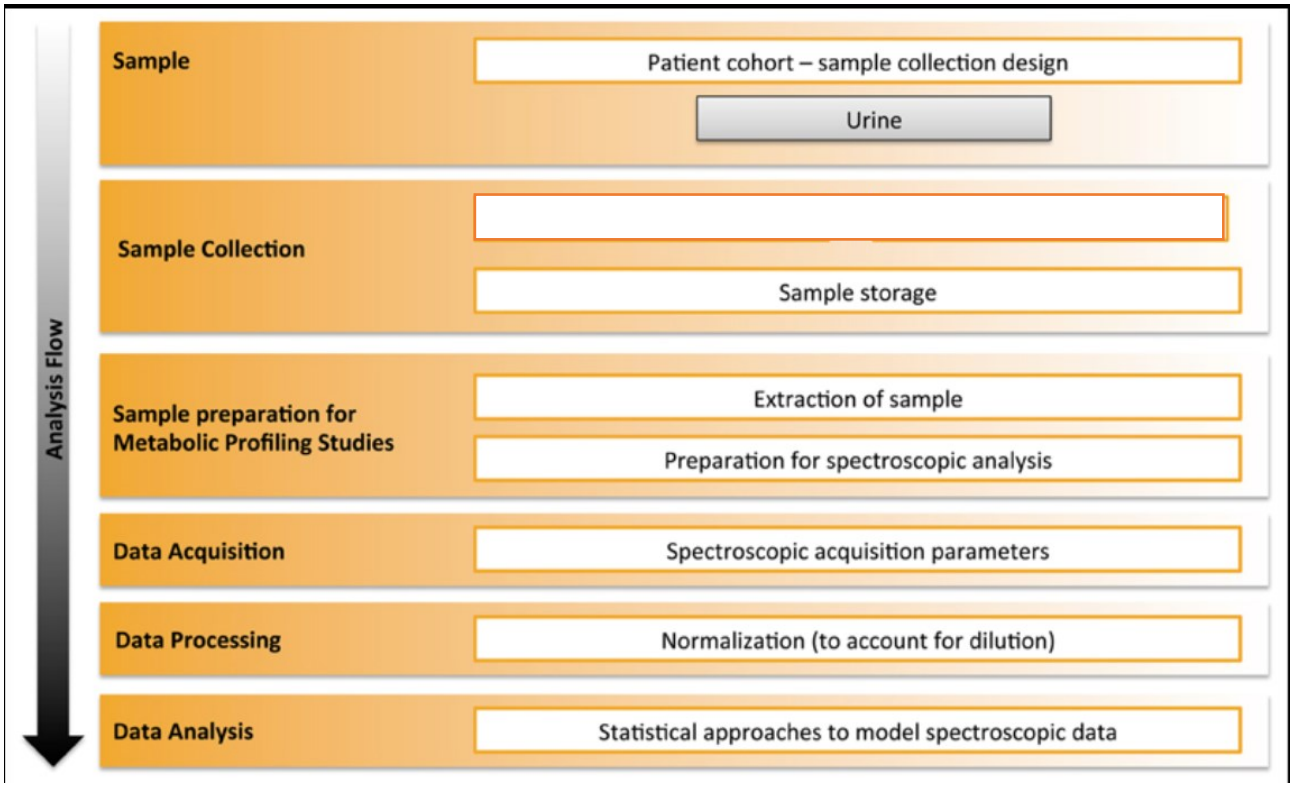


Figure 2. 8 A workflow describing the collection, preparation, and processing of urine samples for mass spectrometry. Adapted from (198) Image reproduced under the Creative Commons Attribution License. https://creativecommons.org/licenses/by/3.0/

2.11 Statistical Analysis

All data were treated as non-parametric. When comparing three groups or more in paired data, Friedman's test was used, and multiple comparisons were controlled for with the false discovery rate (FDR). When comparing only two matching groups, Wilcoxon matched pairs signed rank test was used. For comparing unpaired groups, Kruskal-Wallis test was used to compare more than three groups or more. FDR was also used to control for the multiple comparisons. When comparing only two non-matching groups Mann-Whitney test was used. All data are presented as median with IQR. $p<0.05$ was considered to be statistically significant.

Simple linear regression correlation was undertaken with Spearman's rank test and results are presented with confidence interval, R value and p values.

Principal component analysis (PCA) was carried out using mean-centred and unit variance scaled data.

The on-site medical statistician Dr Sundhiya Mandalia was consulted throughout this project to advise on power calculations and all methods of statistical analysis employed.

3. Cytokines and chemokines in preterm birth

3.1 Introduction

Cytokines and chemokines are small signalling proteins that are produced by an array of cells. Cytokines can be classified by their main function, pro-inflammatory and anti-inflammatory. Well known pro-inflammatory cytokines include TNF, IL-1β, IL-6 and IL-8 and on the other hand anti-inflammatory cytokines include IL-10 and TGF-β (199). There are other signalling proteins that do not quite fit the simplicity of this categorisation which include chemokines, interferons, and growth factors.

Chemokines are a large family of small chemotactic cytokines (200) and are important modulators of leucocyte activation and recruitment from the periphery to sites of inflammation (201). They tend to have rapid local actions, and these are usually short lived- in response to inflammatory stimulus, however they can play a significant role in tissue homeostasis and immune surveillance (201). Over 50 chemokines have been identified and they are often subdivided into four sub-classes, based on their amino-terminal cysteine motif: CC, C, CXC and CX3C chemokines (202).

Cytokines have been shown to play an important role in pregnancy from the very beginning starting with implantation, through to the propagation of inflammation required for labour. An imbalance in cytokine levels has also been associated with pregnancy complications, for example IL-6, IL-8 and TNF-α are noted to be increased in the decidua in pregnancies that unfortunately result in spontaneous miscarriage (199). Placental cytokines are also misaligned in pre-eclampsia where pro-inflammatory cytokines IL-6 and TNF-α are increased whilst anti-inflammatory cytokines IL-4 and IL-10 are reduced (203). Yockey *et al* have summarised current evidence in a review to suggest that that aberrant expression of cytokines and interferons is a common and critical mediator of congenital disorders and fetal loss that occurs in the settings of infections, chromosomal abnormalities, metabolic and autoimmune diseases (204).

Specifically looking at onset of labour, inflammatory cytokines are known to increase in the amniotic fluid towards term and with the onset of term labour, concentrations of IL-1β and

TNF-α are increased (205, 206). IL-6 in amniotic fluid has been a particular cytokine of interest, as it has been raised in amniotic fluid with spontaneous labour(207), is particularly raised in PTB associated with infection, and may even be considered a predictor of PTB in before 34 weeks gestation (208).

The increase in IL-1β in amniotic fluid towards term pregnancy is mirrored in the amnion and chorion along with IL-8 which suggests that this inflammatory transition occurs prior to the onset of labour (209, 210). IL-6 and TNF-α have also been noted to be increased which may suggest that they play a role in chemotaxis of monocytes and other immune cells to the gestational tissues (144, 210).

The choriodecidua has been previously studied in some depth by Hamilton and co-workers (211). They had noted that IL-8 was raised in choriodecidua in labour, at almost a 30-fold increase compared with term no labour. They also showed that $CD56^{+}$ NK cells and T cells are increased in choriodecidua in PTL (211). Another study has proven a rise in CCL8 in choriodecidua in PTL (212, 213) which is commonly known as a monocyte chemoattractant protein, is also a chemoattractant of T cells and NK cells in an inflammatory response.

In contrast to the above gestational compartments, the evidence of inflammation in the placenta seems to be poor (209). There is enough evidence to suggest that placental cells are enabled to respond to inflammatory stimuli such as LPS, IL-1 or pathogenic bacteria by increasing its production of cytokines (IL-1, IL-6, IL-10) and chemokines (CCL8) alike (145).

The current body of evidence is still weak regarding the cytokine and chemokines changes noted with the onset of PTL, especially in the lesser investigated gestational tissues such as choriodecidua. Several studies have looked at changes in gestational tissues in PTL triggered by chorioamnionitis, as this is the leading cause of PTB with one in three preterm babies being born to mothers with an intra-amniotic infection which is largely sub-clinical(213, 214). Chorioamnionitis produces a robust inflammatory response to intra-amniotic infection(215) so it is likely all cytokines, chemokines and immune cell activity will be largely exaggerated. Studying and comparing the cytokine and chemokine responses in both placenta and choriodecidua in different clinical causes of PTB would give us a more intricate picture of their role in triggering labour prematurely.

3.1.1 Hypotheses

1) Choriodecidua is more inflammatory than placenta and amnion in PTL samples specifically in chorioamnionitis and idiopathic labour.
2) Cytokine and chemokines are significantly raised in the CD and placenta of PTL samples triggered by chorioamnionitis.
3) Immunomodulatory cytokines/chemokines will be significantly lower in the CD of PTL samples from the Idiopathic group.
4) Inflammatory cytokines, such as IL-6, IL-8 and TNF-α, will be significantly raised in the CD/Placenta of the chorioamnionitis samples of babies with worse neonatal outcomes.

3.2 Aims

In this chapter, I plan to address the following aims:

1) To compare the cytokine/chemokines changes seen in placenta obtained at preterm non-labouring deliveries with preterm labouring deliveries defined by PTL type:
   - Iatrogenic not in labour, mostly delivery for fetal growth restriction
   - Chorioamnionitis (CA)
   - Abruption
   - Idiopathic
   - Twins not in labour
   - Twins in labour
2) To compare the cytokine/chemokine changes seen in choriodecidua obtained at preterm non-labouring deliveries with preterm labouring deliveries secondary to different pathologies of spontaneous labour (as denoted above).
3) To delve further into the inflammation seen in the choriodecidua compared to the amnion by undertaking an inflammasome assessment of matched samples.

3.3 Sample collection and methodology

The approval for collection of choriodecidua, placenta and amnion were obtained from the Chelsea-Brompton Research Ethics Committee (REC) (REC number: 10/H080/145).

Once fully informed written consent was obtained from the patients, the agreed samples of myometrium, placenta, choriodecidua and amnion were collected from women who underwent a Caesarean delivery for spontaneous PTL due to an identified cause or iatrogenic preterm delivery which was commonly due to fetal growth concerns or breech presentation. The samples were collected on labour ward immediately after delivery of the baby and stored at -80^{o}C within half an hour of the collection of samples. The samples were categorised into groups based on the indication of the delivery using a clear inclusion and exclusion criteria which is summarised in Table 3.1.

The samples collected from the labouring preterm chorioamnionitis, abruption, idiopathic and twins were taken from consented women at Caesarean delivery who had an onset of spontaneous PTL, which was defined as the presence of cervical dilatation (1-3cm dilated) in early labour. The placenta from these patients were sent to histopathology, as was clinical protocol at Chelsea and Westminster Hospital and therefore the presence of chorioamnionitis was confirmed in the CA group, whilst also excluding CA as a pathology in the abruption and idiopathic PTL groups.

The samples collected from the non-labouring groups – preterm non-labouring singleton pregnancies and preterm non-labouring twin pregnancies were taken from consented women who had no signs of labour and were being delivered for either maternal indication such as previous section or fetal indications (fetal growth restrictions or breech presentation).

All placental biopsies were taken randomly from sites between the umbilical cord insertion and placental edge. To avoid decidual contamination, they were taken more than 5mm away from the maternal surface. The amnion and choriodecidua were identified beyond the edge of the placenta and manually separated. The samples were taken midway between the tear-line and/or site of spontaneous rupture and the placental edge.

These samples were collected between 2013 and 2015 from consenting patients on Chelsea and Westminster Hospital labour ward and stored as part of a tissue bank. This was led by Dr Natasha Singh (Clinical research lead consultant). These samples were processed and stored for later analysis by me, Dr Gavin Sooranna, Dr Bronwen Herbert, Dr Maria Fais and Dr Natasha Singh.

In summary, this chapter studies 75 placenta and choriodecidua preterm samples taken from the following groups: preterm non-labour (PTNL=13), preterm non-labour twins (PTNL Tw =14) and spontaneous PTL which include chorioamnionitis (CA = 12), idiopathic (IP = 15), abruption (A = 8), and twins in early labour (TwinsEL = 13).

Table 3. 1 Definition of the distinct PTB phenotypes and their inclusion/exclusion criteria.

Phenotype	Definition and Diagnosis
Preterm Non-Labour	Women who are not in labour at a gestation before 37 weeks who underwent a Caesarean section delivery for maternal or fetal reasons.
Idiopathic	Exclusion criteria: evidence of infection, rupture of membranes >48 hours, abruption, fibroids, multiple pregnancy, previous cervical surgery, mid-trimester loss, previous pelvic inflammatory disease or sexually transmitted disease.
Chorioamnionitis	Maternal pyrexia (temperature 38^0C or more, on at least one occasion) with two or more of the following: foul smelling liquor, tachycardia (>100bpm), fetal tachycardia (>160bpm), uterine tenderness, raised maternal peripheral white cell count (>15000) and raised C-reactive protein.
Abruption	Preterm delivery due to placental abruption: The diagnosis was based on clinical symptoms of vaginal bleeding accompanied by abdominal pain, uterine tenderness, or tetanic contractions with abnormal fetal heart tracing and retroplacental clot at time of delivery.
Twins Non-Labour	Women with twins not in labour who delivered preterm (24-36 weeks) for maternal or fetal reasons.
Twins in early labour	Twins delivered preterm (24-36 weeks), uncomplicated twin pregnancies in spontaneous PTL with no evidence of infection or uterine anomaly.

Inclusion criteria	All women in spontaneous preterm early labour in the distinct PTL phenotypes above without any overlap in clinical signs or symptoms.
Exclusion criteria	Uterine anomaly, maternal infectious disease (hepatitis, HIV, syphilis), polyhydramnios, multiple pregnancies, uterine fibroids, previous pelvic infection, previous cervical surgery.

3.3.1 Multiple cytokine assay

75 samples categorised based on their clinical phenotype were used to undertake a Bio-Plex multiple cytokine assay. The cytokines that were investigated in my chosen tissues of interest, the choriodecidua and placenta were identified by previous work carried out on myometrium by our group. There were twenty cytokines of interest (Table 3.2); 19 cytokines were analysed on a custom-made Bio-Plex 19-plex assay. The last cytokine CCL5 was analysed on a separate assay due to buffer incompatibility. 500µg of protein lysate was used for each well on the 19-plex assay. Further detailed description can be found in the methods section (2.8) and is summarised in Figure 3.1.

Table 3. 2 List of Bio-Plex analytes included in the custom-made Bio-Plex 19-plex assay. CCL5 was on a separate assay.*

Bio-Plex Analytes			
IL-1B	IL-10	CCL2/MCP-1	CCL25/TECK
IL-2	IL-16	CCL5/RANTES*	CXCL1/GROa
IL-4	IFN-g	CCL7/MCP-3	CXCL2/GROb
IL-6	TNF-a	CCL17/TARC	CXCL6/GCP-2
IL-8	CCL1	CCL20	CX3CL1

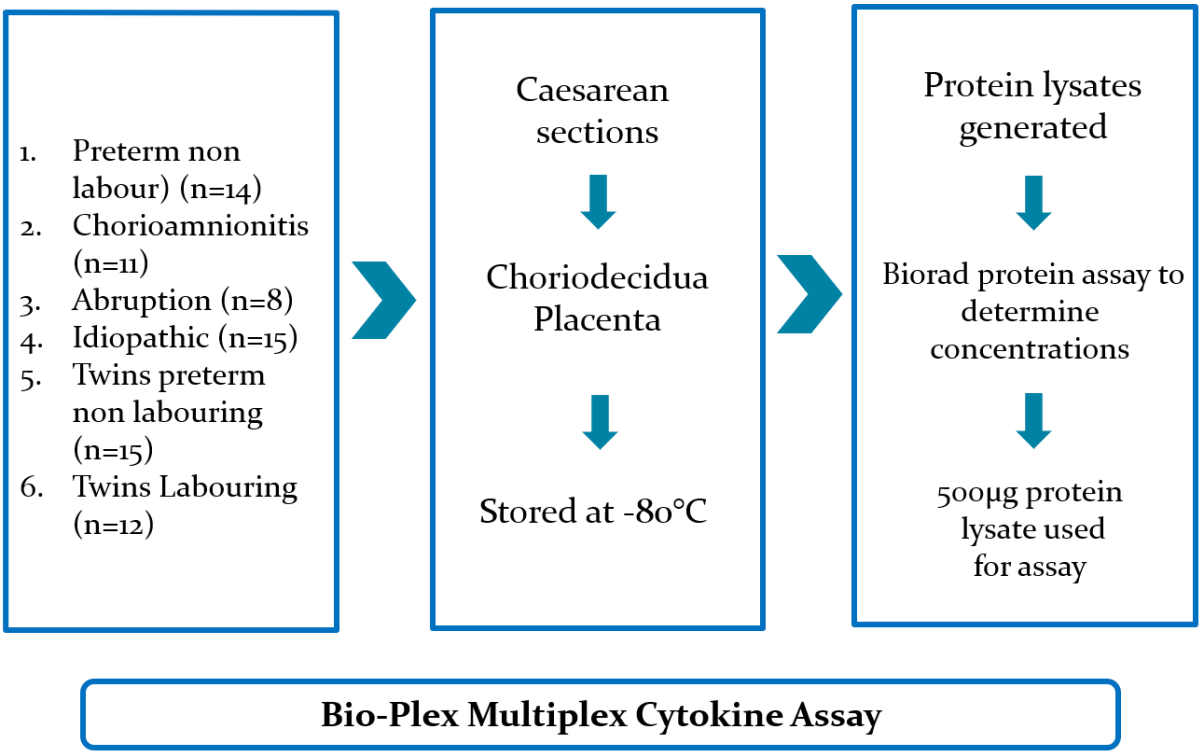


Figure 3. 1 A summary of the method used to collect, store, and process the matched choriodecidua and placenta tissues to analyse cytokines of interest.

3.3.2 Inflammasome multiple assay

Amnion and choriodecidua are adjacent anatomically and evidence have suggested that both could play a role in parturition and possibly in different causes of PTB. In order to further understand the inflammatory roles of these distinct gestational tissues an inflammasome multiple assay was conducted on matched paired amnion and choriodecidua tissue sets. 37 key biomarkers of inflammation from the TNF superfamily proteins, interferon family proteins, Treg cytokines and matrix metallopeptidases (MMPs) were studied via the Bio-Plex Pro™ Human Inflammation Panel, 37 Plex (Table 3.3).

Table 3. 3 37 key biomarkers of inflammation from the TNF superfamily proteins, interferon family proteins, Treg cytokines and matrix metallopeptidases.

Inflammasome plate			
IL-20	IL-6Ra	IFNγ	TSLP
IL-22	IL-8	IFNα2	TWEAK

IL-26	IL-10	IFNβ	APRIL/TNFSF13
IL-27	IL-11	TNF-R1	BAFF/ TNFSF13B
IL-28A/IFNγ1	IL-12 p40	TNF-R2	CD30/TNFRSF8
IL-29/IFNγ2	IL-12 p70	MMP1	CD163
IL-32	IL-19	MMP2	Chitinase-3-like-1
IL-34	IL-2	MMP3	GP130
IL-35	Pentraxin-3	LIGHT (TNFSF14)	Osteocalcin
Osteopontin			

3.3.3 Statistical analyses

All data were treated as non-parametric. For paired data, Wilcoxon matched pair test was used and when comparing three groups or more a Friedman's test, with a Dunn's multiple comparisons post-hoc test. For comparing two unpaired groups, a Mann Whitney test was use and when comparing three groups or more a Kruskal-Wallis test, with a Dunn's multiple comparisons post-hoc test was used. Post hoc tests were necessary to ensure that the findings were valid and reliable, especially when comparing three or more group means. $P < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Human chemokine and cytokine expression profile comparison between CD, amnion and placenta in different clinical subtypes of PTL

Inflammation levels in matched CD, amnion and placental tissues from patients who had PTL due to varying clinical diagnoses were assessed. Demographic and obstetric characteristics of study participants are detailed in Table 3.4 below. This was the first analysis that was undertaken in order to identify whether any one tissue is more pro-inflammatory than the other. In addition, the differences in cytokine and chemokine expression in the different tissues could give us more information on possibly the pathways involved in the differing

clinical subtypes of PTL. The inflammatory response in the placenta, CD and amnion (data kindly provided by Dr Ananya Das) in the PTL-CA, PTL-I and PTL-A group were compared to that in the PTNL group, which was always used as the control throughout statistical analysis.

Table 3. 4 Demographics of women recruited to this study - preterm not in labour (PTNL), idiopathic preterm labour (IPTL), preterm labour secondary to chorioamnionitis (PTL-CA), preterm labour secondary to abruption (PTL-A), preterm twin gestations not in labour (PTNL Tw) and preterm twin gestations not in labour (PTNL Tw) and preterm twin gestation in labour (PTL TwEL). Median and IQR values included.

Preterm CD and Placental Samples						
	PTNL	PTL-I	PTL-CA	PTL-A	PTNL Tw	PTL TwEL
Number of samples	14	15	11	8	15	12
Maternal age (years) (IQR)	31.5 (27.8 – 35.8)	34.0 (28.0 – 37.0)	32.0 (29.0 – 37.0)	29.5 (25.8 - 34.0)	35.0 (30.0 – 42.0)	32.0 (31.0 – 38.0)
Ethnicity						
White British	5	5	4	1	7	5
White other	3	2	1	1	5	3
Asian	1	2	2	0	1	0
Black African	1	1	0	2	0	0
Mixed	2	0	1	0	1	1
Not stated	2	3	0	2	1	0
Any other ethnic group	0	2	3	2	0	3
Gestational age (weeks) (IQR)	33.0 (30.8 – 34.3)	35.0 (34.0 – 36.0)	28.0 (26.7- 31.0)	33.0 (29.6 - 34.8)	35.0 (35.0 – 36.0)	34.0 (33.0 – 36.0)
Parity (n)						
0	10	7	5	4	10	10
1	3	5	4	3	4	2
2	1	2	1	1	1	0
3	0	1	1	0	0	0
Maternal BMI (IQR)	26.0 (23.5 – 28.0)	25.0 (20.0 – 29.0)	24.0 (20.0 – 26.0)	23.0 (21.5 – 24.8)	22.0 (20.0 – 24.0)	21.0 (20.0 - 24.5)

3.4.2 Chorioamnionitis -19-plex and RANTES

It was clear that in CA, there was a significant rise in almost all the cytokines/chemokines analysed across all three tissues when compared to PTNL individuals (Table 3.5 and Table 3.6).

The exceptions occurred predominantly in placenta samples, with TNF-α CCL1, MCP-1/CCL2, RANTES/CCL5, TARC/CCL17, and GCP-2/CXCL6 not showing a significant difference when compared between groups. Contrastingly IL-4 was raised only in choriodecidua and placenta. These findings are well summarised in Figure 3.2.

*Table 3. 5 Cytokine assay results in PTL-CA compared to PTNL in Choriodecidua, Placenta and Amnion. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc test. *** indicate statistical significance (* p<0.05, ** p<0.01, *** p=0.001, **** p<0.0001) ns= not significant. Number documented is fold difference in concentration using PTNL as the control value in each gestational tissue.*

Analyte	**Type**	**PTNL v Chorioamnionitis**			**Source**
		CD	**Placenta**	**Amnion**	
IFN-γ	Immunoregulatory cytokines	*** (6.76)	* (1.15)	*** (7.76)	CD4+ Th1 lymphocytes, CD8+ CTLs, NK cells. B cells, NK, monocytes, macrophages, DCs
TNF-α	Pro-inflammatory cytokine	*** (51.78)	ns (1.43)	*** (12.64)	Macrophages, lymphocytes, neutrophils & mast cells
Interleukins		**CD**	**Placenta**	**Amnion**	
IL-1β	Pro-inflammatory cytokine	*** (311.22)	*** (7.38)	** (736.44)	Monocytes/ macrophages
IL-2	Regulatory cytokine	**** (19.75)	* (2.05)	** (10.96)	T cells
IL-4	Anti-inflammatory cytokine	**** (7.50)	** (2.43)	ns	T & B cells, macrophages, mast cells, basophils, bone marrow stroma
IL-6	Pro-inflammatory cytokine	**** (65.0)	*** (5.95)	*** (102.55)	T cells, monocytes/ macrophages

IL-8	CXC chemokine	**** (41.45)	**** (16.30)	*** (43.04)	T cells, monocytes, neutrophils
IL-10	Anti-inflammatory cytokine	**** (4.56)	*** (1.73)	*** (2.58)	T and B cells, Macrophages
IL-16	Pro-inflammatory cytokine	**** (8.06)	** (1.73)	*(3.57)	Monocytes, mast cells, macrophages, DCs, CD8+ T cells, CD4+ T cells, eosinophil

*Table 3. 6 Chemokine assay results in PTNL compared to PTL-CA in Choriodecidua, Placenta and Amnion. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc test * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001 ****p<0.0001) ns= not significant.*

Chemokines	**Type**	**PTNL v Chorioamnionitis (ratios)**		
		CD	**Placenta**	**Amnion**
CCL1	CC chemokine	*** (1.69)	ns (1.16)	*** (2.25)
MCP-1/CCL2	CC chemokine	**** (17.25)	ns (1.43)	*** (8.53)
RANTES/CCL5	CC chemokine	**** (2.73)	ns (0.95)	Not done
MCP-3/CCL7	CC chemokine	****(5.49)	** (1.62)	*** (13.30)
TARC/CCL17	CC chemokine	* (5.84)	ns (1.10)	Not done
CCL20	CC chemokine	**** (325.28)	*** (3.69)	*** (107.82)
TECK/CCL25	CC chemokine	**** (2.64)	** (1.65)	*** (2.70)
GROa/CXCL1	CXC chemokine	**** (46.19)	** (2.35)	*** (4.31)
GROb/CXCL2	CXC chemokine	**** (56.22)	**** (3.53)	*** (19.31)
GCP-2/CXCL6	CXC chemokine	**** (35.10)	ns (1.25)	** (8.05)
CX3CL1	CX_3C chemokine	**** (15.42)	** (1.43)	*** (6.71)

3.5 Comparison of Amnion and Choriodecidua in the Chorioamnionitis PTL subgroup

The inflammatory status of the individual fetal membranes were assessed in chorioamnionitis matched samples to better understand which fetal membrane compartment may contribute more to the inflammatory trigger of labour, and specifically preterm labour. The majority of samples showed no difference between the two tissues (21/37), eleven (11/37) were lower, and five (5/37) of the analytes were higher in the choriodecidua when compared to the amnion in the PTNL subgroup which is the control group for comparison.

In contrast, in the PTL-CA group, twenty-four (24/37) were higher, none (0/37) were lower, and thirteen (13/37) were similar in the choriodecidua when compared to the amnion. This suggests that CD is a more inflammatory fetal membrane than amnion in the context of CA hence may play a more significant role in the onset of CA-induced PTL. The analytes in detail are summarised in Table 3.7.

Table 3. 7 A comparison of the concentration of different inflammatory markers between matched CD and amnion tissues during PTNL and CA. The red arrows indicate when a significant increase in concentration was noted within the CD when compared to the amnion. The green arrows indicate when a significant decrease was noted within the amnion when compared to the CD.

Analyte	PTNL	Chorioamnionitis
TNF-R1	-----	↑*
TNF-R2	-----	-----
TSLP	↓*	↑**
TWEAK	-----	↑*
APRIL	-----	↑**
BAFF	-----	↑*
CD30	↑**	-----
CD163	-----	↑*
Chitinase	-----	↑**
GP130	↑***	↑**
IFN-α2	↓***	-----
IFN-β	↓**	-----

IFN-γ	-----	↑*
IL-6Ra	-----	↑*
IL-8	↑**	↑**
IL-10	↓**	↑*
IL-11	-----	↑*
IL-12 p40	-----	↑*
IL-12 p70	↓*	-----
IL-19	-----	-----
IL-20	-----	↑**
IL-22	↓**	↑*
IL-26	-----	↑**
IL-27	-----	-----
IL-28A	-----	↑**
IL-29	-----	↑*
IL-32	-----	-----
IL-34	↓**	↑**
IL-35	↓*	-----
LIGHT	-----	-----
MMP1	-----	-----
MMP2	↑*	↑**
MMP3	↓*	-----
Osteocalcin	↑****	↑****
Osteopontin	-----	-----
Pentraxin 3	↓***	↑****

3.6 Idiopathic

3.6.1 Cytokine and chemokine differences between preterm idiopathic and non-labour are predominantly in the choriodecidua

Cytokine assay results from Choriodecidua and Placenta comparing the Idiopathic Preterm Group to the Preterm Non-Labouring group are detailed in Table 3.8 below, chemokines in Table 3.9, and direction of variation in Table 3.10.

*Table 3. 8 Cytokine assay results in PTNL compared to PTL-I in Choriodecidua and Placenta. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc test * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001, **** p<0.0001) ns= not significant.*

Analyte	Type	PTNL v PTL-Idiopathic	
		Choriodecidua	Placenta
IFN-γ	Immunoregulatory cytokines	* (1.66)	ns (0.65)
TNF-α	Pro-inflammatory cytokine	** (1.73)	ns (0.80)
IL-1β	Pro-inflammatory cytokine	ns (0.86)	ns (0.82)
IL-2	Regulatory cytokine	* (3.25)	ns (0.41)
IL-4	Anti-inflammatory cytokine	* (1.55)	ns (0.93)
IL-6	Pro-inflammatory cytokine	* (3.90)	ns (1.43)
IL-8	CXC chemokine	* (5.71)	ns (1.6)
IL-10	Anti-inflammatory cytokine	* (2.34)	ns (1.21)
IL-16	Pro-inflammatory cytokine	* (1.76)	ns (1.33)

*Table 3. 9 Chemokine assay results in PTNL compared to PTL-I in Choriodecidua and Placenta. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc test. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001 ****p<0.0001) ns= not significant*

Analyte	Type	PTNL v PTL-Idiopathic	
		Choriodecidua	Placenta
CCL1	CC chemokine	ns (1.21)	ns (0.88)
MCP-1/CCL2	CC chemokine	** (3.79)	ns (1.17)
RANTES/CCL5	CC chemokine	* (2.00)	ns (0.96)
MCP-3/CCL7	CC chemokine	** (1.71)	ns (0.83)
TARC/CCL17	CC chemokine	ns	ns

		(1.44)	(0.80)
CCL20	CC chemokine	** (2.31)	ns (0.92)
TECK/CCL25	CC chemokine	ns (1.31)	ns (0.85)
GROa/CXCL1	CXC chemokine	* (2.87)	ns (2.13)
GROb/CXCL2	CXC chemokine	** (3.58)	**** (2.00)
GCP-2/CXCL6	CXC chemokine	* (1.50)	ns (1.30)
CX3CL1	CX_3C chemokine	* (2.20)	ns (1.38)

3.6.2 Comparison of Amnion and Choriodecidua in the Idiopathic PTL subgroup

Table 3. 10 A comparison of the concentration of different inflammatory markers between matched CD and amnion tissues during PTNL and Idiopathic PTL. The red arrows indicate when a significant increase or decrease in concentration was noted within the CD when compared to the amnion. The green arrows indicate when a significant increase or decrease was noted within the amnion when compared to the CD.

Analyte	PTNL	Idiopathic
TNF-R1	-----	-----
TNF-R2	-----	-----
TSLP	↓*	-----
TWEAK	-----	-----
APRIL	-----	↑*
BAFF	-----	↑*
CD30	↑**	-----
CD163	-----	-----
Chitinase	-----	-----
GP130	↑***	-----
IFN-α2	↓***	-----
IFN-β	↓**	-----
IFN-γ	-----	-----
IL-6Ra	-----	-----

IL-8	↑**	-----
IL-10	↓**	-----
IL-11	-----	-----
IL-12 p40	-----	-----
IL-12 p70	↓*	-----
IL-19	-----	-----
IL-20	-----	-----
IL-22	↓**	-----
IL-26	-----	-----
IL-27	-----	-----
IL-28A	-----	-----
IL-29	-----	-----
IL-32	-----	-----
IL-34	↓**	-----
IL-35	↓*	-----
LIGHT	-----	-----
MMP1	-----	-----
MMP2	↑*	↑*
MMP3	↓*	-----
Osteocalcin	↑****	↑**
Osteopontin	-----	-----
Pentraxin 3	↓***	-----

3.7 Abruption

3.7.1 Preterm labour secondary to abruption results in minimal cytokine/chemokine changes in both CD and placenta

*Table 3. 11 Cytokine assay results in PTNL compared to PTL-A in Choriodecidua and Placenta. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc test. *** indicate statistical significance (* p<0.05, ** p<0.01, *** p=0.001, **** p<0.0001) ns= not significant*

Analyte	Type	PTNL v PTL-Abruption

		Choriodecidua	Placenta
IFN-γ	Immunoregulatory cytokines	ns (1.12)	ns (0.93)
TNF-α	Pro-inflammatory cytokine	* (2.16)	ns (0.93)
IL-1β	Pro-inflammatory cytokine	ns (1.05)	ns (1.55)
IL-2	Regulatory cytokine	ns (0.28)	ns (1.25)
IL-4	Anti-inflammatory cytokine	ns (0.99)	ns (1.32)
IL-6	Pro-inflammatory cytokine	ns (3.73)	* (2.12)
IL-8	CXC chemokine	ns (3.32)	* (2.55)
IL-10	Anti-inflammatory cytokine	ns (1.20)	ns (1.38)
IL-16	Pro-inflammatory cytokine	ns (1.20)	ns (0.65)

*Table 3. 12 Chemokine assay results in PTNL compared to PTL-A in Choriodecidua and Placenta. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc test. *** indicate statistical significance (* p<0.05, ** p<0.01, *** p=0.001, **** p<0.0001) ns= not significant.*

Analyte	Type	PTNL v PTL-Abruption	
		Choriodecidua	Placenta
CCL1	CC chemokine	ns (0.90)	ns (1.04)
MCP-1/CCL2	CC chemokine	ns (2.43)	ns (1.44)
RANTES/CCL5	CC chemokine	ns (2.01)	ns (1.19)
MCP-3/CCL7	CC chemokine	ns (1.41)	ns (1.02)
TARC/CCL17	CC chemokine	ns (1.04)	ns (0.85)
CCL20	CC chemokine	* (0.89)	* (2.70)
TECK/CCL25	CC chemokine	ns (1.02)	ns (1.22)
GROa/CXCL1	CXC chemokine	* (2.44)	ns (0.89)
GROb/CXCL2	CXC chemokine	**	*

		(2.33)	(1.71)
GCP-2/CXCL6	CXC chemokine	ns (0.91)	ns (1.05)
CX3CL1	CX_3C chemokine	ns (1.16)	ns (1.03)

3.8 Twins Preterm Labour

3.8.1 Cytokine and chemokine differences between preterm twins labour and non-labour are similar in both choriodecidua and placenta

*Table 3. 13 Cytokine assay results in Twins PTNL compared to Twins-L in Choriodecidua and Placenta. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc test. *** indicate statistical significance (* p<0.05, ** p<0.01, *** p=0.001, **** p<0.0001) ns= not significant.*

Analyte	Type	Twins PTNL v Twins-L	
		Choriodecidua	**Placenta**
IFN-γ	Immunoregulatory cytokines	** (1.75)	* (1.42)
TNF-α	Pro-inflammatory cytokine	ns (1.80)	* (1.83)
IL-1β	Pro-inflammatory cytokine	* (5.47)	ns (0.73)
IL-2	Regulatory cytokine	ns (2.25)	** (2.60)
IL-4	Anti-inflammatory cytokine	* (2.76)	* (11.8)
IL-6	Pro-inflammatory cytokine	ns (1.38)	Ns (1.37)
IL-8	CXC chemokine	* (3.13)	* (1.92)
IL-10	Anti-inflammatory cytokine	** (2.09)	** (1.60)
IL-16	Pro-inflammatory cytokine	ns (3.12)	ns (1.11)

Table 3. 14 Chemokine assay results in Twins PTNL compared to Twins-L in Choriodecidua and Placenta. Statistical testing was undertaken using Friedman's test with a Dunn's multiple comparisons post hoc

*test. *** indicate statistical significance (* p<0.05, ** p<0.01, *** p=0.001, **** p<0.0001) ns= not significant.*

Analyte	Type	Twins PTNL v Twins-L	
		Choriodecidua	Placenta
CCL1	CC chemokine	ns (1.08)	ns (1.09)
MCP-1/CCL2	CC chemokine	* (2.28)	ns (0.84)
RANTES/CCL5	CC chemokine	ns (1.60)	ns (1.06)
MCP-3/CCL7	CC chemokine	ns (1.22)	ns (1.26)
TARC/CCL17	CC chemokine	ns (1.36)	* (1.49)
CCL20	CC chemokine	* (2.06)	** (2.48)
TECK/CCL25	CC chemokine	ns (1.07)	* (1.36)
GROa/CXCL1	CXC chemokine	* (3.76)	ns (1.48)
GROb/CXCL2	CXC chemokine	** (2.11)	* (1.65)
GCP-2/CXCL6	CXC chemokine	ns (1.21)	ns (0.86)
CX3CL1	CX_3C chemokine	* (2.17)	*** (1.65)

3.8.2 Comparison of Amnion and Choriodecidua in the Twins

Table 3. 15 Comparison of the concentration of different inflammatory markers between matched CD and amnion tissues during Twins PTNL and Twins PTL. The red arrows indicate when a significant increase or decrease in concentration was noted within the CD when compared to the amnion. The green arrows indicate when a significant increase or decrease was noted within the amnion when compared to the CD.

Analyte	Twins PTNL	Twins PTL
TNF-R1	↑*	↑*
TNF-R2	-----	↑*
TSLP	↓**	-----
TWEAK	-----	↑*
APRIL	-----	↑**
BAFF	↑*	↑*

CD30	-----	↑**
CD163	-----	-----
Chitinase	-----	↑*
GP130	↑*	↑*
IFNα2	↓**	↓***
IFNβ	↓*	↓*
IFNy	-----	-----
IL-6Ra	↑*	↑*
IL-8	↑**	↑**
IL-10	↓*	-----
IL-11	-----	↑*
IL-12 p40	-----	-----
IL-12 p70	↓*	↓*
IL-19	-----	↑**
IL-20	-----	-----
IL-22	↓*	↓**
IL-26	↑*	-----
IL-27	-----	-----
IL-28A	-----	↑**
IL-29	-----	-----
IL-32	-----	-----
IL-34	-----	-----
IL-35	-----	-----
LIGHT	-----	-----
MMP1	↓**	↓**
MMP2	-----	↑*
MMP3	-----	-----
Osteocalcin	↑****	↑**
Osteopontin	-----	↑*
Pentraxin 3	-----	-----

3.9 Cytokines associated with adverse neonatal outcomes

Neonatal outcomes were assessed by generating a combined score of adverse neonatal outcome (including neonatal death, chronic lung disease requiring home oxygen on discharge, and intraventricular haemorrhage) related to the occurrence of an adverse neonatal outcome and to tissue cytokine levels (Table 3.16). Five babies from the CA group had raised scores based on their neonatal outcomes (Table 3.17). Interestingly within the other sub-groups, there was only 1 adverse neonatal outcome in the abruption (n=8) group, which was BPD, all other sub-groups had no adverse neonatal outcomes.

Table 3. 16 Neonatal outcomes of the preterm babies born secondary to chorioamnionitis. (PPROM – preterm premature rupture of membranes, RDS – respiratory distress syndrome, PDA – patent ductus arteriosus, HTN – hypertension, NEC - necrotising enterocolitis, NND – neonatal death, IVH – intraventricular haemorrhage). The Apgar score is an accepted and convenient method for reporting the status of the newborn infant immediately after birth and the response to resuscitation if needed (American Academy of Paediatrics, 2015). The score is undertaken at 1 and 5 minutes of life, a 5-minute Apgar score of 0 to 3 correlates with neonatal mortality in large populations. A low 5-minute Apgar score clearly confers an increased relative risk of cerebral palsy, reported to be as high as 20- to 100-fold over that of infants with a 5-minute Apgar score of 7 to 10 (American Academy of Paediatrics, 2015). The score is calculated by assessing colour of baby, heart rate, reflex irritability, muscle tone and respiration.

Gestation	**Indication**	**Fetal weight/centile**	**Apgars**	**Neonatal complications**	**Neonatal outcomes**
B3 29/40	CA (PPROM)	1520g/11-25th	8,8	RDS, sepsis	Well
B4 33/40	CA (PPROM)	2234g/51-75th	8,8	RDS, sepsis, hypoglycaemia	Well
B5 27/40	CA (PPROM)	924g/5th	5,8	PDA, jaundice, sepsis	Chronic lung disease needing O2 on discharge
B6 32+6/40	CA (PPROM)	1740/26-50th	9,9	Jaundice, sepsis	Well
B7 26/40	CA (PPROM)	900g/<5th	7,9	PDA	Chronic lung disease needing O2 on discharge
B10 26+5/40	CA (PPROM)	895g/<5th	7,9	RDS, PDA	Well
B11 28/40	CA	1260g/6-10th	5,8	RDS, overwhelming sepsis, persistent pulmonary HTN	Bilateral grade III IVH, NND Day 3

B12 28/40	CA (PPROM)	1430g/11-25th	6,7	RDS, jaundice, sepsis,	Well
B13 26/40	CA	1081g/6-10th	6,5	RDS, suspected NEC, sepsis	IVH grade II, chronic lung disease at 28/7
B14 31+6/40	CA	1780g/26-50th	6,10	RDS	IVH Grade I
B15 30/40	CA	1200g/6-10th	4,8	RDS, PDA, jaundice, sepsis	Well

Table 3. 17 Comparison of the good outcomes versus bad outcomes group amongst the chorioamnionitis group.

Neonatal outcome	Good	Adverse	Significance
Number of babies	6	5	
Median gestational age	31+3/40	27/40	ns (p=0.183)
Mean fetal weight	1503g	1189g	ns (p=0.258)

The only cytokine which was significantly raised in the choriodecidua samples from mothers whose babies had the worst neonatal outcome was IL-8 (Figure 3.2).

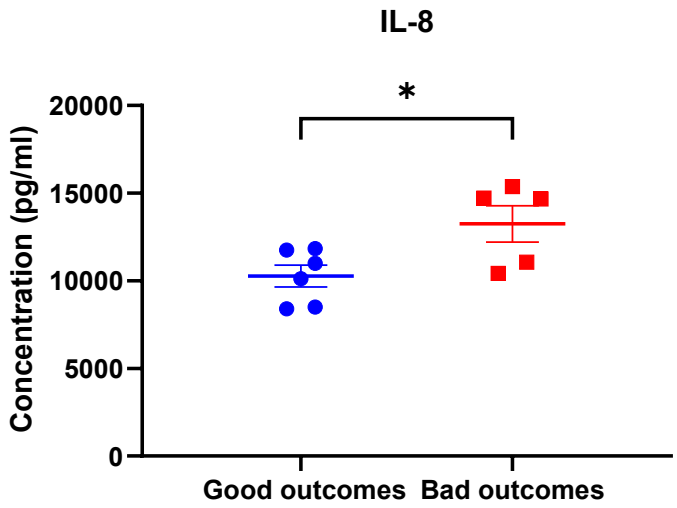


*Figure 3. 2 IL-8 concentration in each neonatal outcome group. Adverse outcome n=5, Good outcome n=6 (*p<0.05). Supplementary cytokine data can be seen in appendix 7.4.*

3.10 Discussion

This chapter details the findings of an extensive study assessing cytokine and chemokine levels in different preterm delivery pathologies. Additionally, cytokine and chemokine levels were compared between gestational tissues to identify which tissue demonstrated the highest inflammatory cytokine levels to focus future work.

3.10.1 Cytokines and chemokine distribution in Chorioamnionitis

Chorioamnionitis is an inflammatory process which effects the chorion and amnion, which can be secondary to sterile intra-amniotic inflammation or more commonly intra-amniotic infection (215). Results from the chorioamnionitis preterm subgroup concur with this, with most cytokines and chemokines being significantly raised across the three gestational tissues when compared to the PTNL group and this is well summarised in Figure 3.3.

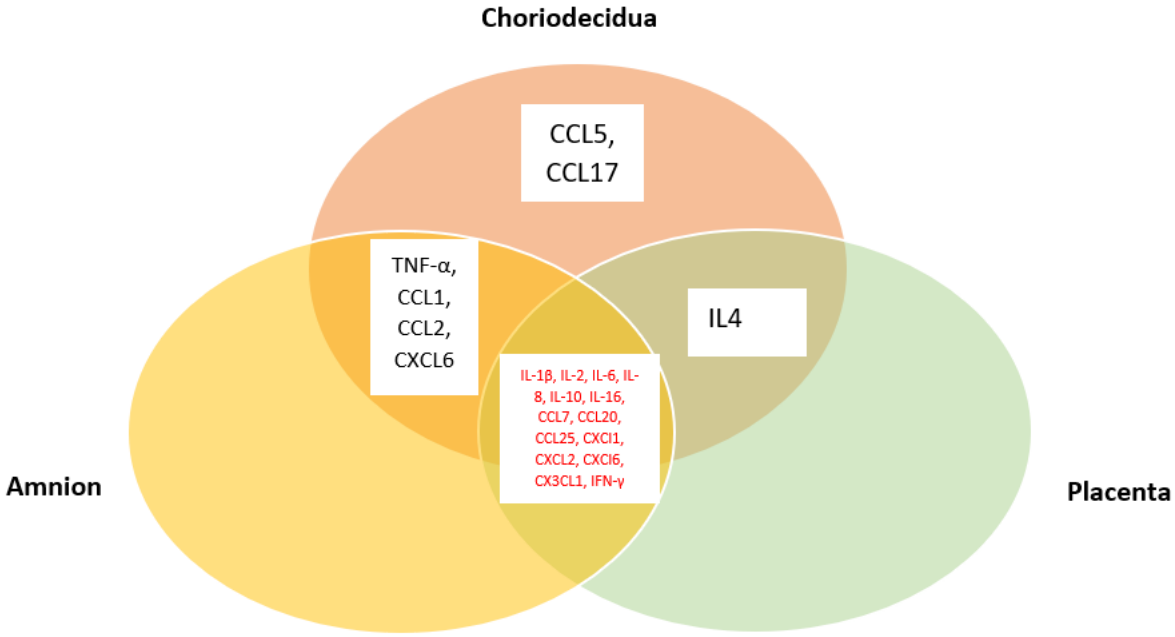


Figure 3. 3 Venn diagram summarising which cytokines/chemokines were significantly raised in the three tissues – choriodecidua, placenta and amnion in PTL subgroup of chorioamnionitis. The analytes that were globally significantly raised in all three tissues are highlighted in red.

However, whilst all three gestational tissues had significantly increased levels of all the cytokines/chemokines tested for, the CD was the most significantly concentrated with each cytokine/chemokine. The placenta was found to be the least inflammatory tissue, closely followed by the amnion, with 14 cytokines/chemokines significantly increased in the placenta versus 18 cytokines/chemokines in the amnion when compared to PTNL samples. In contrast, all 21 cytokines/chemokines analysed were significantly raised in the CD.

These results suggest that the CD is a more inflammatory gestational tissue than its neighbouring amnion and placenta. The CD is where the maternal and fetal components initially interact, suggesting that immunological changes in this interface may precede those in the surrounding tissue. Vento-Tormo and colleagues showed that many molecular and cellular mechanisms operate to generate a physiologically peaceful decidual environment (112) and alteration at this interface may lead to pathological pregnancies, although this is still not very researched.

Interestingly, IL-4 was only significantly raised in placenta and CD. This is often released by macrophages, T cells, B cells, mast cells (216), suggesting these cells may pay a role at the maternal-fetal interface in infection associated PTB. Identifying cell population changes in the placenta and CD may give us more information on which exact cells may be contributing to the increased levels of IL-4. IL-4 is well recognised for its anti-inflammatory activity and can downregulate the production of a variety of cytokines and chemokines such as IL-1β, TNF-α and IL-8 (217, 218). The significant increase in the placenta and CD during chorioamnionitis would suggest that this response is negative regulatory feedback to the global rise in pro-inflammatory cytokines and chemokines seen in all three gestational tissues. Such IL-4 negative feedback circuits have been seen in allergic airway response to protect the airways from inflammatory damage following allergen exposure (219). Bryant *et al* showed that IL-4 downregulates the lipopolysaccharide mediated inflammatory response by gestational tissues; whilst this was noticeably clear in an explant tissue model where the tissues were pre-treated with IL-4, when tested on explants post exposure to LPS, the anti-inflammatory effect of IL-4 was diminished (220). This is likely reflected in a real-life clinical setting, where the IL-4 would rise post infection and hence would not be enough to contain or control the inevitable acute inflammatory process. It is interesting that IL-4 does not seem to significantly

rise in the amnion, and this may be due to receptor concentration. IL-4 is noted to work via the IL-4 receptor alpha-chain dependent manner (220) and it is possible that IL-4 was not significantly increased in amnion as amniotic epithelium may have less IL-4 receptors compared to placenta and CD(221).

Unlike IL-4, IL-10 is significantly raised in all three gestational tissues. IL-10 is a well-recognised anti-inflammatory cytokine which plays a central role in infection by limiting the immune response to pathogens, thereby preventing damage to the host (222). It has been demonstrated that induction of IL-10 often occurs together with inflammatory cytokines, although pathways that induce IL-10 may negatively regulate these pro-inflammatory cytokines(222). The placenta, chorion and maternal decidua have all been shown to express IL-10 mRNA and secrete IL-10 protein, which is increased in human amniotic fluid during late gestation (223). Explant model experiments have shown that IL-10 is increased by LPS(224), *C.albicans*, *G.vaginalis* and *S. agalactiae,* which are pathogens associated with intra-amniotic infection especially in the choriodecidua(223). This supports the global rise in IL-10 observed from the data in the CA-PTNL group.

Interferon-γ is an extremely versatile cytokine which is known to play a significant anti-microbial and anti-viral role. It can initiate pro-inflammatory pathways in tandem with immune cells and other cytokines such at IL-4, promoting an immune-protective environment (225). This immunoregulatory and immuno-protective cytokine was significantly raised across all three gestational tissues, but especially in CD and amnion. Interferon-γ has been shown to be lower in the plasma of women who present with PPROM (226) which may suggest it has an immunomodulatory role in procuring a full-term pregnancy, or at the least that its reduced levels may increase the chances of intrauterine infection leading to rupture the membranes. Further work needs to be undertaken to consider this possibility.

Most other cytokines and chemokines were significantly increased in all three tissues, as seen in Figure 3.3, which was expected as chorioamnionitis is an acute state of inflammation, caused by infection. Chorioamnionitis has been shown by several studies to be associated with raised TNF-α, IL-1β, IL-6 and IL-8 (227-229). These cytokines along with interferon gamma, IL-4, IL-6, IL-10, and C-X-C-motif chemokine ligand-1 (CXCL1) have been shown to cause fetal injury in animal models (230). Our data complimented this finding as IL-8 was

significantly raised in the CD of the babies born with worse neonatal outcomes. A similar trend was seen for IL-6 (p=0.068), but no other inflammatory markers were associated with the worse neonatal outcomes. These data are consistent with the findings of McCartney and co-workers (231). They noted that in a non-primate model that IL-6 and IL-8 specifically in amniotic fluid were superior predictors of fetal lung injury and they were most associated with fetal lung scores. Another study also noted that umbilical cord levels of IL-6 were 2.1-fold higher in neonates exposed to clinical chorioamnionitis than among those exposed to maternal hyperthermia alone which is not mirrored when looking at maternal levels of IL-6 (232). This suggests that there may be a fetal production of IL-6, but this needs further research; our data indicates that IL-6 levels are evidently raised at the maternal-fetal interface too.

All 11 chemokines tested for in all three tissues are significantly higher in the CD in the chorioamnionitis group. This was similar in the amnion where all 9 chemokines that were measured were increased. In the PTL-CA group cytokine/chemokine levels in the CD were significantly greater than in the amnion. In contrast, the placenta demonstrated less inflammatory change with only 6 chemokines of 11 measured being increased.

MCP-1 and MCP-3 are potent attractants and activators of monocytes/macrophages and increases in MCP-1 have been described in cervical and amniotic fluid in women with intra-amniotic infection and PTL (233). It has been shown in both human and animal models to be raised during chorioamnionitis (234, 235). These chemokines are increased in amniotic fluid when there is histological evidence of chorioamnionitis (236). However, a recent study showed that MCP-1 and MCP-3 are not significantly increased in the decidua or placenta when comparing term labour and term non-labour (237), suggesting that these immune changes are due to a pathological change associated with PTL, as opposed to physiological labour.

CCL17 and CCL22 participate in recruiting Tregs to the site of inflammation (238) and both are significantly raised in the CD of the CA group, indicating that these chemokines are involved in triggering the adaptive immune system in the presence of infection associated PTL. CCL17, CCL22 and CCL2 are increased in uterine tissues obtained from mice after intra-peritoneal LPS administration by Arenas-Hernandez and colleagues who believe that infection causes an

imbalance of the innate and adaptive immune cells at maternal-fetal interface (239). CCL17 was not significantly risen in the placenta in the mouse studies.

CCL20 also known as macrophage inflammatory protein-3 is a chemokine which elicits its effects on target cells by binding and activating the chemokine receptor CCR6 (240). It is strongly chemotactic for lymphocytes and weakly attractant to neutrophils (241). CCL20 has been shown to be present in amniotic fluid in uncomplicated pregnancies with concentration increasing with gestation. This is vastly increased in presence of intra-amniotic infection mirrored by the CCL20 levels in all three tissues in the CA group. In addition to this finding, Hamill and colleagues noted that CCL20 was significantly increased with the onset of term labour which suggests it encourages lymphocyte trafficking to the uterus to play a role in the inflammatory trigger of parturition (242).

CCL25, also known as thymus expressed chemokine (TECK), is chemotactic of thymocytes, macrophages and dendritic cells (243). It acts via the chemokine reception CCR9 (244). CCL25 is a well-researched chemokine in other inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease and asthma(245), and there are various studies confirming its role in propagating and driving inflammation (246, 247). Although there is no recent evidence of CCL25's role in preterm or term labour, given its potential to drive inflammation it is unsurprising that it is significantly increased in all three tissues in response to chorioamnionitis.

CXCL1, CXCL2 and CXCL6 are members of CXC chemokine family all three chemokines are well known attractants of neutrophils (248). Although their roles are unclear in the process of labour, their ability to respond to all types of infection is well described (249-251). During an active infection, the process of neutrophil recruitment is highly regulated as excessive neutrophils recruitment can cause collateral tissue damage and disease (252) equally, too few neutrophils may fail to suppress the infection and allow it to overwhelm the host (248). This suggests that an excessive neutrophil recruitment in chorioamnionitis may result in excess inflammation and the onset of PTL.

CX3CL1/fractalkine is a large chemokine which is a unique member of the CX3C family and is expressed by inflamed endothelial cells, macrophages, neurons, and glial cells. It has been

shown to mediate leukocyte capture and adhesion of monocytes, $CD8^+$ T cells, and $CD16/56^+$ NK cells (253). It has been implicated in obstetric disorders such as pre-eclampsia, GDM and miscarriage (254-256). My data shows CX3CL1 was significantly increased in all three tissues with highest significance seen in the CD in the CA group. This corresponds with data from a Japanese research team that found using a mouse model that CX3CL1 was significantly raised in amnion epithelial cells and placenta with PTL and specifically with chorioamnionitis. This was not seen with term labour in the mouse model. They even demonstrated that the deficiency of its receptor CXC3R1 may prevent LPS-induced PTL by suppressing intrauterine macrophage recruitment(257). This implies the increased CXC3L1 observed in PTL-CA may drive the recruitment of activated/inflammatory macrophages that may play a role in the onset of PTL.

3.10.2 Cytokine and chemokine distribution in Idiopathic Labour

PTL secondary to an idiopathic cause is the most interesting and probably the least understood clinical phenomenon amongst all PTL causes. Unlike chorioamnionitis, idiopathic PTL on whole is not as inflammatory and there are other more subtle processes happening. This clinical picture has been somewhat mirrored in the cytokines and chemokines. While in the CA group there was a significant increase in nearly all the cytokines/chemokine assessed, especially in the CD, idiopathic PTL was associated with an increase in 16 of 20 cytokines/chemokines in the CD. Even more interestingly, only CXCL2 was significantly increased in the placenta.

IL-2 and IL-4 were both significantly higher in the CD in the idiopathic PTL group and, although there is a paucity of data in this area of research, Gomez-Lopez and colleagues has found similar changes in amniotic fluid(258). They noted that these cytokines with IL-13 were significantly elevated along with elevated fetal CD4+ T cells in the amniotic fluid of idiopathic PTL patients (258). They have as a result proposed a new mechanism of disease for a subset of idiopathic PTL, in which fetal T cells mediate a unique inflammatory response in the amniotic cavity, partially mediated by IL-2, IL-4, and IL-13, which precedes PTL. Further work would need to be undertaken to truly consider whether cytokines produced by fetal cells in the amniotic fluid could migrate into the CD.

IL-10 is significantly higher in the CD, this cytokine is a potent inhibitor of the immune response (259). IL-10 at low levels has been associated with PTL (260-262) which is in contrary to our data in the CD when compared to the control group PTNL. This may be due to an overdrive to re-establish an immunotolerance which is a hypothesis previously proposed by other researchers (263). IL-10 can induce Tregs which are known to promote a tolerant microenvironment at the maternal-fetal interface (264). With idiopathic PTL, this tolerance may have been lost, which in turn may cause an increase in IL-10 to promote Treg activity, as well as other tolerogenic cells including dendritic cells and macrophages (265). This has been recently demonstrated by Gomez-Lopez *et al*, who showed the functional Tregs are reduced at the maternal-fetal interface in a subset of women with idiopathic PTL. However, this was not seen in term labour, suggesting that some cases of PTL are mediated through a distinct mechanism (142). The increase in IL-10 in the CD of idiopathic PTL indicates there could be a larger proportion of IL-10 producing cells, such as Tregs, present in the tissue compared to PTNL women.

CXCL1 and CXCL2 were both very significantly increased in the CD of idiopathic labour. As discussed before these chemokines are chemoattractant to neutrophils which are immune cells typically associated with infection induced PTL. However, CXCL1 and CXCL2 can be produced by a variety of cells including macrophages, monocytes and epithelial cells (266, 267). The high levels of theses chemokines may be evidence of monocyte infiltration of the CD which has been a well-known underlying trigger for labour (268) (269) and even PTL. This is supported by the fact that MCP-1 and MCP-3 were both significantly higher in the CD which are also potent attractants of monocytes. Another possibility to consider is that idiopathic PTL is predominantly caused by sterile inflammation, which, if like other organs such as the liver, may require infiltration of neutrophils and monocytes for repair and healing (270, 271).

Another interesting finding was that IL-1β was not significantly increased in the CD (or placenta) in idiopathic PTL. This cytokine is pro-inflammatory cytokine seen in infection associated PTL and reflected in our data too (Table 3.5) (93, 210, 272). A lot of research has been undertaken into the role of IL-1β in term and PTL, as far back as 1989 (273), however its role in idiopathic PTL is not well established. Our finding may simply illustrate that the main

inducer of IL-1β is an infective trigger, which was not present in the idiopathic PTL patients, IL-1β was not raised in either tissue.

3.10.3 Cytokine and Chemokine Distribution in Preterm Labour Caused by Abruption

Placental abruption (PA) is a relatively rare complication of pregnancy yet is a very serious condition which can impact both maternal and fetal health. It is caused by the early separation of a placenta from the lining of the uterus, and this mostly occurs before 37 weeks gestation, hence it being a relevant cause of PTL. The exact aetiology of PA is unknown, however certain risk factors are well understood which include smoking, cocaine use, maternal age over 35, PA in previous pregnancy, hypertension, pre-eclampsia, multiple pregnancies, and trauma to the abdomen such as a road traffic accident or fall (274, 275).

There is limited work looking at cytokines/chemokines relating to PA, however there is a general impression that PA, along with its associated conditions of pre-eclampsia and fetal growth restriction, have a process of inflammation as part of its underlying causes (276).

The immunological alterations that occur in PA remain mostly unknown (274) and our data (Tables 3.11 and 3.12) shows that there is very little cytokine/chemokine signalling occurring in the PA group compared to the PTNL group. Of the 20 cytokines/chemokines that were analysed, only 4 (TNF-α, CCL20, CXCL1 and CXCl2) were significantly raised in the CD and placenta (IL-6, IL-8, CCL20 and CXCL2). This may be because the physical and sudden alteration that occurs in abruption bypasses the immune-mediated aspect of labour. Three of the cytokines are heavily associated with labour, however IL-8 is probably the most interesting, as it has previously been shown to increase in decidual cells secondary to thrombin. This increase in IL-8 attracts neutrophil migration and this association of decidual haemorrhage of abruption and neutrophil infiltration has been shown by Lockwood and colleagues(277). In contrast to this, our data shows that IL-8 was significantly raised in the placenta and not the CD; this may be because of the sampling technique we used. The CD that we collected was not specifically the decidua basalis which could be where the increase in IL-8 may have occurred, as this is likely the epicentre for a PA. Small quantity of decidual basalis may have also been collected when sampling the placenta which may explain the significantly higher IL-

8 levels in the placenta group. Another possibility is that due to the sudden separation of the placenta from the uterus, the increase in IL-8 may be more concentrated within the placenta.

The increased levels of IL-6 in CD and CXCL1 in placenta may also be secondary to the effect of Thrombin which has been demonstrated by Mhatre and colleagues (278). This effect was not seen with TNF-α which they also measured. Although TNF-α's role in PA has not been clearly demonstrated, its role has been studied in relation to PET which is all part of the placental syndrome pathology as show in Figure 3.4.

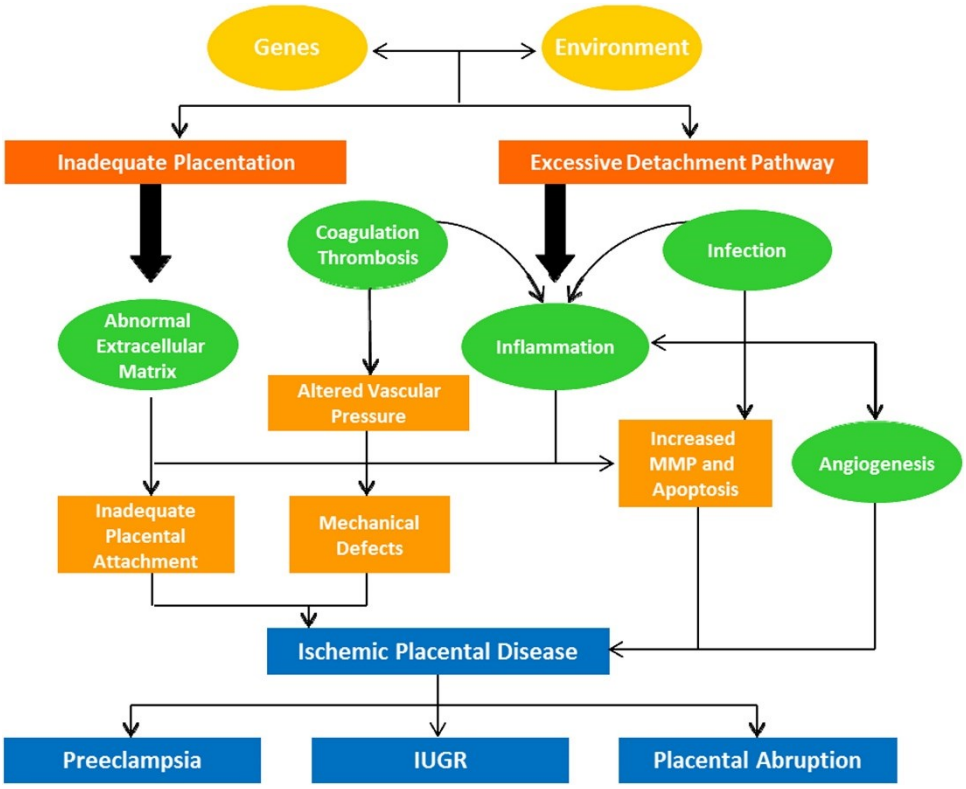


Figure 3. 4 Conceptual model for ischaemic placental disease (Ananth, 2014). Image reproduced with permission of the rights holder Elsevier, © Elsevier (IUGR -intrauterine growth restriction, MMP – matrix metalloproteinase)

It is clear when considering the published data so far, that the immunological changes seen with PA are mainly driven by Thrombin and although my thesis is not focussed on PA, it would seem considering neutrophils as an immune cell of interest in PTL could be fruitful.

3.10.4 Cytokine and Chemokine Distribution in Preterm Labour in Twin Pregnancies

Preterm birth in twin pregnancies is much higher than in singleton pregnancies. It is acknowledged that spontaneous preterm birth occurs in twin pregnancies in 60 out of 100 births (445) which is at least 8 times higher than the preterm birth rate in singletons in the UK. Additional factors with a twin pregnancy can increase the risk of PTL including chronicity (279) and intrauterine death of one twin (280). Due to the obstetric overburden on resources preterm birth places which is significantly contributed to by the increasing rates of multiple pregnancies(280) research into twin pregnancies is an ever-growing area. Much work has been done into the effects of over-distension in twin pregnancies and our group has previously shown that uterine distension is associated with an inflammatory pulse that precedes and correlates with PTL (281).

My data shows that 10 out of the 20 cytokines/chemokines analysed were significantly raised in the CD in the labouring twins' group. This number was mirrored in the placenta, in which 10 out of 20 cytokines/chemokines were significantly higher, albeit a few different ones. There are quite limited data on decidual cytokines in twin pregnancies, however IL-8, which was significantly increased in both the CD and the placenta, has been noted to be associated with spontaneous PTL in twin deliveries (282, 283). In addition, Lee *et al* found that CCL2 was also higher in the mid-trimester samples of amniotic fluid in women who went on to have preterm twin deliveries and this association was seen in our CD cohort too.

IL-10 plays an important anti-inflammatory role and was noted to be significantly raised in both CD and placenta in the TwL cohort. There is evidence that IL-10 is an important regulator of decidual NK cell function (284, 285) and although there is little published evidence on IL-10 in the decidua in twin pregnancies regarding PTB, Agra and colleagues recently looked at IL-10's possible role in pre-eclampsia (286). They noted that there was no significant increase in decidual IL-10 in pre-eclampsia which may suggest that its lack of anti-inflammatory response may contribute to the development of pre-eclampsia(286). In contrast, IL-10 is significantly higher (Table 3.13) in both CD and placenta of TwL group which suggests a negative feedback response in which IL-10 is increased to counteract the inflammatory effect of other cytokines such as IL-8 and IL-1β (287).

CCL2 was significantly higher in the CD from the TwL group, and this chemokine has been shown by our group to increase in the myometrium of a non-human primate model with

overdistension (281) and previously also in a rat model (288). CCL20 was also noted to be increased in the CD and the placenta of the TwL group which is interesting as our group had seen this previously in the myometrium in PTL patients and had hypothesised that this effect may well be due to mechanical stretch (289). The unanswered question is of course whether these chemokines initially increase in the maternal-fetal interface, triggering an influx of inflammatory cells and its associated cytokines which finally act to trigger labour via increasing myometrial contractility.

Similar to idiopathic PTL, CXCL1 and CXCL2 are significantly increased in the CD, which as described before can be produced by macrophages and monocytes, and it is also chemoattractant to neutrophils. It is interesting that in both non infection related PTL i.e., idiopathic and TwL, CXCL1 and CXCL2 were significantly elevated. I think this emphasises that these cells may play an important role in the decidua when it comes to the pathological trigger of PTL in the absence of infection.

CXC3L1 is significantly increased in both CD and placenta in TwL group, this is a chemokine that can enable migration of NK cells and is known to also augment cytolytic activity of NK cells (290). This further suggests that NK cells could play a significant role especially at the maternal-fetal interface in the process of PTL.

3.11 Comparing key biomarkers of inflammation in amnion and choriodecidua

The purpose of undertaking this inflammatory marker panel was to understand whether the inflammation process was greater in the CD than in the amnion, as both these tissues are anatomically adjacent to one another. One of my hypotheses was that choriodecidua is a more inflammatory gestational tissue than its neighbouring amnion and placental tissues. Chorioamnionitis is a highly inflammatory condition, however when making the simple comparison of these biomarkers in the CD versus the amnion, there is heightened inflammation in the CD with 23 out of the 37 biomarkers analysed being significantly higher in the CD compared to amnion. This suggests that CD as the gestational tissue at the maternal-fetal interface plays a significant role immunologically in chorioamnionitis associated PTB.

In the idiopathic PTL group only four biomarkers (Figure 3.5) were significantly raised in the CD which were APRIL, BAFF, MMP-2, and Osteocalcin. APRIL (proliferation inducing ligand) and BAFF (B cell activation factor) are members of the TNF superfamily which can be produced by macrophages, monocytes, dendritic cells, activated T cells, NK cells, and neutrophils (291). This is suggestive that these cells may play a role in the decidua as a trigger of idiopathic PTL. Although little evidence can be found regarding APRIL and BAFF in pregnancy, it is interesting that in asthma it has been noted APRIL and BAFF can be produced by infiltrating inflammatory immune cells, which promotes the survival of these cells in the airways during inflammation. There is also some evidence to suggest that BAFF may inhibit T regulatory cells although further work is required to confirm this (292). It is possible that APRIL and BAFF may play a similar role in the inflammatory process triggering idiopathic labour, hence they are significantly increased in the CD of idiopathic PTB women.

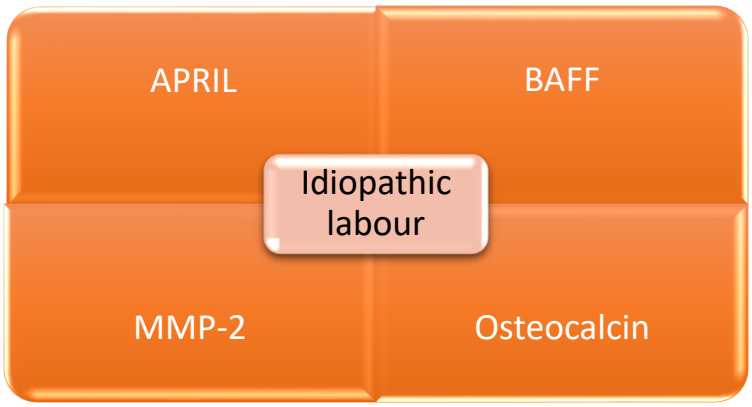


Figure 3. 5 Biomarkers that had increased significantly in the CD in the Idiopathic group. APRIL (proliferation inducing ligand), BAFF (B cell activation factor) and MMP-2 (matrix metalloproteinase-2) were significantly increased with p<0.05, Osteocalcin was significantly increased with p<0.01

MMP-2 is a matrix metalloproteinase which becomes activated to degrade the matrix of the chorion, amnion, and cervix. This causes weakening and later rupture of the membranes and cervical softening, supporting dilatation, myometrium contractions, and placental detachment (293). Its role in PTB has been investigated, however there are varying findings; one study has shown that slightly lower levels of vaginal MMP-2 is associated with PTB (293) whereas other studies have shown increased human placental levels and mice myometrial

levels of MMP-2 is associated with PTB respectively (294, 295). There has also been evidence suggesting that MMP-2's action may be varied based on the location within the gestational tissue, and that its activity was highest in the decidua albeit in term samples (296). This finding is supported by the difference we have noted between our matched amnion and CD samples in all three groups that were analysed (CA, Idiopathic and TwL). This globally increased levels of MMP-2 in decidua across all the labouring groups suggests that it may play a role in membrane rupture, contractions, and potentiating labour.

Osteocalcin is an osteoblast-specific secreted protein which acts as a hormone by stimulating insulin production and increasing energy expenditure and insulin sensitivity in target organs (297). Osteocalcin has been shown in other parts of the body to increase IL-6 (298), a myokine which is also a cytokine that is well known to be associated with labour. Interestingly in adipocytes Osteocalcin can also reduce the production of IL-10 (299). The increased level of Osteocalcin in the CD could be acting to reduce IL-10 production in the CD, in turn reducing the inhibition of inflammatory signalling and promoting changes that lead to labour.

Looking at the TwL group, more inflammatory biomarkers (16/37) were increased in the CD than compared to the Idiopathic group. There were also significantly reduced levels of some biomarkers (5/37) in the amnion. Focusing on only the biomarkers that had differed in concentration compared to the control twin non-labouring group, 10 biomarkers (seen in Figure 3.6) were increased in the CD whilst none were changed in amnion.

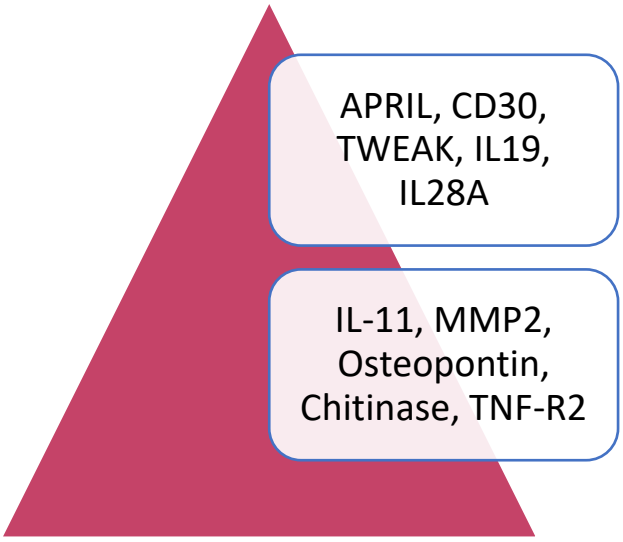


Figure 3. 6 Biomarkers that had increased significantly in the CD in the Twin Labouring Group – the top group were significantly increased with p<0.01 and the bottom group were significantly increased with p<0.05.

CD30 is another member of the TNF receptor family. Much of research into CD30 has been focused on its role in haematological cancers and autoimmune diseases such as Rheumatoid Arthritis and Inflammatory bowel disease, where serum CD30 levels are known to be high. CD30 is expressed on activated B, T cells and NK cells (300). It can stimulate T cells to produce cytokines such as IL-2, TNF and IFN-γ (301). The increased CD30 expression in the CD of twin pregnancies may be due to activation of lymphocytes in response to the distension of tissues, and the increased proportion of activated cells may further promote PTL signals.

TWEAK is a TNF superfamily member and can act as a multi-functional cytokine (302). It has been shown in a mouse model to participate in parturition activation via interaction with furin which is involved in uterine activation in labour (303). In research into asthma, TWEAK has been noted to induce both CCL2 and IL-8 which are all associated with the potential to drive inflammation needed to trigger labour (304).

IL-19 belongs to the IL-20 subfamily of cytokines, which includes IL-19, IL-20, IL-22, IL-24, and IL-26, and is primarily produced by leukocytes (monocytes, macrophages, B cells). Its immunological role is still unclear (305). There are studies that suggest it has an anti-inflammatory role by inducing monocytes to produce IL-10 (306, 307), others have suggested it is pro-inflammatory cytokine via its positive induction of TNF-α and IL-6 (308). Interestingly

IL-19 has been studied within the fetal membranes before, albeit at term when it was noted that IL-19 is present in both chorion and amnion. Menon *et al* also identified that IL-19 has a stimulatory effect on IL-6 but an inhibitory effect of TNF-α, however they noted that this effect was dose dependent (309) so the significantly increased levels of IL-19 seen in our data, may in fact tip its role to be pro-inflammatory contributing to the trigger of labour in these twin pregnancies. More recently new evidence has suggested that IL-19 is an essential regulator of neutrophil development, significant enough to consider it a treatment for neutropenia (Xiao *et al*, 2021). This is an interesting discovery in the context of PTB, as neutrophils have been suggested to be a key innate immune cell in the decidua producing other pro-inflammatory cytokines such as IL-1B and IL-8 (93, 310).

IL28-A is a member of the IL-10 cytokine family (311). There has been little work looking specifically at IL28A levels in gestational tissues; a couple of studies have measured IL28A levels in maternal serum, one finding no difference in IL28A serum levels with the incidence of PTB (312), and another, in active term labour, found lower serum levels of IL28A compared to that during the third trimester (313). This study contrasts with the change noted in the CD in the TwL group and this may be due to several reasons including sample (serum vs gestational tissue), gestation (term vs preterm) and number of babies (singletons vs twins).

IL-11 belongs to the family of IL-6 cytokines and is known to have an immunoregulatory role (314). IL-11 has been a cytokine of interest as it is known to be present in the endometrium and its deficiency is associated with infertility and miscarriage (315). In contrast a murine model has shown that an increase of IL-11 in the decidua can be associated with PTB (316). This suggests that IL-11 is only required in early post-implantation and its natural decline is associated with term labour (317). *In vitro* studies have shown that IL-11 can directly modulate macrophage activity through inhibition of TNF-α, IL-1β and IL-12 production (314). High levels of IL-11 in the decidua may be an immunoregulatory response to inflammation associated with PTL.

Osteopontin is a chemokine like protein which is known to have different biological functions in different stages of pregnancy (318). It has been shown to be an important chemokine for embryo implantation (319) and placental development (320). In the decidua, Osteopontin is secreted by trophoblast cells and immune cells including NK cells and macrophages (321). It

can also induce the migration of immune cells such as T cells, macrophages and dendritic cells and participate in various inflammatory reactions. It can also up-regulate IL-12 and down-regulate IL-10 (322). The increase seen here in Osteopontin in the CD in twin pregnancies may be a consequence of increased number of NK cells and macrophages in the decidua, which may propagate inflammation further by attracting other immune cells such as T cells and dendritic cells.

Chitinases are enzymes which belong to the 18 glycosyl hydroxylase (GH18) family, a class of ancient, conserved evolutionary enzymes (323). The potential role of chitinases has been identified in the manifestation of various allergies and inflammatory diseases (324). They exhibit their roles in both innate and adaptive immunity (325) and are a potential biomarker of activated macrophages in tissue (326). Increased chitinases could be an indication of macrophages becoming activated, switching from its tolerogenic state to possibly a more pro-inflammatory role in the decidua to trigger labour (327).

TNF-R2 is a member of the TNF receptor superfamily (328). TNF is a is a pleiotropic inflammatory cytokine which acts via TNF-R1 or TNF-R2 (329). Recent work has shown that TNF-R2 is preferentially expressed by highly suppressive and replicating Tregs and TNF-R2 signalling leads to the activation and proliferation of Tregs (330). The increased levels of TNF-R2 seen in the CD of TwL group may be a reactive immune response counteracting inflammation by activating Tregs.

3.12 Strengths and Limitations

Although the work presented in this chapter covers a vast range of inflammatory markers mainly in the form of cytokines and chemokines in different gestational tissues, it only suggests which immune cells may be involved in the process and importantly trigger of labour, especially as many of the inflammatory markers can be induced by different immune cells or even non-immune cells. An attempt was made at identifying the cells that may have been present in the stored tissue using immunohistochemistry however undertaking it on frozen fetal membranes (which were not snap frozen) led to some concern regarding quality of tissue. Hematoxylin and eosin stain of a sample set of 24 CD was undertaken and reviewed by Professor Sebire and team at UCL Gos institute of Child Health. Unfortunately, due to the

poor material quality and high artefact it was agreed that undertaking further immunohistochemistry on these samples would not be advisable, as the immunostaining would not be reliable and deemed inadequate for histological diagnosis. Therefore, further work using these samples was abandoned.

Another limitation of this study is that CD was a combination of both decidua and chorion, and ideally these tissues should have been sampled separately and more specifically.

The study nevertheless allowed us to review matched tissues (CD, placenta and amnion) of a large cohort of 75 patients subdivided into smaller cohorts based on their clinical indication for delivery. Using large panels enabled us to analyse for 19 inflammatory markers which were based on our group's earlier work on myometrium (Sivarajasingam *et al*, 2016) whereas the other 37 inflammatory markers were undertaken using a pre-designed Human Inflammation panel.

3.13 Summary

CD seems to be overall a more inflammatory gestational tissue when compared to placenta and amnion (Figure 3.7).

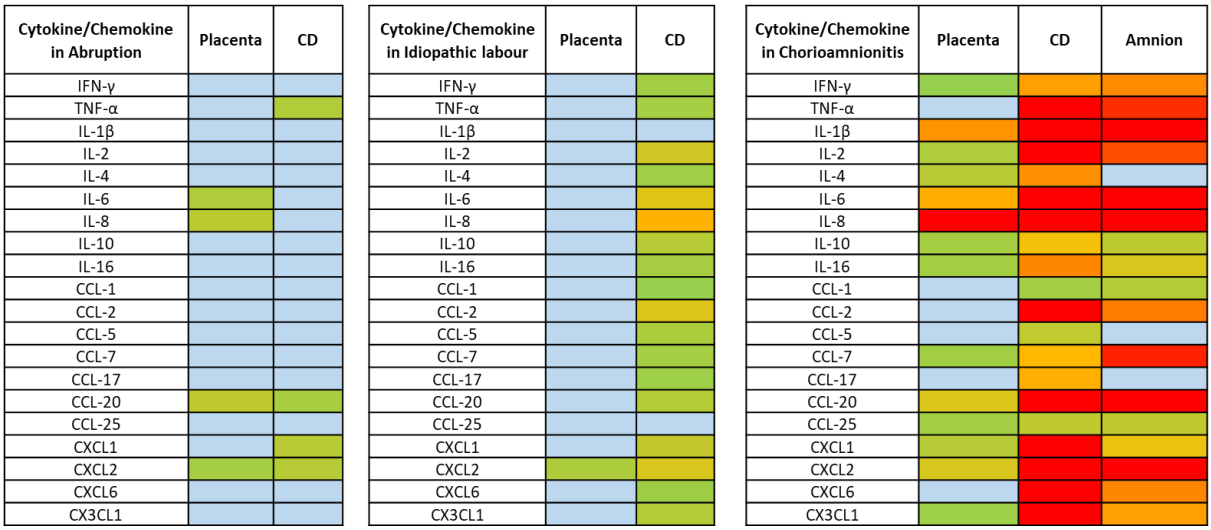


Figure 3. 7 Heatmap summarising the cytokine/chemokine changes in placenta, CD and amnion (where undertaken) in singleton pregnancies complicated by abruption, idiopathic labour and chorioamnionitis. Light blue colour indicates not significant. Green to red indicates statistical significance. Green = up to 5x ratio of cytokines/chemokines when compared to its respective PTNL sample. Orange = up to 10x ratio of cytokines/chemokines when compared to it respective PTNL sample. Red = more than 10x ratio of cytokines/chemokines when compared to its respective PTNL sample.

Cytokines and chemokines on whole were significantly increased in the CD, placenta, and amnion in the CA group, with 14/19 analytes being significantly increased in all 3 gestational tissues. In the idiopathic group, contrasting to my hypothesis, immunoregulatory cytokines such as IL-2, IL-4 and IL-10 were significantly raised in the CD, with no change seen in matched placental samples. This highlights that idiopathic PTB is not a simple switch in immune tolerance and a far more complex process requiring a lot more work. IL-8 levels in the CD were significantly associated with worse neonatal outcomes in the CA group, however this significance was not seen in relation to IL-6 or TNF-α which may be due to the small number of patients studied within each neonatal outcome group (good outcome n=5, adverse outcome n=6).

Considering all the cytokines and chemokines discussed in this chapter and following review of current literature on immune cells (Figure 3.8) that these inflammatory messengers may induce or respond to, monocytes, NK cells (lymphocytes), T cells and neutrophils were repeatedly represented. Figure 3.9 summarises that not only did CD have a higher increment

in cytokines and chemokines when PTL was triggered by CA than placenta but also that there was more activation of neutrophils and monocytes to counteract the infective trigger. Figure 3.10 highlights that a very similar cohort of cytokines/chemokines were increased in CD of idiopathic labouring women; IL-1β did not feature which is well known to be more activated by infection (273). There was a significant rise in 1L-10 which may be to activate Tregs in a negative feedback loop to increase immune tolerance (67). These findings formed the basis for my next chapter looking at immune cells using flow cytometry.

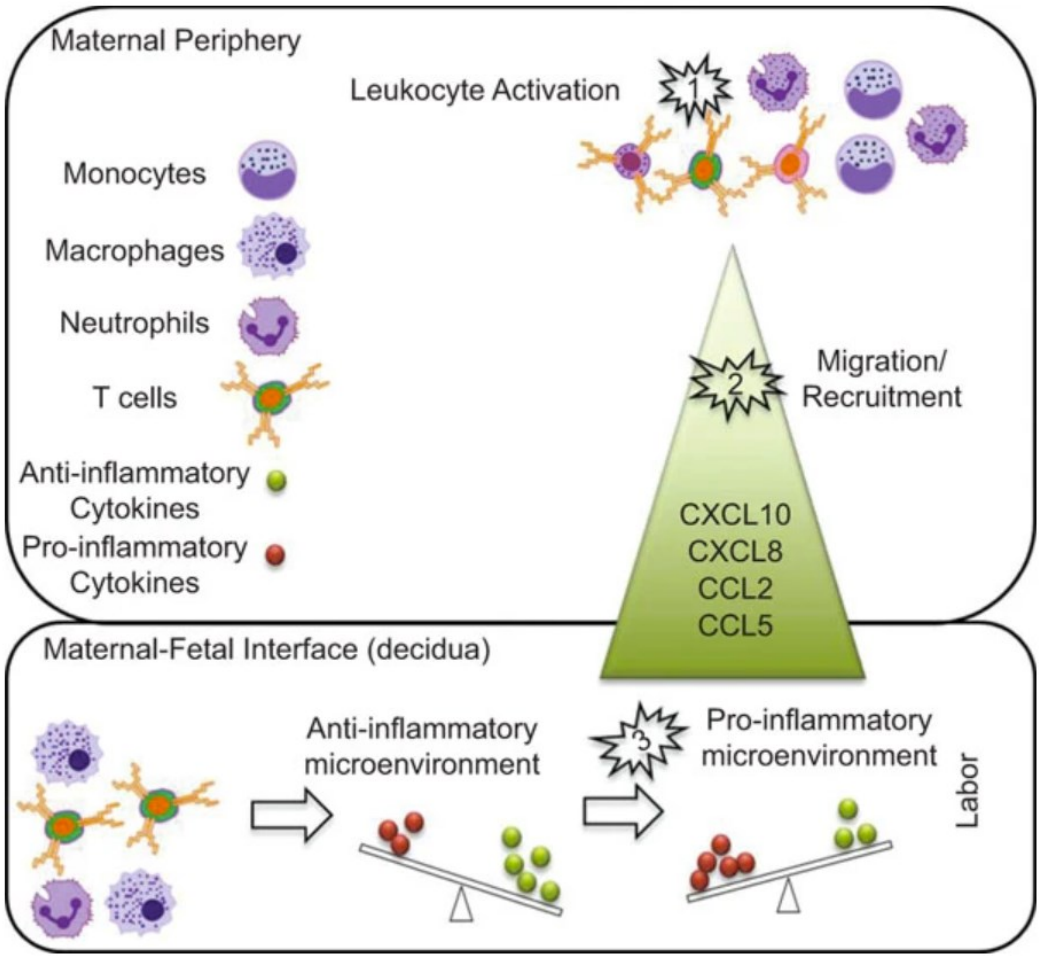


Figure 3. 8 A suggested pathway leading to PTL or term labour focusing on the maternal-fetal interface (Gomez-Lopez, StLouis, Lehr, Sanchez-Rodriguez, & Arenas-Hernandez, 2014). Reproduced under the Creative Commons CC-BY-NC-SA license.

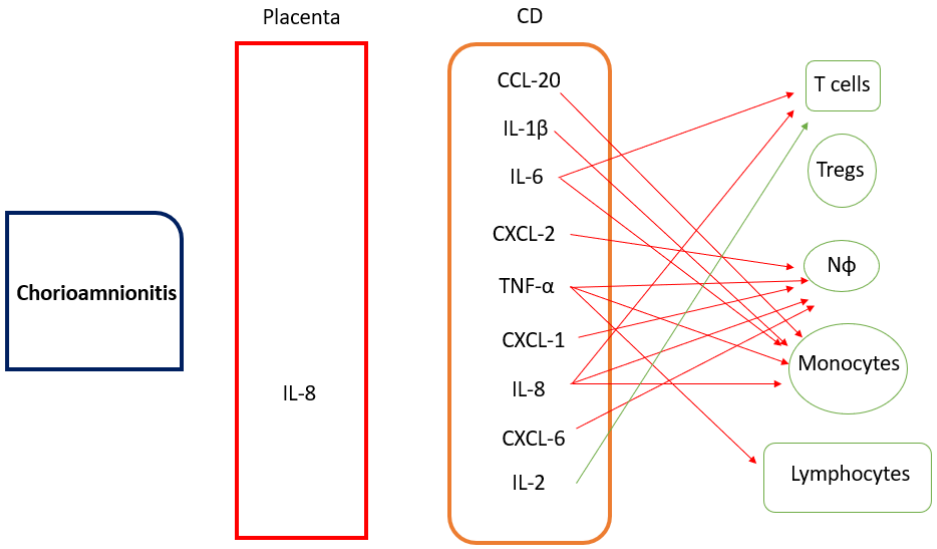


Figure 3. 9 A summary of the cytokines/chemokines that were increased in placenta and CD in chorioamnionitis by at least 10 times the concentration seen in PTNL samples. Red arrows indicate a pro-inflammatory effect, green arrows indicates a regulatory effect. Nϕ = Neutrophils.

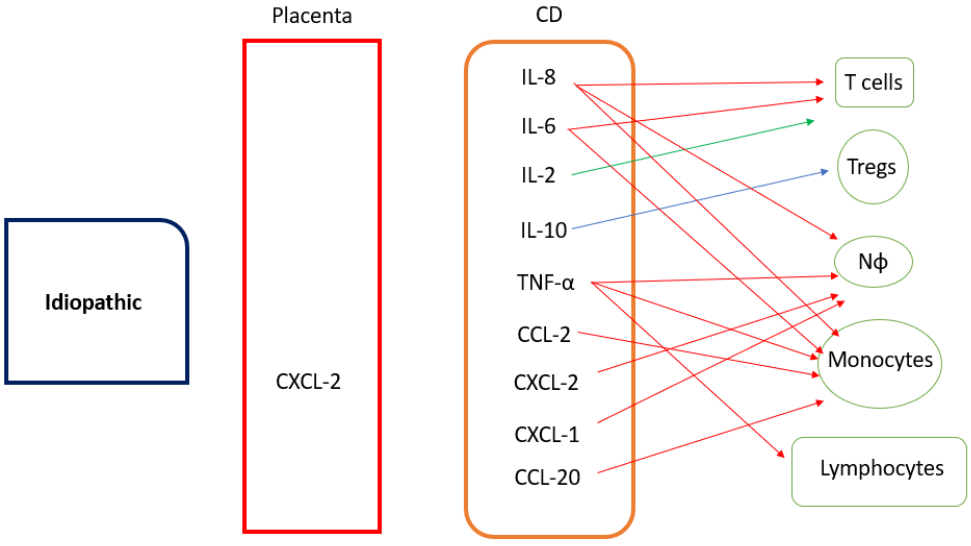


Figure 3. 10 A summary of the cytokines/chemokines that were increased in placenta and CD in idiopathic labour by at least 10 times the concentration seen in PTNL samples. Red arrows indicate a pro-inflammatory effect, green arrow indicates a regulatory effect, blue arrow indicates an anti-flammatory effect. Nϕ = Neutrophils.

4. Immune cells in the decidua during term and preterm labour

4.1 Introduction

In both humans and murine pregnancies labour is held to be an inflammatory process, potentially driven by fetal antigen exposure or innate immune system stimuli (141). As a result, much work has been focused on characterising immune response in peripheral blood, as not only is it easier to collect maternal blood samples but there is a drive to identify a possible predictive marker in peripheral blood samples. Nevertheless, to understand subtle immunological changes that may precipitate both term and preterm labour, studying the local inflammation at the maternal-fetal interface would be both more informative and relevant.

The decidua is commonly understood to be the maternal-fetal interface and is developed from endometrial cells in early pregnancy (331). The decidua can be divided into three different parts depending on its anatomical location – decidua basalis, decidua parietalis and decidua capsularis (332). The decidua basalis develops from the endometrium where the embryo implants and this covers the basal plate of placenta. Decidua parietalis lines the fetal membranes (Figure 4.1) and the decidua capsularis covers other parts of the uterus (332).

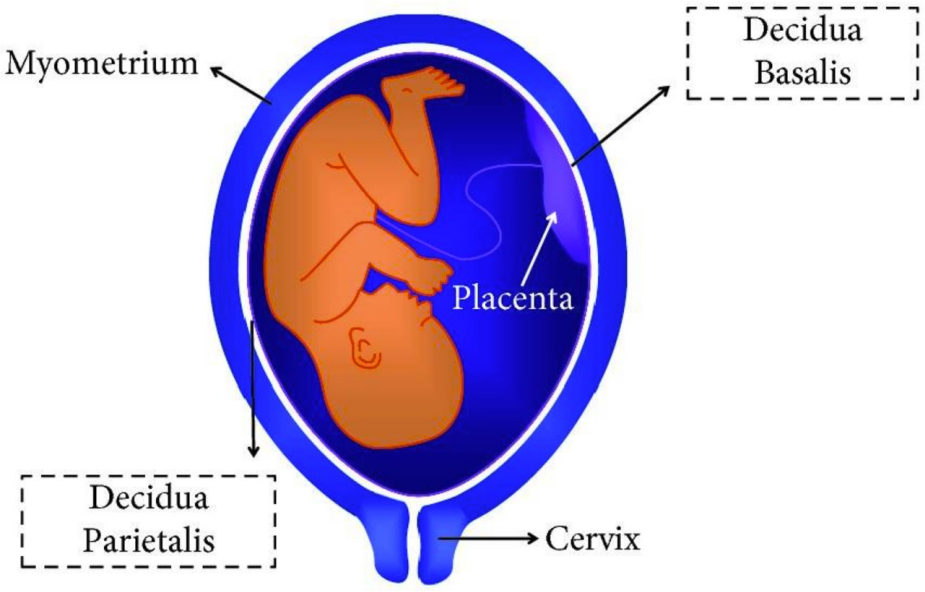


Figure 4. 1 The anatomical position of the decidua parietalis and decidua basalis (Slutsky et al., 2019) Image reproduced under the Creative Commons Attribution License. https://creativecommons.org/licenses/by/3.0/

For a pregnancy to carry to term successfully, a tightly regulated equilibrium of immune cells is required at the maternal-fetal interface. The decidua basalis, due to its location, contains immune cells of both maternal and fetal origin, it is therefore considered as the major site of the most numerous and complex maternal-fetal cellular interactions (333). This was my reason to sample the decidua basalis for my immune cell work. In addition, Jacobs *et al* confirmed that fetal membranes without the decidua contained a small proportion of immune cells, however these membranes did show a significant increase in all $CD45^{+}$ cells in term labour compared to term not in labour (334). This finding may be further explored and understood by studying the decidual immune cells, which are denser in proportion than the fetal membranes (334).

4.1.1 Immune cells in the decidua

The decidua is known to harbour several immune cells which has been described in several studies (55, 192, 333). In the first trimester, it is believed that up to 40% of all decidual cells are leukocytes (24, 115). NK cells constitute up to 70% of the decidual leukocytes (335). They play a crucial role in the initiation and resolution of inflammation (336) and are detected in all phases of pregnancy. Contrasting to peripheral NK cells which are 90% $CD56^{dim}$ NK cells,

decidual NK cells are predominantly CD56^{bright} NK cells, which produce a variety of cytokines with weak cytolytic activity (28), suggesting that NK cells have a regulatory role in the decidua.

Decidual NK cells have been shown to be involved in several aspects of pregnancy (Figure 4.2) including implantation (337), placentation (115) and even fetal development (338). As a result, they have been implicated in several pathological conditions including endometriosis, recurrent pregnancy loss, and pre-eclampsia (335). Nevertheless, their role in parturition is still incompletely understood. It has been suggested that NK cells play a role in regulating labour as expression of the single cell signatures of NK cells was upregulated in women with spontaneous labour at term compared to gestational age matched controls without labour (339). Aside from this recent study, most recent work looking at decidual NK cells has been focused on early pregnancy, IVF, and implantation failures (340, 341). However, what is very interesting is that there is a proposal that NK cells and their role in recurrent miscarriages may also predict risk of preterm birth and other obstetric conditions (342).

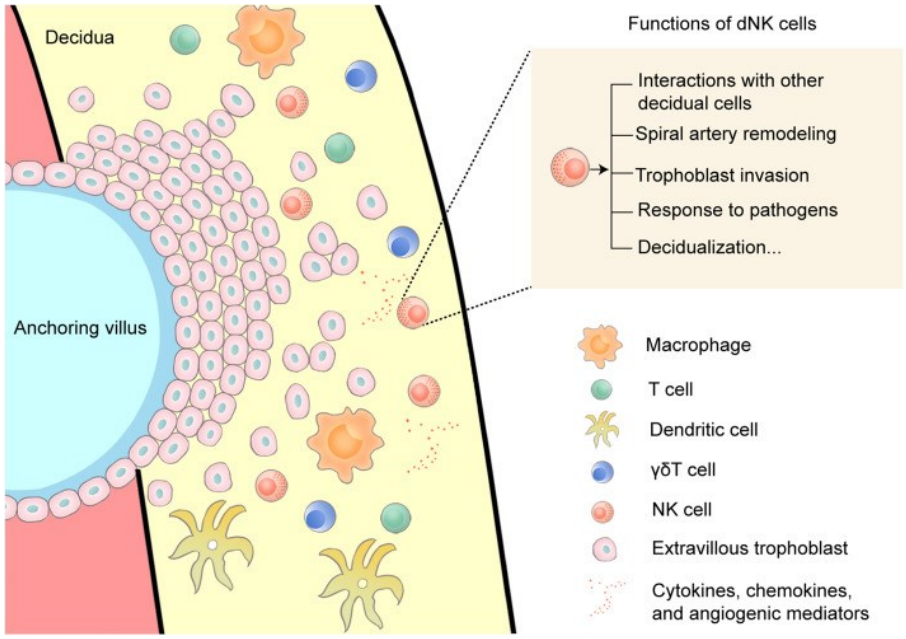


Figure 4. 2 Function of decidual NK cells in pregnancy (L. Li, Feng, Zhou, Liu, & Li, 2021). Image reproduced under the Creative Commons Attribution license. https://creativecommons.org/liceanse/by/3.0/

Macrophages are innate immune cells that reside in tissues where they detect and ingest dead cells and debris. They also process and present antigens but are phenotypically and functionally heterogeneous (55). Monocytes are the sole precursors to tissue macrophages (343). Monocytes can be subdivided into classical, intermediate, and non-classical subsets. Classical monocytes ($CD14^+CD16^-$) have a vital role in combating pathogens via phagocytosis, generation of oxygen species (ROS) and they produce cytokines in response to pattern recognition receptors (344). In contrast to this, non-classical monocytes ($CD14^-CD16^+$) are poorly phagocytic but are efficient cytokine producers (344). Intermediate monocytes ($CD14^+CD16^+$) have been suggested as being monocytes in transition from classical to non-classical; they exhibit both phagocytic functions and anti-inflammatory effects (345). Monocytes have been implicated in the pathogenesis of PTB and an elevated levels of $CD14^+$ monocytes have been observed in the peripheral blood of mothers who delivered prematurely (346). Kim *et al*. have also shown that an increased levels of intermediate monocytes in maternal blood are associated with PTB (347).

At the maternal-fetal interface, monocyte-derived macrophages are important for defence against infection and, M2 macrophages, may be crucial to tissue remodelling and immunomodulation during pregnancy (348). They are also well recognised to become activated in labour in response to decidual inflammation (349) to enable tissue remodelling and clear apoptotic debris (343).

T cells are a major component of the adaptive immune system and can be divided into multiple subpopulations that may possess diverse functions (333). Interestingly they become the primary immune cells of the decidua in the third trimester, mainly due to the decline in decidual NK cells (53). T cell subsets can be classified into $CD4^+$ helper T cells and $CD8^+$ cytotoxic T lymphocytes which are effector $CD8^+$ T cells (116). $CD4^+$ T cells include 4 main subsets which are Th1, Th2, Th17 and regulatory T (T_{reg}) cells according to their cytokine profiles (333). It has been shown that there is a significant increase in the proportion of $CD4^+$ T cells in term labouring decidua compared to term non labouring decidua (350). In addition, studies have shown that a proportion of the maternal T cells found at the maternal-fetal interface display an exhausted or dysfunctional phenotype at term pregnancy (192, 351). This

further demonstrates a mechanism in which effector T cells are precluded from disrupting normal gestation.

T_{reg} cells are a special subset of T effector cells in the decidua which creates a tolerogenic microenvironment through the production of cytokines such as IL-10 (352). Their immunosuppressive functions are primarily exerted by direct cell-cell interactions with the target cell, consumption of IL-2 and the release of anti-inflammatory molecules (353). Decreased T_{reg} frequencies in both peripheral blood and the decidua have been shown to be associated with pregnancy complications such as recurrent pregnancy loss (354) and pre-eclampsia (353).

Neutrophils are a major group of innate immune cells which have been thoroughly studied in pregnancy. They are typically involved in acute inflammation; phagocytic in nature and release granules containing lytic enzymes and produce reactive oxygen intermediates with antimicrobial activity (89). They are normally short lived, however there is evidence to suggest they may survive longer in the presence of certain cytokines such as IFN-γ and TNF-α (86). Neutrophils may also have an anti-inflammatory/suppressive effect as has been shown in tumour microenvironment and chronic disease (99). Neutrophils have been shown to interact with cells of both the innate and adaptive immune systems, in particular macrophages, T cells and NK cells (86, 99, 100, 355).

Hamilton *et al* have shown that neutrophils are higher in decidua in women with preterm labour associated with chorioamnionitis than in women with term gestations (with and without labour) and in women with spontaneous preterm labour/birth without chorioamnionitis (211). Neutrophils have been seen to be important in infection associated preterm labour and pathological preterm premature rupture of membranes (356). The recruitment of maternal neutrophils may be due to decidual derived chemokines such as CXCL8 and these neutrophils release inflammatory mediators and MMPs in both term and preterm labour (229, 357).

4.2 Hypotheses

1) $CD56^{bright}$ NK cells decrease in the decidua with the onset of term labour compared to term non-labouring decidua.
2) Intermediate monocytes are increased in decidua with the onset of term labour compared to term non^{-}labouring decidua and in preterm samples.
3) T_{reg} cells are depleted in the decidua with the onset of term labour compared to term non-labouring decidua.
4) Neutrophils will be depleted in the decidua with the onset of term labour compared to term non-labouring decidua.

4.3 Aims

In this chapter I plan to look at NK cells, monocytes, T cells and neutrophils using flow cytometry. I will specifically identify the proportional differences of these immune cells in placenta, decidua, maternal and cord blood in different clinical settings:

1) Term Non-Labour (TNL)
2) Preterm Non-Labour (PTNL)
3) Term early labour (TeL)

Sub-groups of the immune cells will be looked at to consider their functional role in the different clinical settings. These include:

1) NK cells – $CD56^{bright}CD16^{+}$, $CD56^{+}CD16^{+}$, $CD56^{-}CD16^{+}$
2) Monocytes – Non-classical ($CD14^{-}CD16^{+}$), Intermediate ($CD14^{+}CD16^{+}$), Classical ($CD14^{+}CD16^{-}$)
3) T cells – $CD4^{+}$ T cells, $CD8^{+}$ T cells, $CD4^{+}T_{reg}$, $CD8^{+}T_{reg}$
4) Neutrophils – $CD15^{+}CD16^{+}$

The choice of immune cells to be investigated on a flow cytometry panel was based on two factors; current literature and further focused by the cytokine/chemokine results observed in chapter three.

4.4 Sample collection and methodology

The approval for collection of choriodecidua, placenta and amnion were obtained from the Chelsea-Brompton Research Ethics Committee (REC) (REC number: 10/H080/145).

Once fully informed written consent was obtained from the patients, the agreed samples of placenta, decidua, maternal and fetal blood were collected from women who underwent a caesarean delivery. The women who had term non labour delivery were women who attended for elective caesarean sections for obstetrically uncomplicated reason such as previous caesarean delivery, maternal request, and malpresentation such as breech baby. The term early labour samples were collected from women who went into early labour before their planned caesarean section date. PTNL samples were collected from women who underwent a caesarean section for fetal reasons such as fetal growth restriction. Exclusion criteria for recruitment included multiple pregnancy, induction of labour, augmentation of labour and vaginal bleeding. The tissue was collected immediately after delivery of the placenta by me and Dr AnnieBelle Sassine (PhD student) in the labour ward, Chelsea and Westminster Hospital and processed within two hours of delivery.

Decidual basalis (DB) tissue was collected centrally from the maternal aspect of the placenta and matched placental tissue 1x1x1cm was collected directly beneath the DB biopsy site. Matched cord blood (20ml) was drawn from the cord using a needle and syringe and placed in lithium heparin vacutainers. Matched maternal blood (20ml) was also collected in lithium heparin vacutainers just prior to anaesthetic at time of cannulation by the anaesthetist in charge of the patient to minimise patient discomfort. All samples were kept at room temperature and processed as soon as possible, within 4 hours of collection, and all analysis was completed on non-cryopreserved samples.

4.4.1 PBMC/Cord Blood Processing

All blood samples were overlayered on to a Ficoll-Hypaque gradient for density gradient centrifugation. Whole blood was gently layered onto Histopaque (Sigma-Aldrich) at 1:1 ratio in a sterile 50 mL tube and centrifuged at 2000 revolutions per minute (RPM) (800 relative centrifugal force (RCF)) for 20 minutes at room temperature, with no braking at deceleration,

to separate the blood components into layers. The mononuclear layer was recovered and washed once in 40ml PBS and centrifuged at 1800 RPM (700 RCF) for 10 minutes. The supernatant was discarded, and the cells were resuspended in 50ml of PBS for counting.

4.4.2 Decidua Basalis/Placenta Processing

Following collection of the tissues, excess blood was removed from the tissues using sterile gauze and then the tissue was placed in 20ml PBS in a sterile Falcon/centrifuge tube. Once samples were back in the laboratory villous tissue and blood vessels were further removed from the DB to maximise purity of the maternal-fetal interface and placental tissue. Once the tissues were optimally cleaned the DB and placenta are placed in 50ml of PBS in a Falcon/centrifuge tube. The tissue was then put through a two-stage disaggregation process, firstly mechanical disaggregation followed by enzymatic disaggregation (described in detail in Chapter 2 Materials and Methodology).

The digested tissue was then passed through a 70µm cell strainer and suspended in 20ml FACS wash buffer (FWB). This was then gently layered onto 20ml Histopaque (Sigma-Aldrich) at 1:1 ratio in a sterile 50 ml Falcon/centrifuge tube and centrifuged at 2000 rpm (800 RCF) for 20 minutes at room temperature, with no braking at deceleration, to separate the tissue components into layers. Leucocytes are found in the interface between the density gradient media and the FWB. This mononuclear layer was carefully aspirated using a plastic Pasteur pipette and washed once more in 40ml FWB and centrifuged at 1800rpm for 10 minutes at room temperature. The supernatant was discarded, and the cells were re-suspended in 5ml of FWB for counting.

4.4.3 Cell Counting

Twenty µl of cell solution was added to 20µl of Trypan blue dye (Sigma-Aldrich) and 10µl pipetted into a KOVA Glasstic® slide (Hycor Biomedical Inc, Edinburgh, UK). Using microscope cells were counted in 2 sets of 9 square blocks and the average number calculated. The average was multiplied by 2 to account for the Trypan blue dilution, by the volume of cell solution (V), and by the KOVA chamber factor (F = 10,000) to reach the total cell count. Thus, formula used to determine total number of cells in sample was as follows: Av x 2 x F x V = T

4.4.4 Statistical analyses

All data were treated as non-parametric. When comparing three groups or more in paired data, Friedman's test was used, and multiple comparisons were controlled for with the false discovery rate (FDR). When comparing only two matching groups, Wilcoxon matched pairs signed rank test was used. For comparing unpaired groups, Kruskal-Wallis test was used to compare three groups or more. FDR was also used to control for the multiple comparisons. When comparing only 2 non-matching groups Mann-Whitney test was used. When comparing between patient cohorts, TNL were our control group, PTNL and TL groups were compared to TNL patients to evaluate gestation-related and labour-related changes. $P<0.05$ was considered statistically significant. Simple linear regression correlation was undertaken with Spearman's rank test and results are presented with confidence interval, R value and p values.

Statistical analyses were performed with GraphPad Prism version 9 (GraphPad, San Diego, CA, USA).

4.5 Flow Panel

One flow cytometry panel was designed to study the cells discussed above. The decision for a single panel was based on total cell number collected from DB. It took nearly 6 months to develop and finalise a cell collection protocol which consistently provided 1 million live cells from the DB; hence I limited staining to a single flow panel. This panel (Figure 4.3) was designed with the guidance of Mrs Parisa Amjadi, FACS facility manager, Centre for Immunology and Vaccinology. The aim of this flow cytometry panel was to stain for the immune cells which were identified to be key from the work undertaken and discussed in Chapter 3.

Fluorochrome	BV786	BV711	BV605	BV421	AmCyan	FITC	PE	APC	PerCP	APC-H7	PE- Cy7
Looking for	CD56	CD4	CD8	CD127	Aqua	CD14	CD15	CD16	HLA DR	CD3	CD25
Significance	NK cells	Lymph/ T cells	T cells	T regs	Live/ Dead	Mono	Neut	NK	General activation	Lymph/ T cells	T regs

Figure 4. 3 Flow cytometry panel design

Details of surface staining and titrations are detailed in Chapter 2 Materials and Method section 2.9.2. All samples were run as soon as they were processed which meant samples were run on different days. To ensue sample acquisition was longitudinally comparable, the flow cytometer was optimised using the Perfetto (194) method. This provided target MFI values to adjust the detectors to each day using fluorescent beads. This adjustment ensured consistent acquisition of sample data throughout the length of this project.

4.6 Results

4.6.1 Demographics of patients included in this study

Below is a table summarising relevant demographics of each woman recruited into this study. There was no difference in maternal age between the three groups. Gestation was significantly different between the TNL and PTNL groups.

Table 4. 1 The demographics of the patients who were consented and sampled for these experiments.

	PTNL (n=9)	TNL (n=15)	TEL (n=6)	P value
Age (years)	34 IQR (30.5 - 36.5)	35 IQR (32.0 – 41.0)	33 IQR (26.0 – 38.5)	ns
Gestation (weeks)	33.0 IQR (30.4 – 36.3) ****	39.0 IQR (39.0 – 39.3)	38.0 IQR (37.9 – 38.7)	**** p<0.0001
Ethnicity: White British White Other	 2 3	 5 6	 1 3	

Black African	2	2	0	
Asian	2	1	1	
Other	0	0	1	
Parity				
0	6	3	1	
1	3	8	3	
2	0	2	1	
>2	0	2	0	
Women with:				
1) Previous term births	3	12	4	
2) Previous preterm births	0	0	0	
3) Miscarriages <16 weeks	2	4	0	
4) Miscarriages >16 weeks	0	0	0	
5) Early TOP<16 weeks	1	0	0	
6) Late TOP >16 weeks	0	0	0	
PTNL reasons for birth:				
1) Fetal growth restriction	3	N/A	N/A	
2) Pre-eclampsia	3			
3) Other causes	3			

4.6.2 Natural killer cells in term non-labouring women

Natural killer cells were gated for using the gating strategy indicated in Figure 4.4.

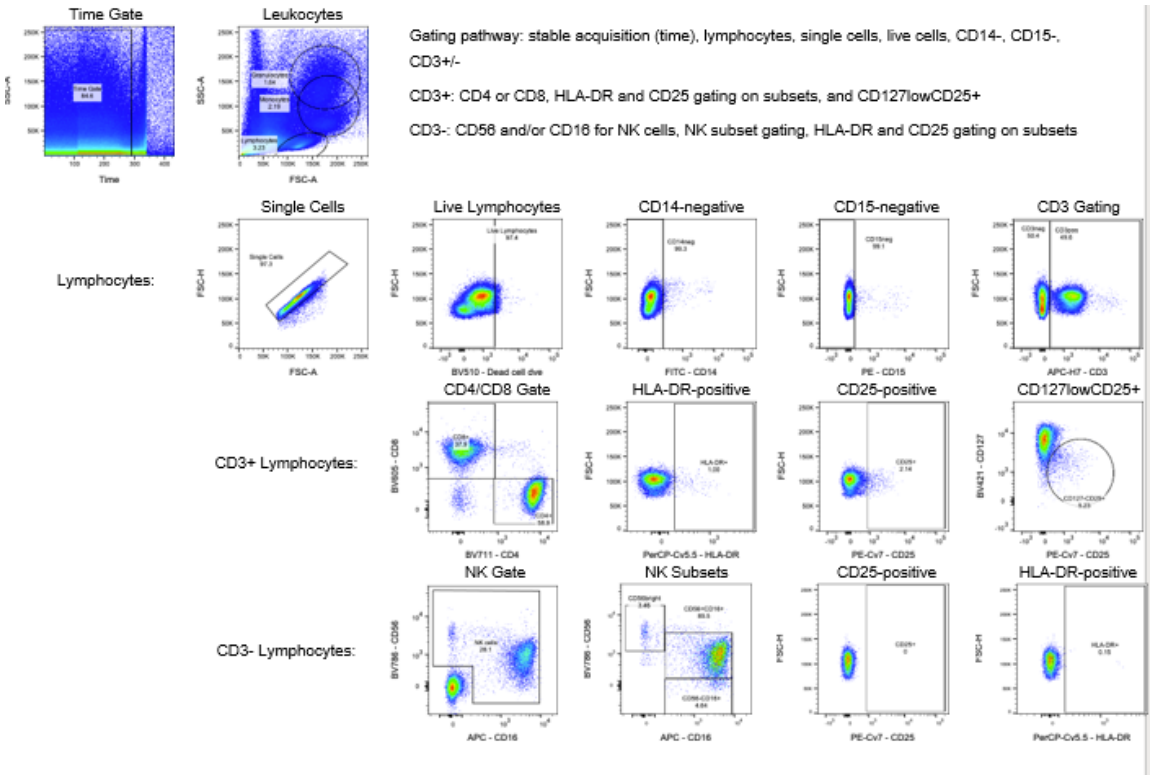


Figure 4. 4 NK cells were identified by gating initially for lymphocytes. This was divided into CD3- cells which was further gated for NK cells and finally subdivided into the three sub-groups – CD56brightCD16-, CD56+CD16+ and CD56-CD16+.

Term non labour samples were used as the control group to which both PTNL and TEL samples were compared to. Term non labour samples were taken from completely healthy elective caesarean section patients uncomplicated by any pregnancy pathology, hence deemed most likely to represent the immune-quiescent environment of a term pregnancy. Comparison of both the other groups to TNL should highlight the differences which may be involved in triggering the inflammatory process that is labour.

Total NK cells were significantly higher in both the decidua and placenta compared to peripheral blood (PB) and cord blood (Figure 4.5). NK cells found most in the decidua were $CD56^{bright}$ NK cells whereas the majority in placenta, PB and cord blood were $CD56^{+}CD16^{+}$ cells. $CD56^{bright}$ NK cells were significantly higher in the decidua than in the other three compartments. $CD56^{+}CD16^{+}$ NK cells were significantly higher in PB and significantly lower in the decidua in comparison. $CD56^{-}CD16^{+}$ NK cells were low in all four compartments.

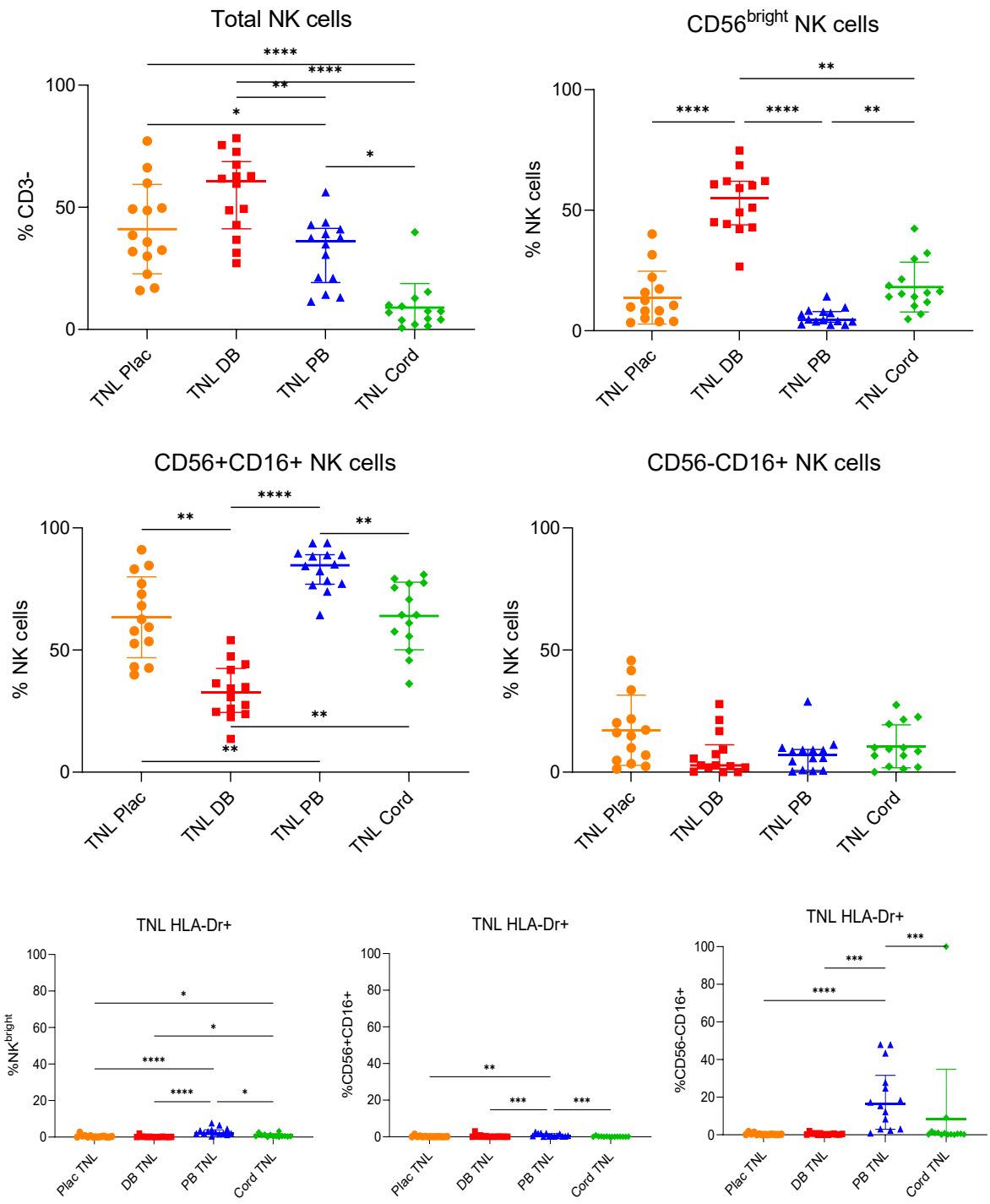
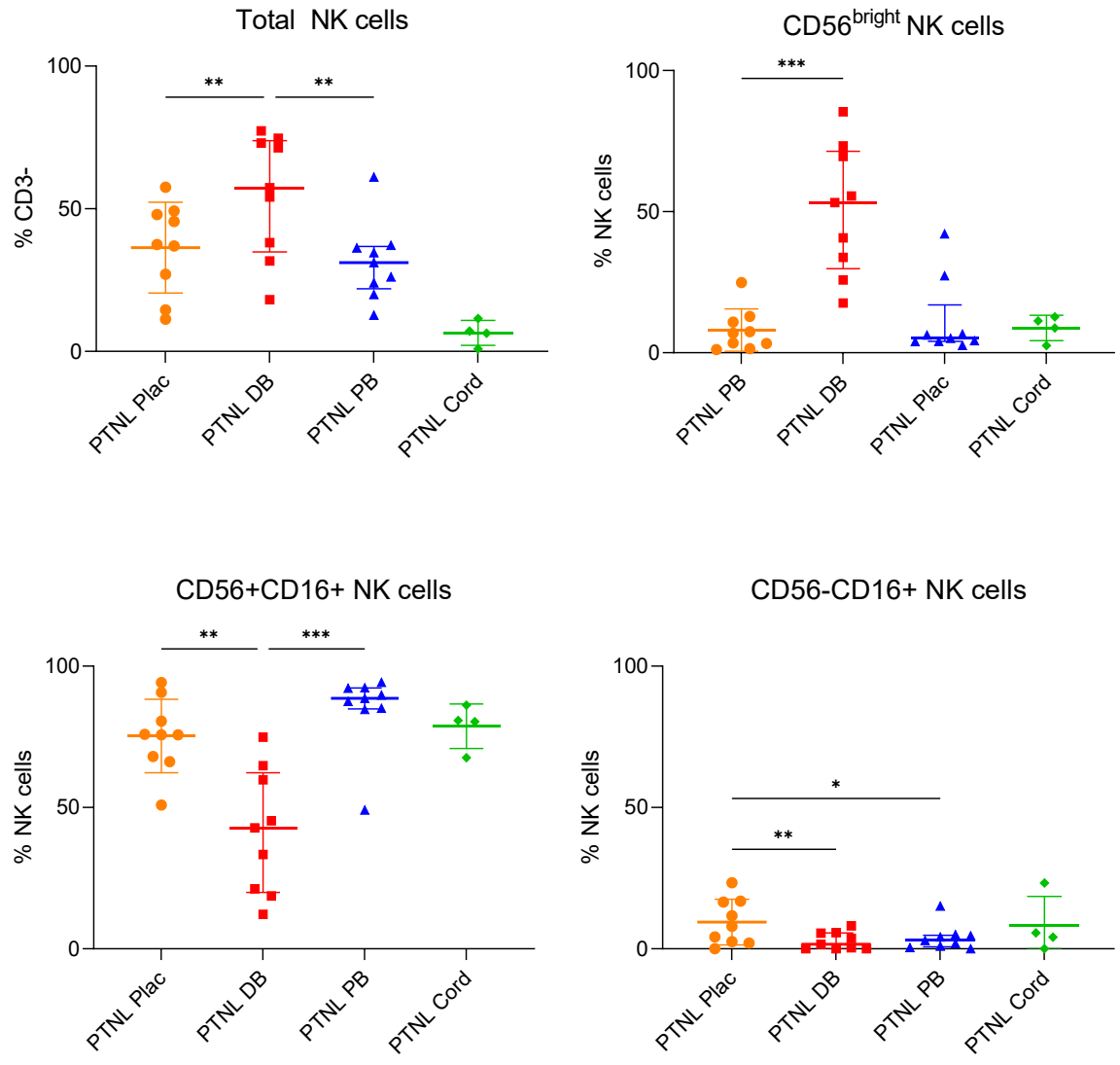


*Figure 4. 5 Proportion of NK cells (%), its subsets and HLA-DR expression in term non labouring samples identified in placenta, decidua (DB), peripheral blood mononuclear cells (PB) and cord blood. P value is based on the non-parametric Friedman's test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p<.001). Error bars indicate median and interquartile ranges. TNL n=15*

4.6.3 Natural killer cells in preterm non labouring women

In PTNL women total NK cells were significantly higher in the decidua when compared to the placenta and peripheral blood (Figure 4.6). CD56^{bright} cells were still the predominant NK cell group in decidua and were significantly higher than in placenta ($p<0.001$) whereas CD56^{+}CD16^{+} cells were the predominant NK group in the other three compartments. In contrast to TNL, in PTNL CD56^{-}CD16^{+} NK cells were significantly higher in the placenta than decidua and PB.



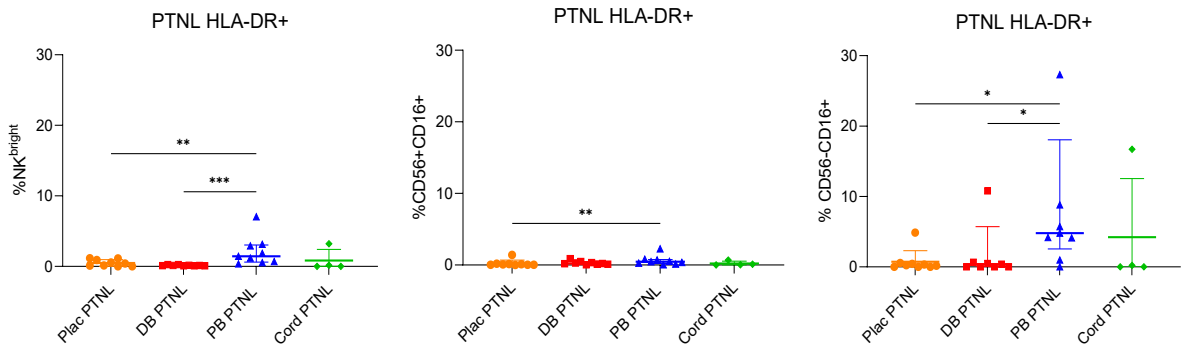
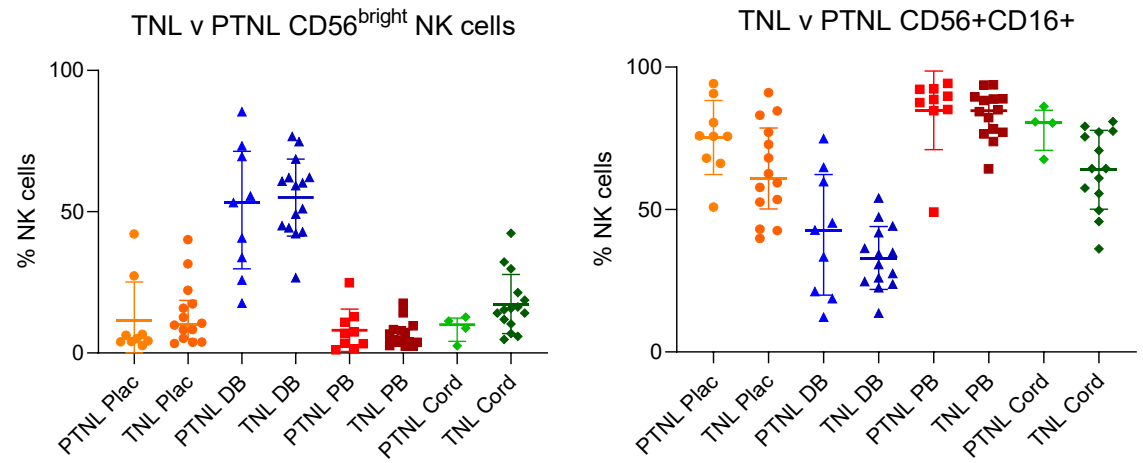


*Figure 4. 6 Proportion of NK cells (%) their subsets and HLA-DR expression in preterm non labouring samples identified in placenta, DB, PB and cord blood. P value is based on the non-parametric Friedman's test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. PTNL n=9*

On the whole, the total NK cells were present in similar proportions in all compartments (Figure 4.7). $CD56^{bright}$ NK cells were present in similar proportions in all compartments. $CD56^{-}$ $CD16^{+}$ NK cells were present in low proportions in all four compartments from PTNL to TNL. There were no significant differences noted in any NK cell group in any compartment when comparing PTNL to TNL.



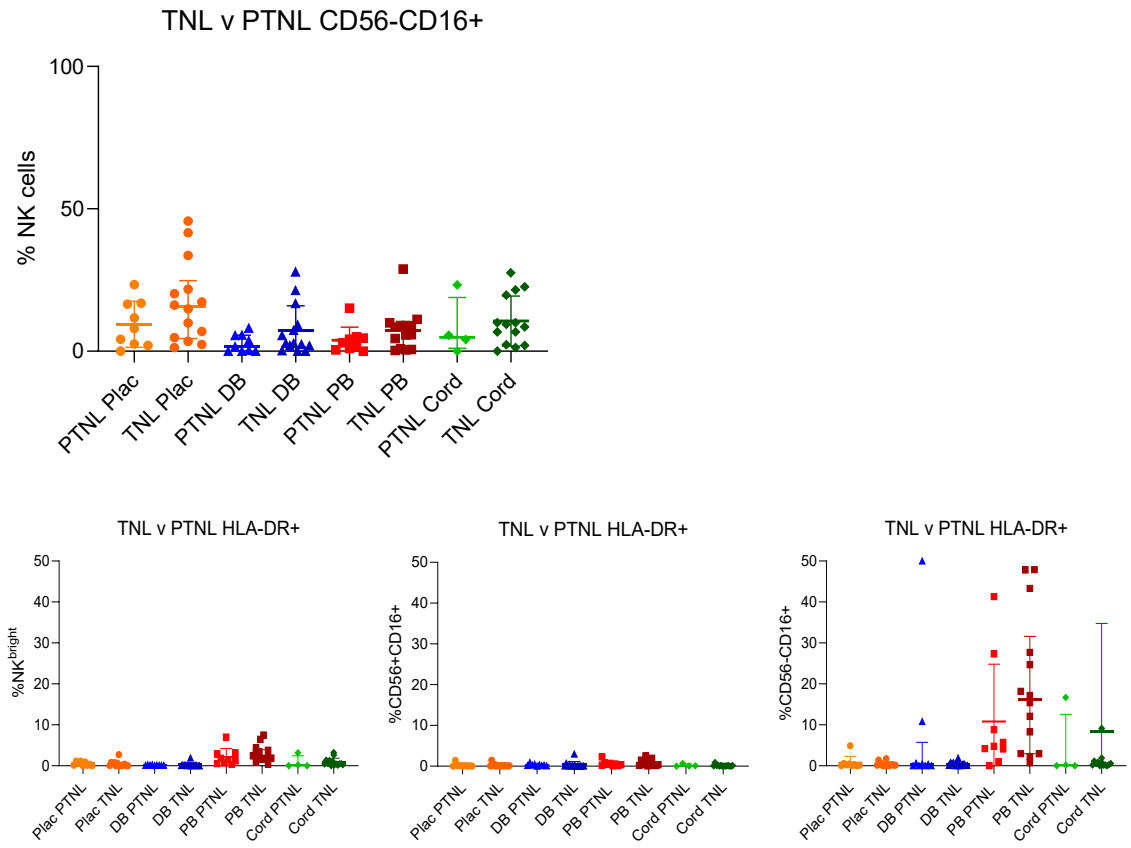


*Figure 4. 7 Proportion of NK cells (%), their subsets and HLA-DR expression in preterm non-labouring samples compared to TNL samples in placenta, DB, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p<0. 001), error bars indicate median and interquartile ranges. TNL n=15, PTNL n=9*

and decidua when comparing TEL to TNL (Figure 4.8). This contrasted with PB where there were higher total NK cells. $CD56^{bright}$ NK cells were lower in decidua (not significant) when comparing TEL to TNL $CD56^{+}CD16^{+}$ NK cells were on whole higher (not significant) in placenta when comparing TEL to TNL. $CD56^{-}CD16^{+}$ NK cells were on whole unchanged across all four compartments with the onset of labour.

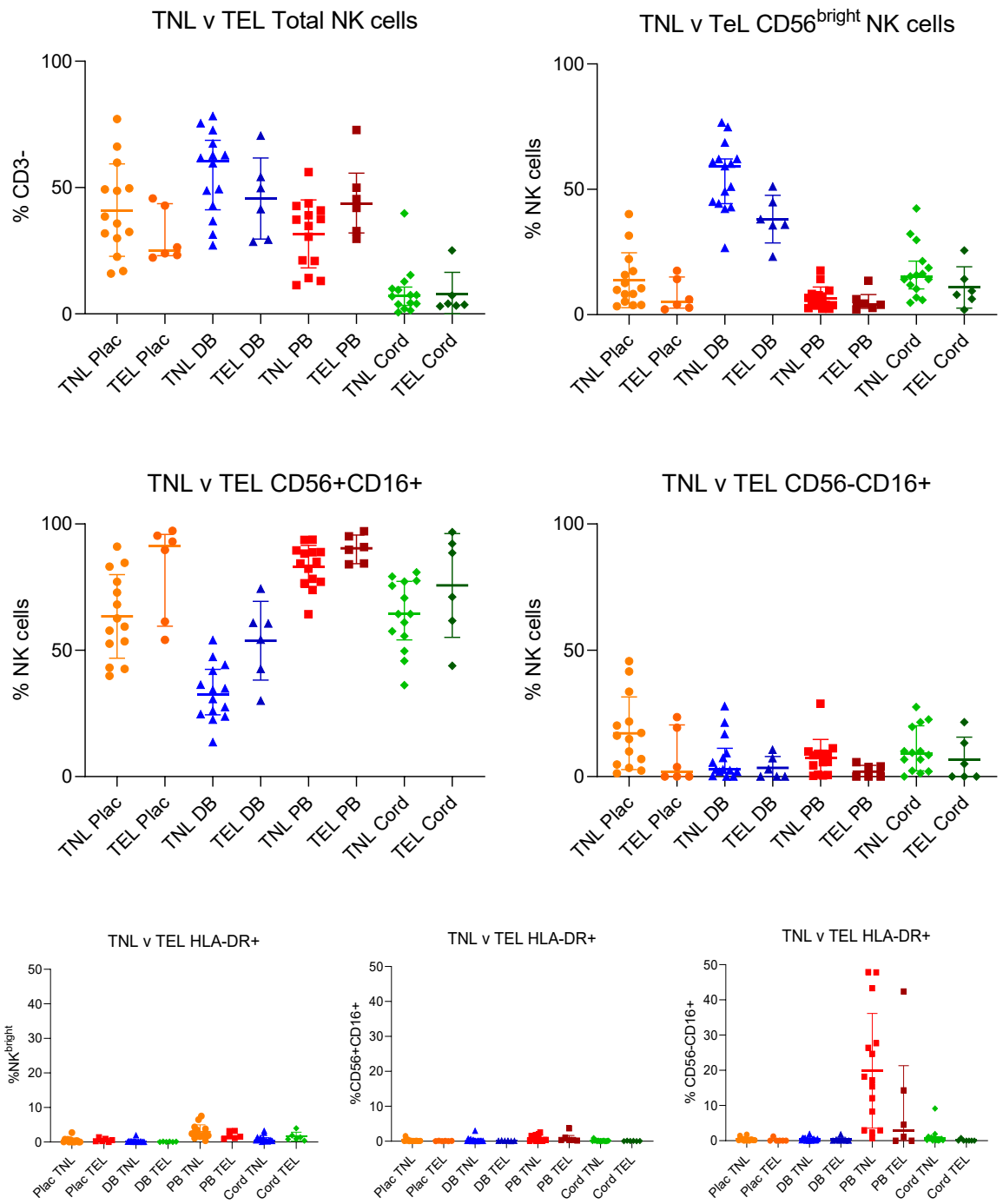


*Figure 4. 8 Proportion of NK cells (%), their subsets and HLA-DR expression in term early-labouring samples (TEL) compared to TNL samples in placenta, DB, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. TNL n=15, TEL n=6*

4.6.6 Monocytes in TNL samples

Monocytes were gated for using the gating strategy (358) shown in Figure 4.9.

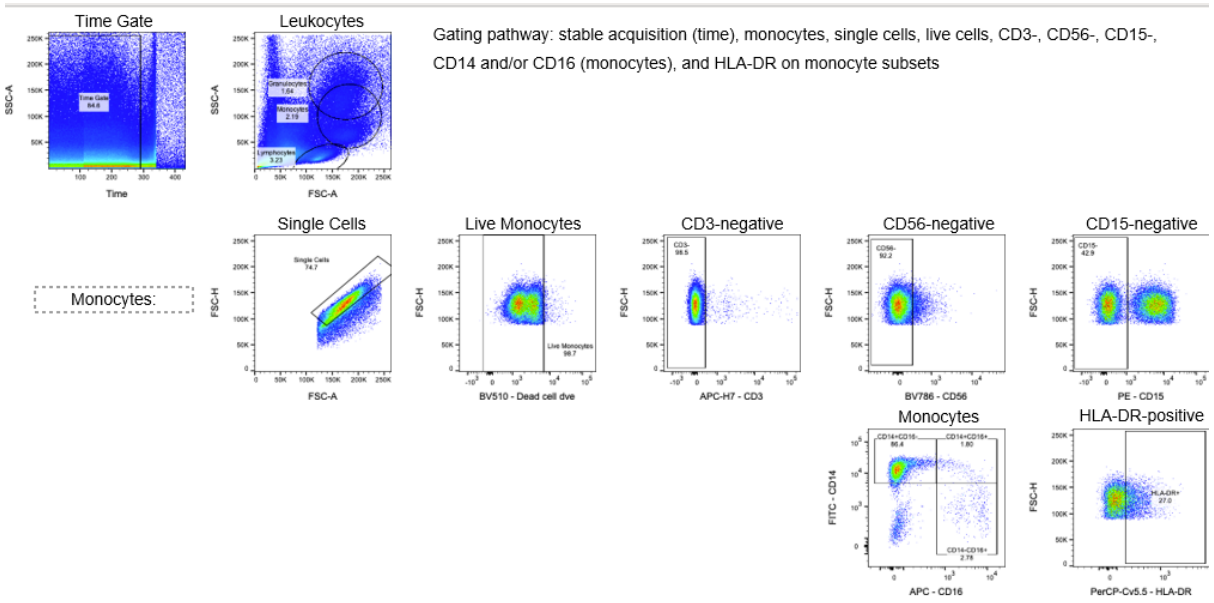


Figure 4. 9 Gating strategy for monocytes. Live monocytes were gated to be CD3 negative, CD56 negative and CD15 negative. The monocytes were then gated with CD14 and CD16 to identify the three sub-groups of monocytes.

The predominant monocyte subset in all four compartments were the classical monocytes which was significantly higher in cord blood compared to PB ($p<0.05$, Figure 4.10). Intermediate monocytes were significantly lower in cord blood than the other three compartments (placenta $p<0.001$, decidua $p<0.001$, cord $p<0.01$). Non-classical monocytes were significantly higher in PB than in the other three compartments (placenta $p<0.01$, decidua $p<0.01$, cord $p<0.05$).

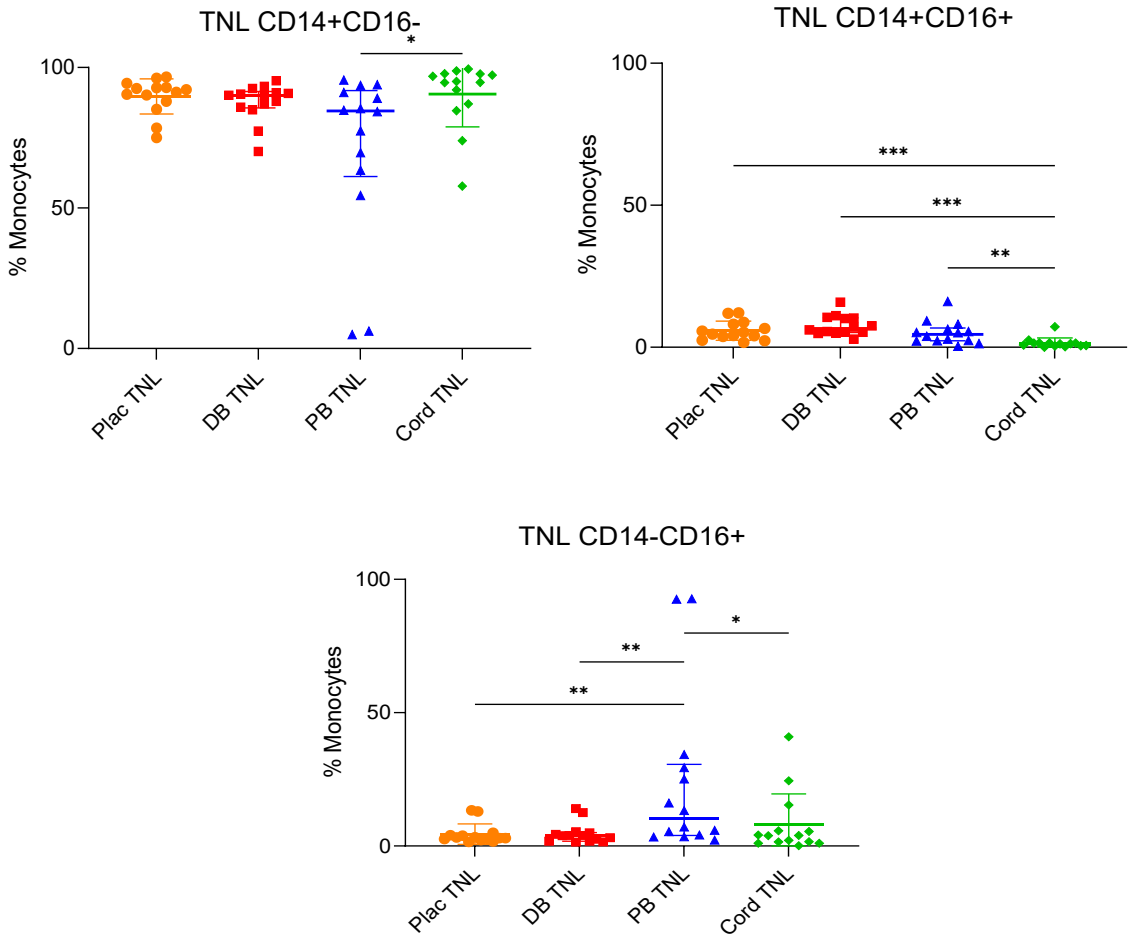


*Figure 4. 10 Proportion of monocytes subsets (%) in term non-labouring samples (TNL) in placenta, DB, PB and cord blood. P value is based on the non-parametric Friedman's test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. TNL n=15*

4.6.7 Monocytes in PTNL samples

In PTNL the same distribution of monocytes was observed as in TNL, with classical monocytes being the predominant sub-group in all compartments (Figure 4.11). There were no significant differences seen in any monocyte subset between the four compartments.

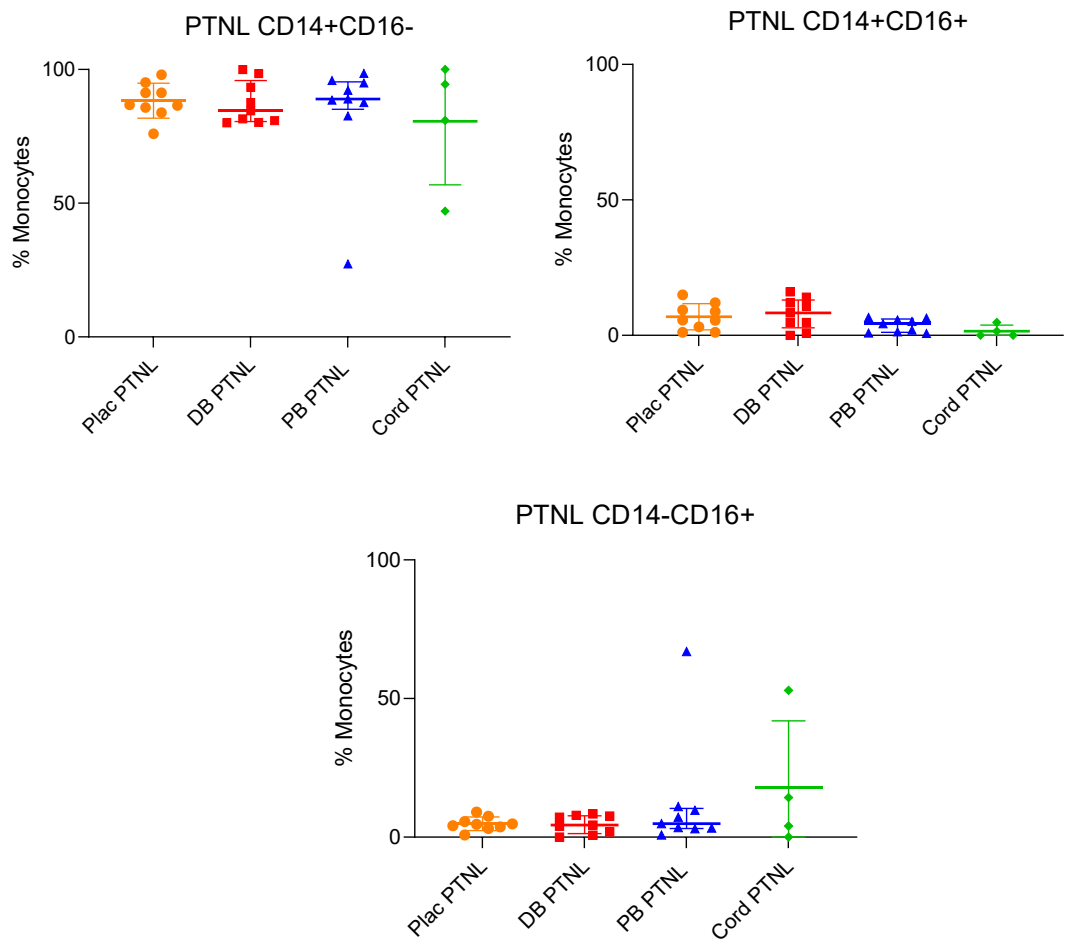


*Figure 4. 11 Proportion of monocytes subsets (%) in preterm non-labouring samples (PTNL) in placenta, DB, PB and cord blood. P value is based on the non-parametric Friedman's test with multiple comparisons controlled for with false discovery rate. *Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. PTNL = 9.*

4.6.8 Monocytes – Comparison of PTNL to TNL

There were no significant differences in any of the monocyte subsets in any of the four compartments when comparing TNL to PTNL (Figure 4.12). However, there was a significant difference between the level of HLA-DR expression on classical monocytes in PB at term compared to PTNL.

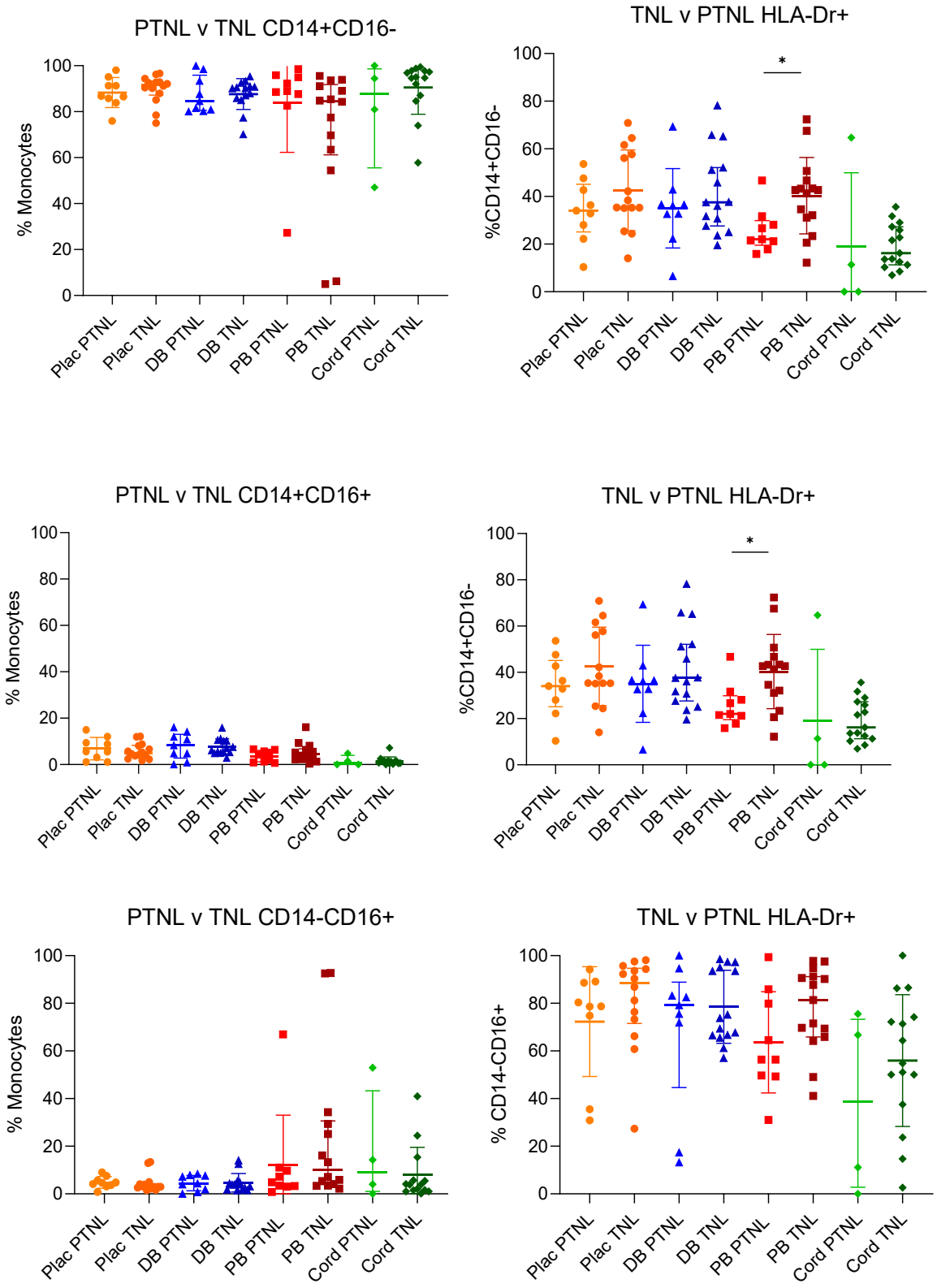
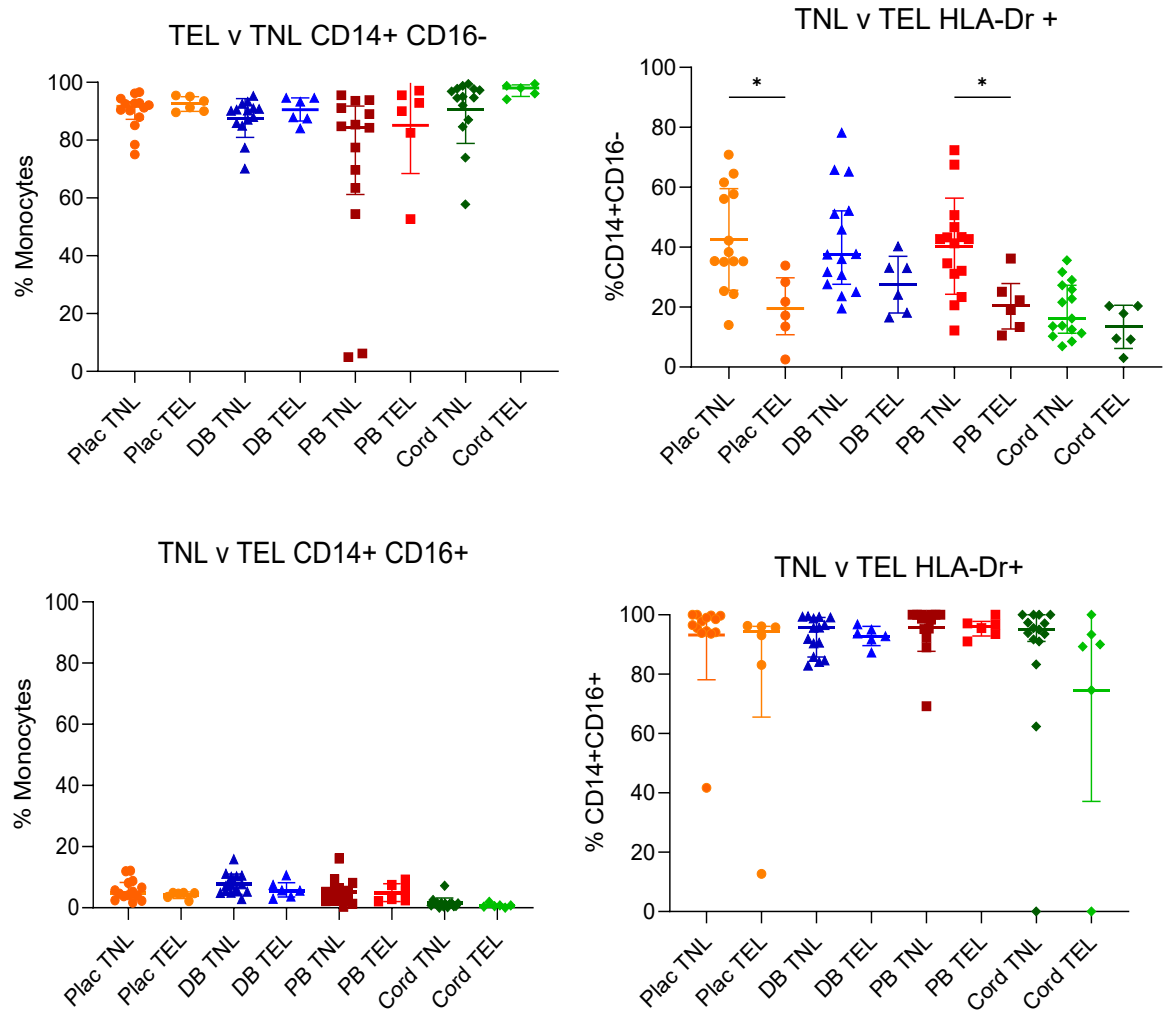


*Figure 4. 12 Proportion of monocytes (%) subsets in preterm non-labouring samples (PTNL) compared to TNL samples in placenta, DB, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile*

4.6.9 Monocytes – Comparison of TEL to TNL

There were no significant differences seen in any of the monocyte subsets across all four compartments when comparing TEL to TNL (Figure 4.13). There was significant reduction in HLA-DR expression on classical ($CD14^{+}CD16^{-}$) and non-classical ($CD14^{-}CD16^{+}$) monocytes in placenta when comparing TNL to TEL. There was also a significant reduction in HLA-DR expression on classical monocytes in PB with the onset of labour.



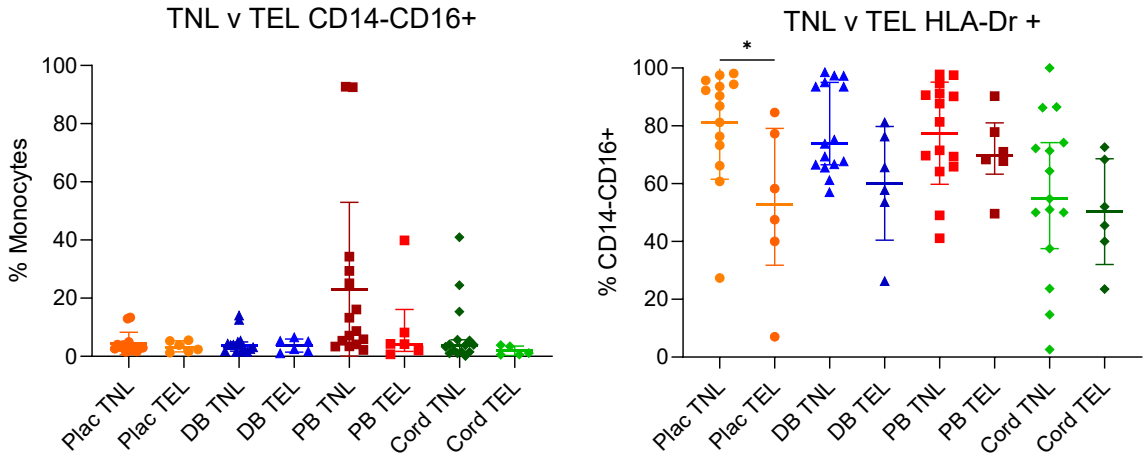


*Figure 4. 13 Proportion of monocytes (%) subsets in term early-labouring samples (TEL) compared to TNL samples in placenta, decidua, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. TNL n=15 TEL n=6.*

4.6.10 T cells in TNL

T cells were gated for using the gating strategy (358) shown in Figure 4.14.

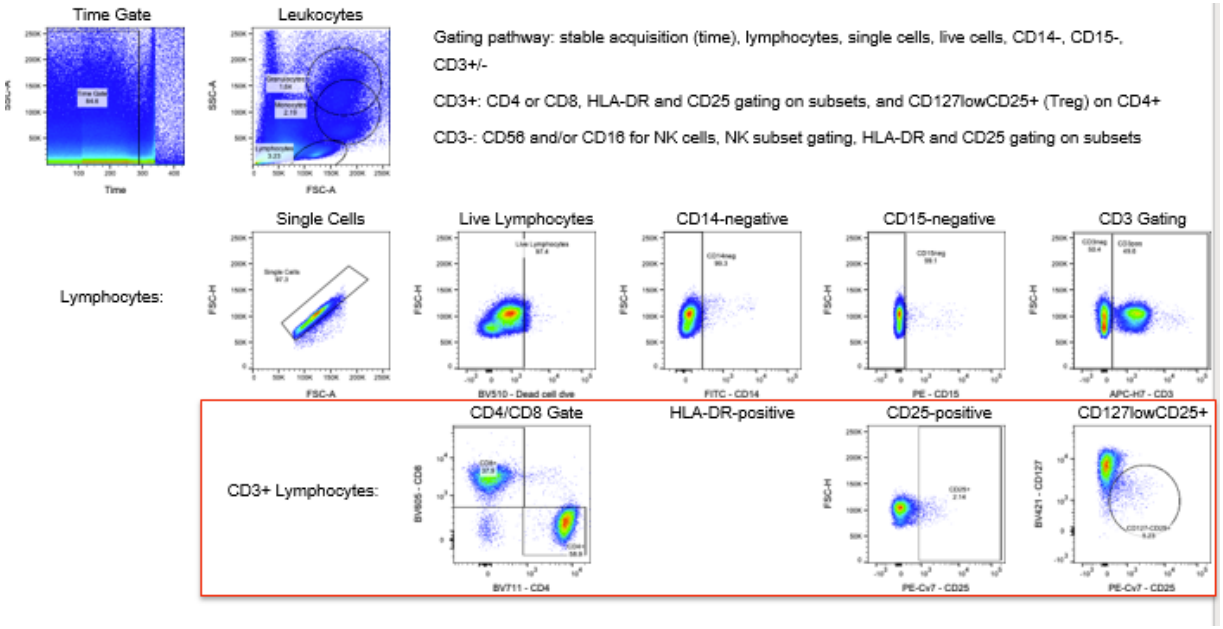
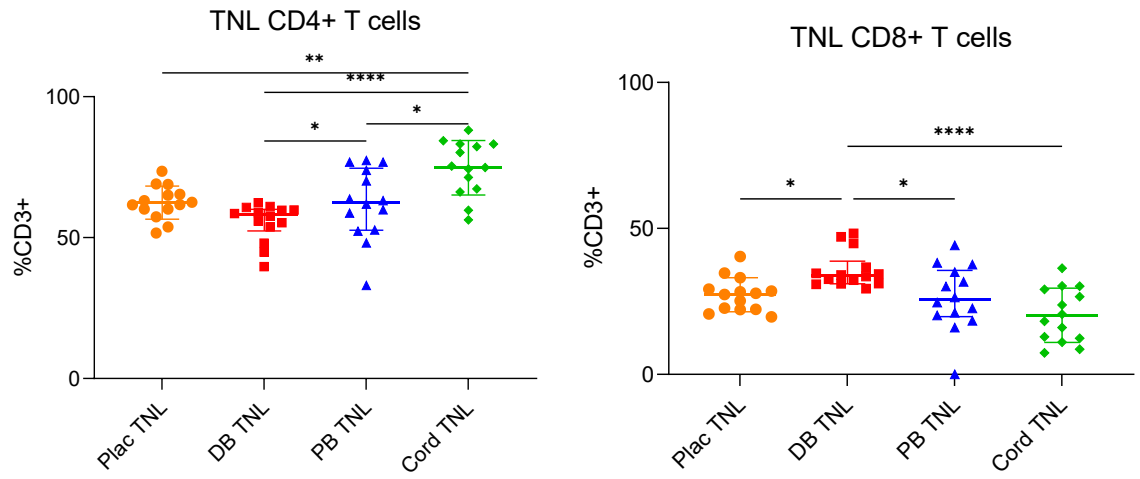


Figure 4. 14 Gating pathway used to identify T cells and Treg cells. Lymphocytes were gated for CD3+ cells which were further subdivided into CD4+ and CD8+ T cells. Treg cells were defined as CD4+ cells that were CD127lowCD25+ cells.

$CD4^+$ T cells were the predominant T cell group in all four compartments (Figure 4.15). $CD4^+$ T cells were found significantly higher in cord blood when compared to the three other compartments (Placenta $p<0.01$, DB $p<0.001$, PB $p<0.05$). $CD8^+$ T cells were significantly higher in decidua when compared to placenta ($p<0.05$), PB ($p<0.05$) and cord blood ($p<0.001$). T_{reg} cells were significantly higher in decidua than in PB ($p<0.01$) and cord blood ($p<0.05$). Placenta had significantly higher proportion of T_{reg} cells than PB ($p<0.05$).



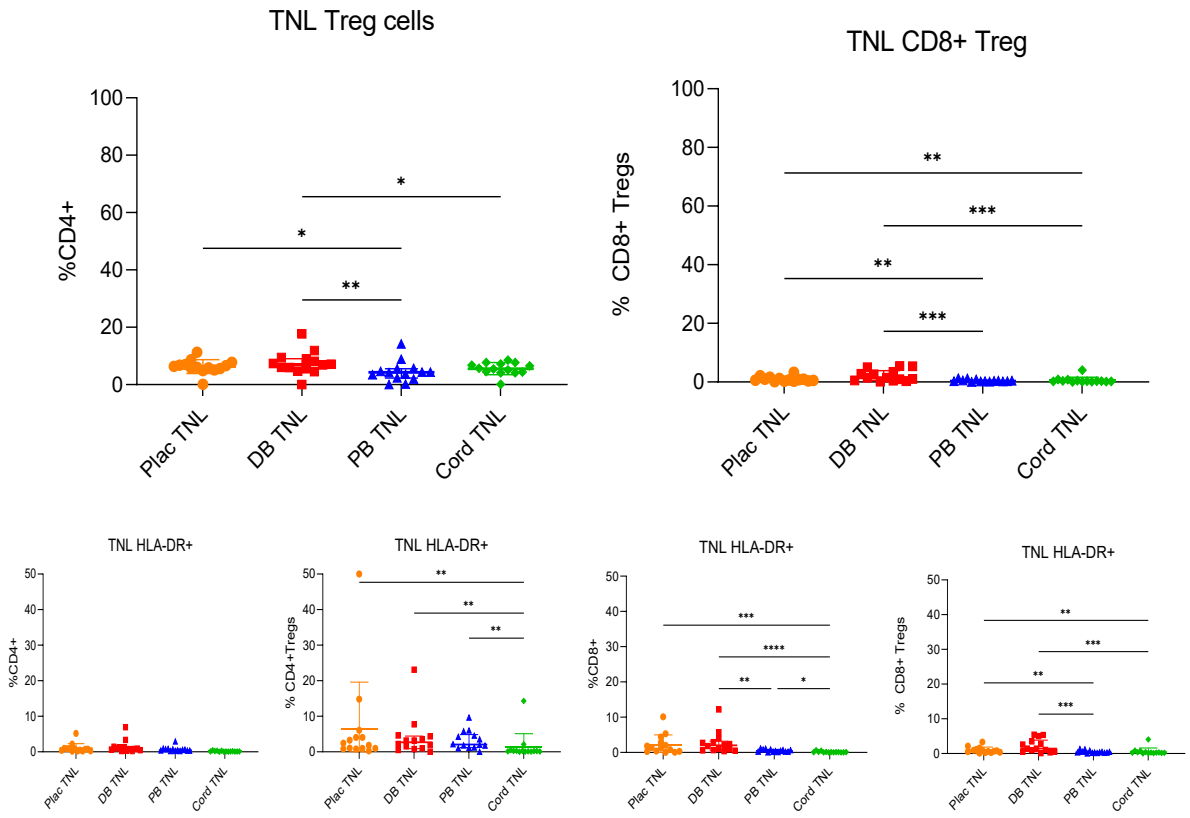
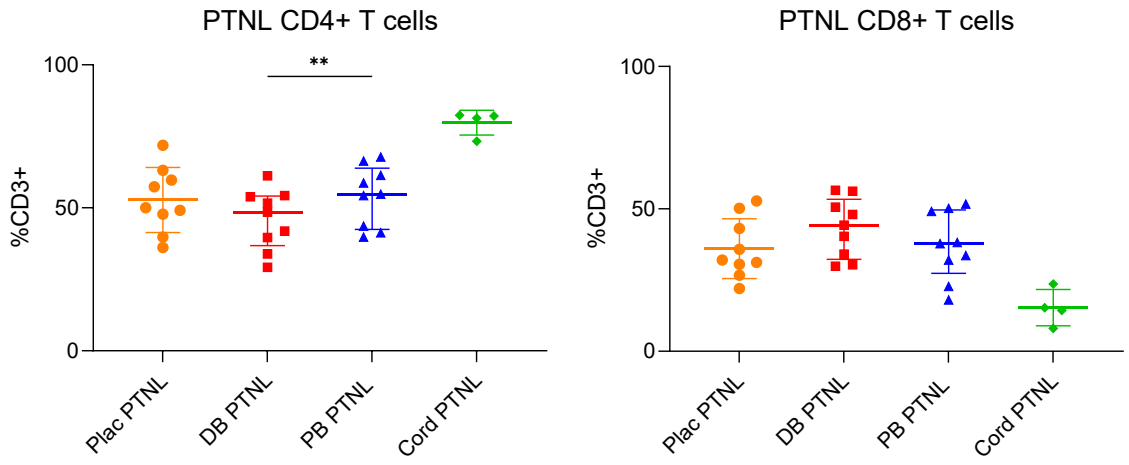


*Figure 4. 15 Proportion of T cells (%) and HLA-DR expression in term non-labouring samples (TNL) in placenta, DB, PB cells and cord blood cells. P value is based on the non-parametric Friedman's test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. TNL n=15.*

$CD4^+$ T cells were significantly higher in PB than DB in PTNL, mirroring TNL. T_{reg} cells were significantly higher in DB than placenta (p<0.01, Figure 4.16).



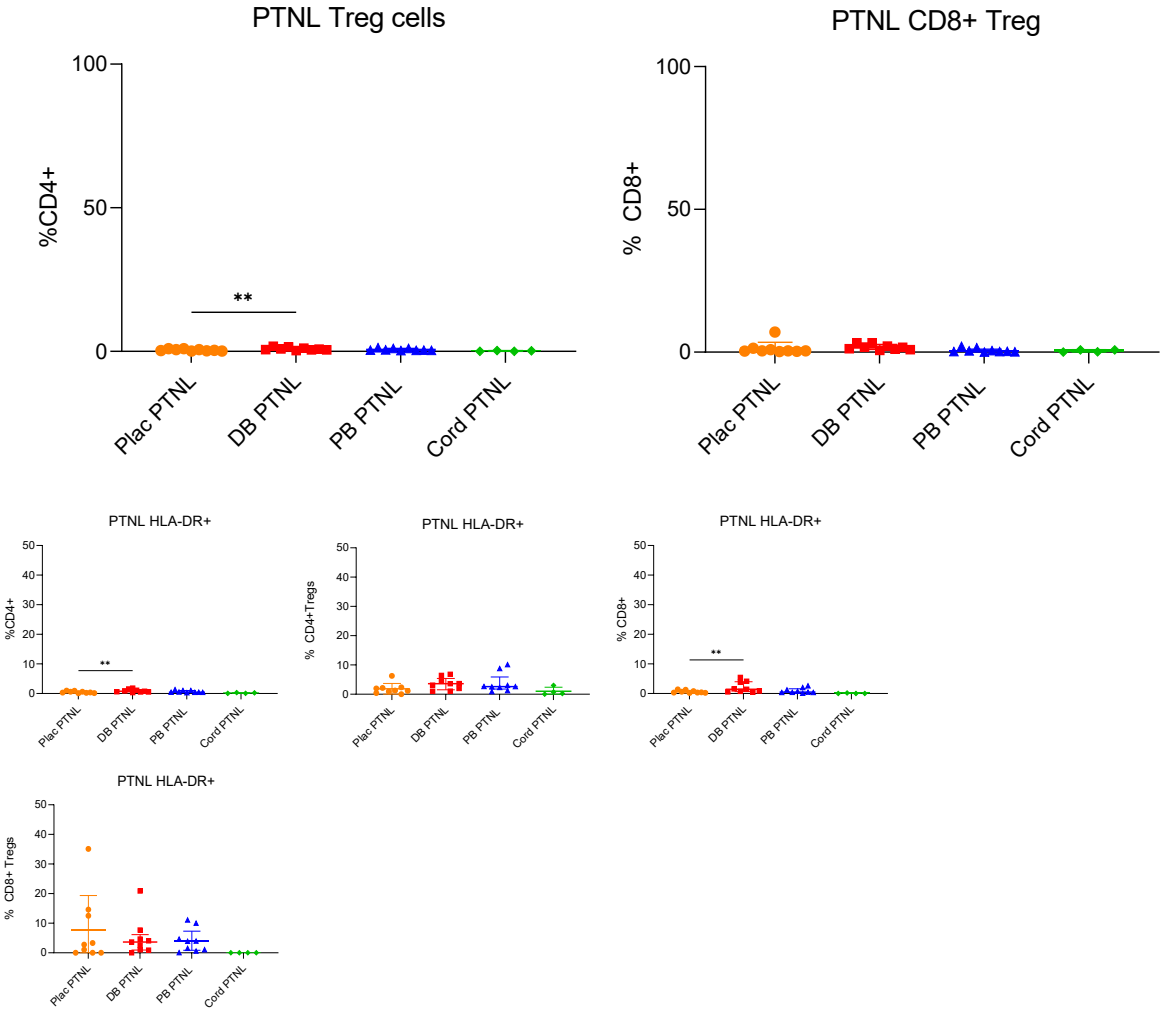


*Figure 4. 16 Proportion of T cells (%) and HLA-DR expression in preterm non-labouring samples (PTNL) in placenta, DB, PB and cord blood. P value is based on the non-parametric Friedman's test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. PTNL n=9*

4.6.12 T cells – Comparison of TNL to PTNL

There was no significant difference in $CD4^+$ T cells or $CD8^+$ T cells in any compartment when comparing PTNL to TNL (Figure 4.17). Treg cells were significantly higher in all compartments in TNL when compared to PTNL (placenta p<0.001, DB p<0.001, PB p<0.05, Cord p<0.001). HLA-DR expression of T cells was also compared between TNL and PTNL; no significant difference was found (Figure 4.17).

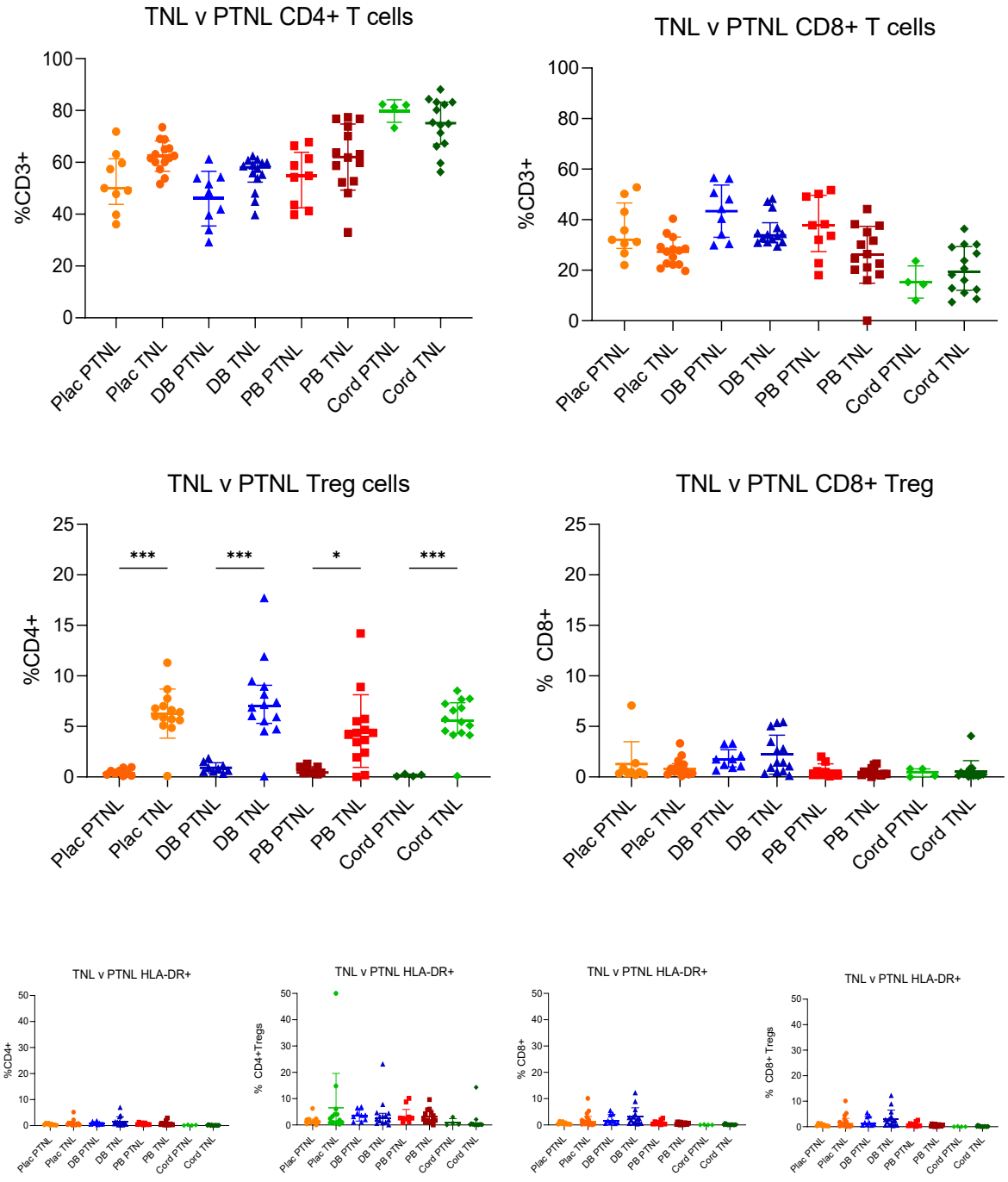
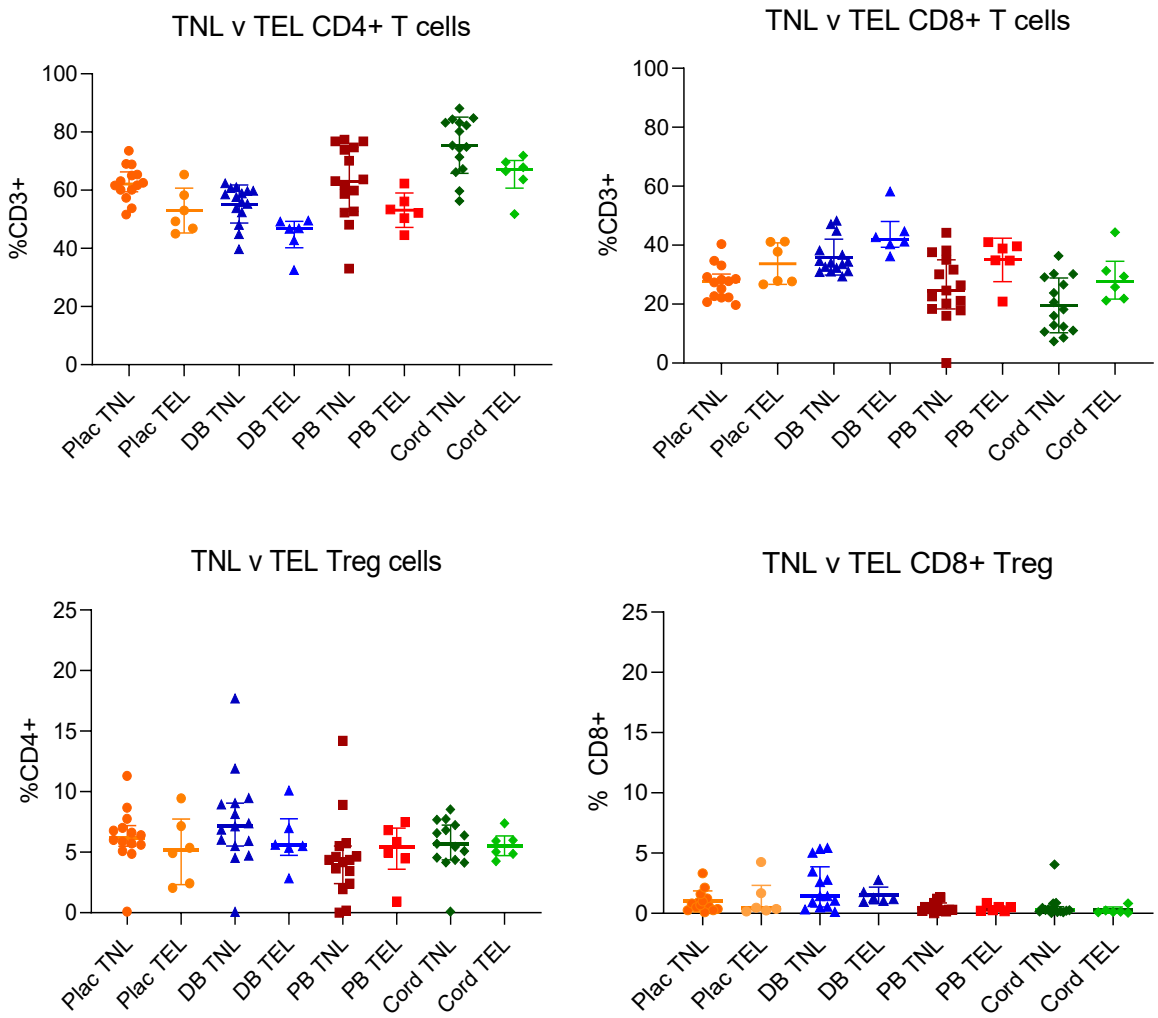


*Figure 4. 17 Proportion of T cells (%) and HLA-DR expression in preterm non-labouring samples (PTNL) compared to TNL samples in placenta, DB, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. TNL n=15, PTNL n=9*

4.6.13 T cells – Comparison of TNL to TEL

On the whole, when comparing TEL to TNL, $CD4^+$ cells were lower in proportion in all four compartments (Figure 4.18). This reduction however was not statistically significant. There was no significant difference seen in $CD8^+$ T cells or T_{reg} cells. HLA-DR expression of T cells was also compared between TNL and TEL; no significant difference was found (Figure 4.18).



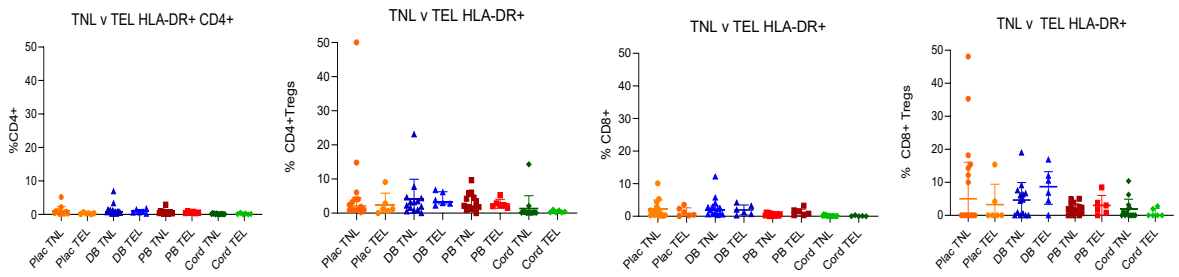


*Figure 4. 18 Proportion of T cells (%) and HLA-DR expression in term early-labouring samples (TEL) compared to TNL samples in placenta, DB, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001), error bars indicate median and interquartile ranges. TNL n=15, TEL n=6.*

4.6.14 Neutrophils in TNL and PTNL

Neutrophils were gated for using the gating pathway (358) in Figure 4.19.

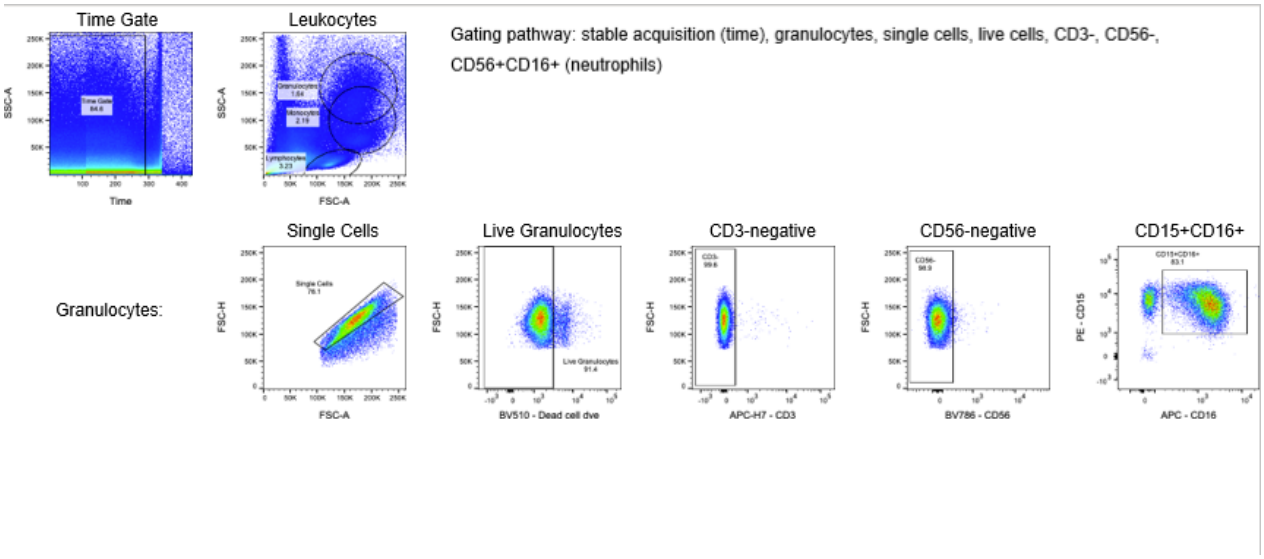


Figure 4. 19 Gating pathway used to identify neutrophils from live granulocytes. Neutrophils were identified by gating CD3-CD56- granulocytes that were CD15+CD16+.

At TNL, cord blood had significantly lower neutrophils than DB and placenta. This difference was not seen at PTNL.

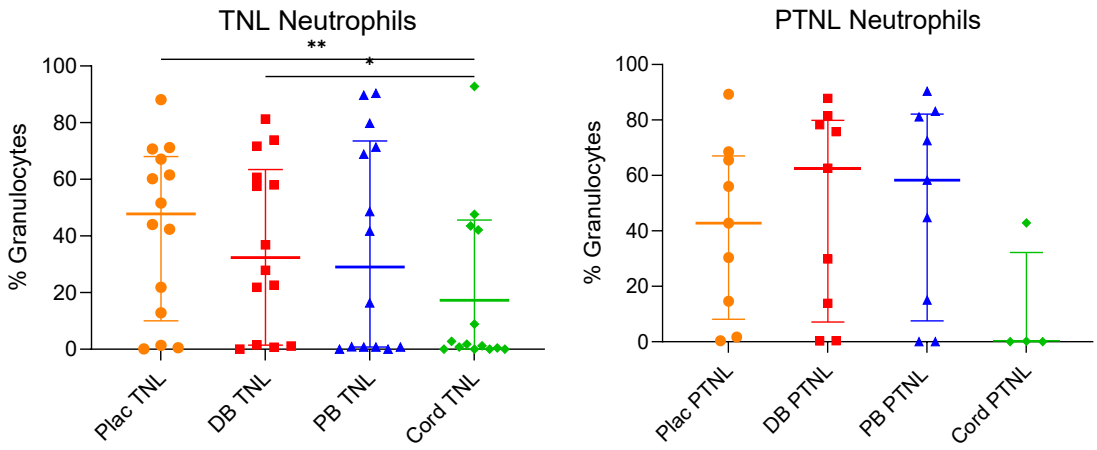


*Figure 4. 20 Proportion of neutrophils in TNL (n=14) and PTNL (n=9) in placenta, DB, PB and cord. P value is based on the non-parametric Friedman's test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001). Error bars indicate median and interquartile ranges.*

4.6.15 Neutrophils – Comparison of TNL to PTNL

There were no significant differences noted between any of the four compartments when comparing PTNL to TNL samples (Figure 4.21).

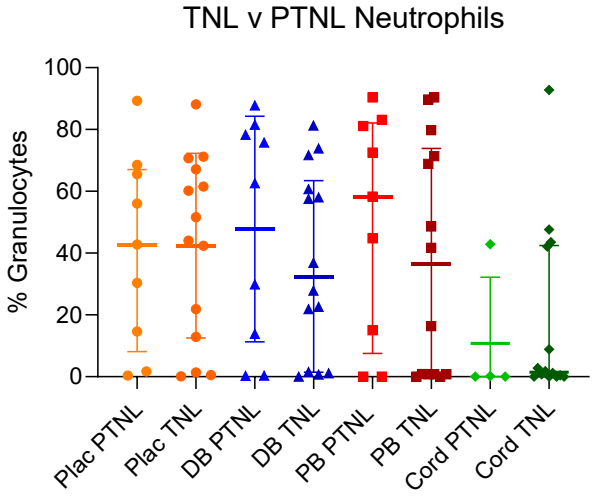


*Figure 4. 21 Proportion of neutrophils (%) in preterm non-labouring samples (PTNL) compared to TNL samples in placenta, DB, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, *** p=0.001). Error bars indicate median and interquartile ranges TNL n=14, PTNL n=9*

4.6.16 Neutrophils – Comparison of TNL to TEL

There were no significant differences noted between any of the four compartments when comparing TEL to TNL samples (Figure 4.22).

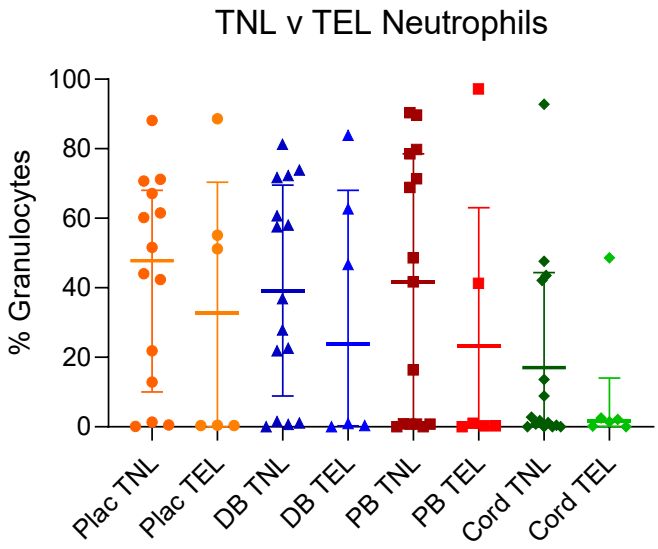


*Figure 4. 22 Proportion of neutrophils (%) in term early-labouring samples (TEL) compared to TNL samples in placenta, DB, PB and cord blood. P value is based on the non-parametric Kruskal-Wallis test with multiple comparisons controlled for with false discovery rate. * Indicates statistical significance (* p<0.05, ** p<0.01, ** p=0.001). Error bars indicate median and interquartile ranges TNL n=15, TEL n=6.*

4.6.17 Correlation between CD4+ T_{regs} and NK^{bright} cells

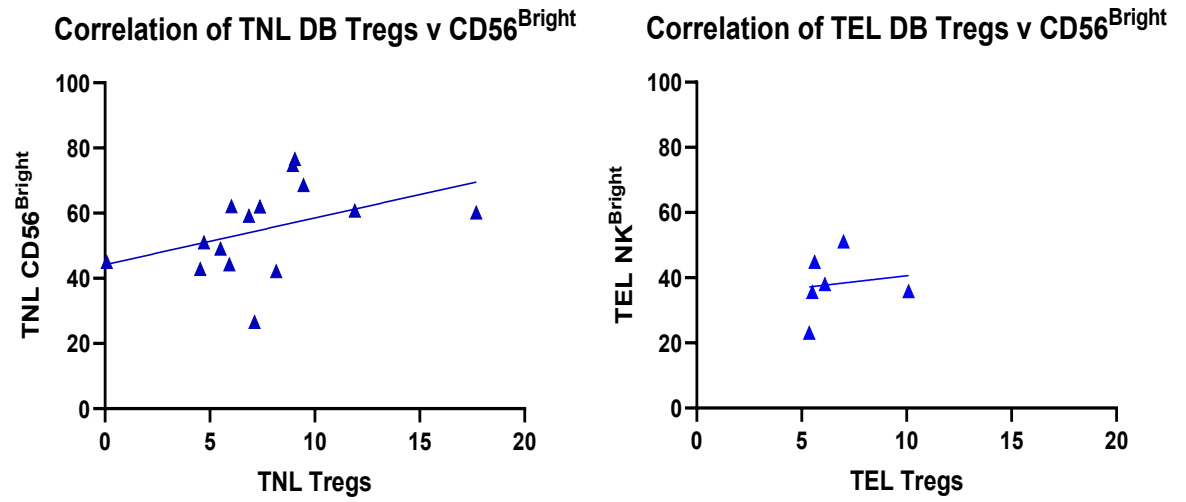


Figure 4. 23 Simple linear regression graphs correlating CD56bright cells to Tregs in DB in TNL and TEL. Spearman's rank correlation revealed statistically significant correlation in TNL (r=0.53,95% CI 0.01--0.83 p=0.04). In TEL there was no significant correlation (r=0.60, p=0.24)

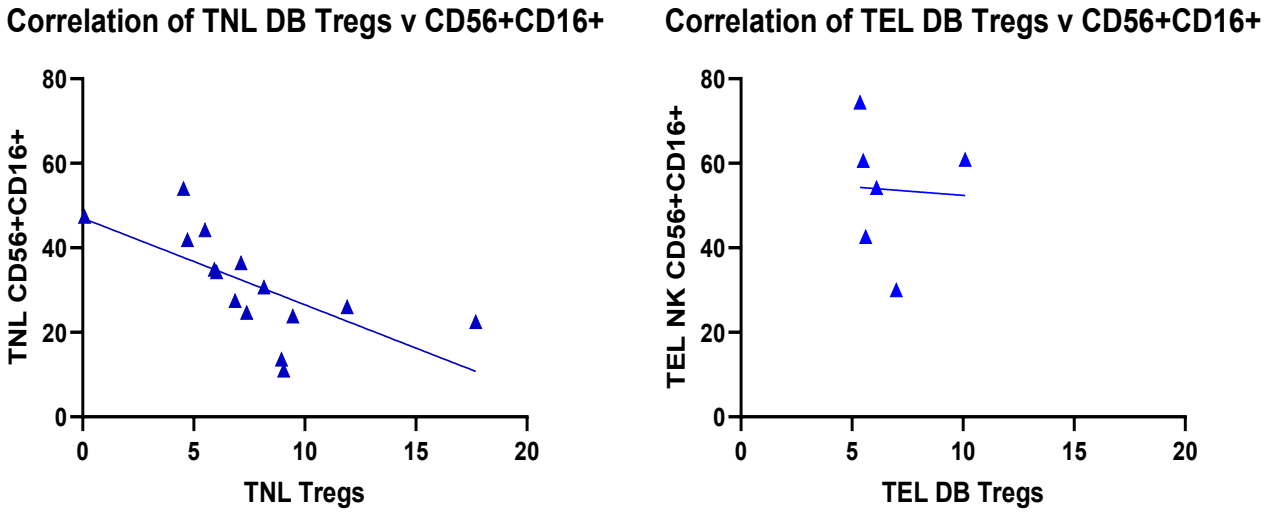


Figure 4. 24 Simple linear regression graphs correlating CD56+CD16+ NK cells to Tregs in TNL and TEL. Spearman's rank correlation revealed a significant correlation in TNL (r=-0.88, 95% CI -0.96 - -0.66, p<0.0001). In TEL there was no correlation (r=-0.37, p=ns).

4.6.18 Summary of NK cell subsets in TNL compared to TEL

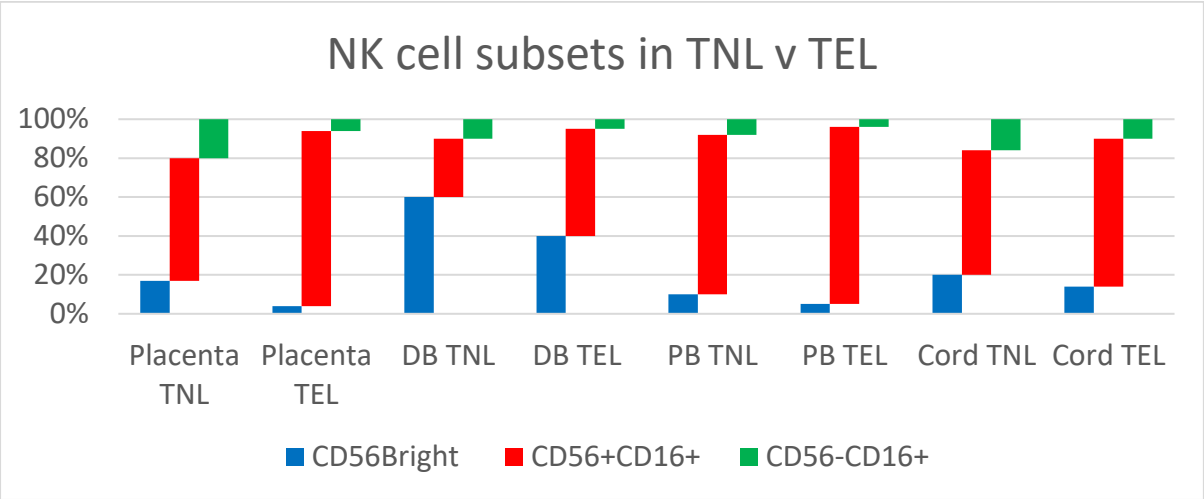


Figure 4. 25 Change in NK cell subset proportions in placenta, DB, PB and cord with the onset of term labour.

4.7 Discussion

This chapter looks specifically at NK cells, monocytes, T cells and neutrophils in four different compartments: placenta, decidua, PB and cord blood in samples collected from women undergoing caesarean sections who were in one of three clinical groups: TNL, PTNL and TEL.

4.7.1 NK cell subset proportions alter with the onset of labour in DB

My data showed that on average around 60% of $CD3^-$ lymphocytes in the decidua are NK cells which has been reported before (57). In both term and preterm non labouring decidua, $CD56^{bright}$ NK cells were the predominant NK cell subset. This has been evidenced well in literature (335) and $CD56^{bright}$ cells have been shown to have an important role in immune tolerance at the maternal-fetal interface (57). In contrast to this $CD56^+CD16^+$ cells remained high in term non labouring PB, cord blood and placenta. $CD56^+CD16^+$ NK cells are efficient killers with poorer cytokine production potential (335) and are the commonest NK cell subset in PB (336). The fact that $CD56^+CD16^+$ NK cells are significantly higher in placenta than DB emphasises the unique immunological role DB plays and maintains throughout normal pregnancy.

In PTNL, the distribution of the NK cell subsets is maintained throughout the four compartments, but interestingly $CD56^{-}CD16^{+}$ NK cells were noted to be significantly higher in placenta than DB and PB. This may be because several of the PTNL deliveries were associated with fetal growth restriction; Fu and colleagues had noted that NK cells can promote fetal development by secretion of growth promoting factors (338). Having a significantly higher level of the cytolytic NK cell subset in the placenta may be associated to the pregnancy pathology as opposed to physiological gestation differences. There was no significant difference noted in NK cells across the four compartments when comparing TNL to PTNL. This would suggest that on the whole NK cells are maintained in proportion throughout the third trimester unless an inflammatory or pathological trigger occurs.

On comparison of TNL to TEL, although there were no significant differences, there was a trend seen in the $CD56^{bright}CD16^{-}$ NK cells in DB. $CD56^{bright}CD16^{-}$ NK cells had reduced on average from approximately 60% in TNL to 40% in TEL in DB (Figure 4.25). These changes are not seen in neighbouring placental tissue and remain unique to DB. This suggests that the immune tolerance that these NK cells are well known to create (359) may be withdrawn to facilitate the pro-inflammatory trigger of labour. This is inversely mirrored in the $CD56^{+}CD16^{+}$ NK subset which is increasing (albeit not significantly) in DB with the onset of term labour. It is possible that the increase in $CD56^{+}CD16^{+}$ NK cells in DB is due to recruitment from PB, along with other leukocytes as part of the inflammatory process of labour (110, 111, 350). This possible switch in NK cells from the predominant tolerant $CD56^{bright}CD16^{-}$ to the cytolytic $CD56^{+}CD16^{+}$ NK cell group, and the inevitable functional shift (53), specifically in DB (Figure 4.25) may play a role in the loss of immunological tolerance required for labour to occur. If further TEL patients were recruited a significant difference in $CD56^{bright}CD16^{+}$ NK cells with the onset of labour may have been seen.

HLA-DR expression on NK cells was analysed which did not show any significant differences in any subset in any compartment between TNL and PTNL or TEL (Figure 4.7/4.8). It did however show that at term, PB had significantly higher expression of HLA-DR than placenta, DB and cord blood in all three subsets of NK cells (Figure 4.5). NK cells that express HLA-DR are functionally active, they can produce inflammatory cytokines, degranulate and easily proliferate in response to stimuli (360). $HLA\text{-}DR^{+}$ NK cells produce more IFN-γ (361) and *in*

vitro have been shown to regulate the adaptive immune response, by interaction with $CD4^+$ and $CD8^+$ T cells through several co-stimulatory molecules and MHC class I and II (360). Their increased presence in PB has been associated with several medical conditions including HIV-1, multiple sclerosis and autoimmune conditions such as systemic lupus erythematosus (SLE), however their role is not defined (360). Their ability to intensify inflammation via production of IFN-γ is particularly interesting as IFN-γ was significantly raised in CD in idiopathic labour (Chapter 3.6.1, Table 3.8) and it is possible that peripheral HLA-DR^+ NK cells may contribute to this by migrating into the gestational tissues. Overall, increased levels of HLA-DR^+ NK cells in PB at term could contribute to the pro-inflammatory state required for labour to be triggered.

4.7.2 Monocytes – HLA-DR^+ classical monocytes are significantly reduced following the onset of labour in placenta and PB

Monocytes are short-lived circulating cells, which arise from myelo-monocytic precursors in the bone marrow (24). They can be sub-divided into three sub-groups based on their expression of CD14 and CD16. The main subset, classical monocytes are identified as being $CD14^+CD16^-$ and make up over 90% of all monocytes. The second subset is the non-classical monocytes, and this is identified by having low expression of CD14 and high expression of CD16. The third subset, appropriately named intermediate monocytes have high expression of both CD14 and CD16.

In TNL, classical monocytes were the most represented subset of monocytes in all four compartments which would fit with the fact that this is the main subset of monocytes (362). In the gestational tissues, intermediate monocytes made up the highest proportion of monocytes after classical monocytes, whereas in both PB and cord blood, non-classical monocytes were the more represented than intermediate monocytes (Figure 4.26).

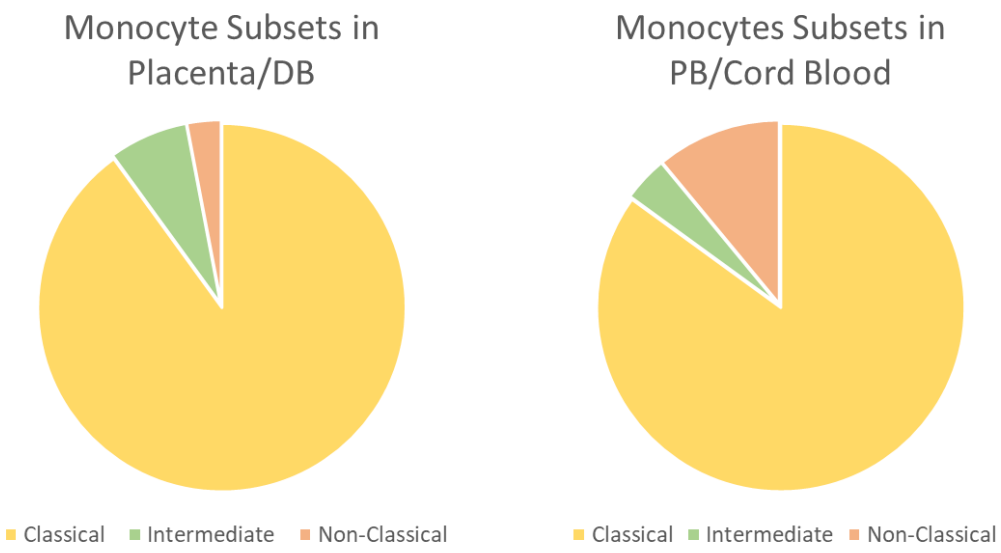


Figure 4. 26 Pie charts exhibiting the average proportion of classical, intermediate, and non-classical monocytes in placenta/DB compared to PB/cord blood at term non labour

My data shows that intermediate monocytes reside in the gestational tissues more so than non-classical. This subset typically is derived from classical monocytes, and they essentially behave like premature non-classical monocytes (344). They can produce inflammatory mediators in response to bacteria and can expand during infection and antigen presentation (363). Their increased presence in the gestational tissues, specifically in the DB, may suggest they have an immunological role to have both an anti-inflammatory effect and phagocytic function (345). They may also be present to replenish tissue resident macrophages, specifically M2 macrophages which are immune regulators and anti-inflammatory in function (348).

In PTNL, the same distribution of monocyte subsets was maintained across all four compartments; like TNL, there were no significant differences in any of the monocyte subsets. This again suggests that the monocyte subsets are maintained at similar proportions throughout third trimester. When comparing TNL to PTNL, there were no significant difference in monocytes subsets, however when looking further into HLA-DR expression, a significant difference was identified. HLA-DR expression was significantly higher ($p<0.05$) in classical monocytes in PB at term than at preterm gestation. This increased expression may contribute to antigen presentation to $CD4^+$ T cells (364). In Rheumatoid arthritis, increased HLA-DR expression on classical monocytes indicates disease stability and suppressed activity

(364), so if a similar mechanism is acting here then the increased peripheral HLA-DR^{+} classical monocytes could contribute to an immunological quiescence at term gestation.

It was interesting to see that when comparing TNL to TEL, although there was no significant difference in monocyte subsets across all four compartments, there was a significant difference noted in HLA-DR expression. The expression levels of HLA-DR seen at TNL in classical monocytes in PB was lost with the onset of early labour (PB $p<0.05$). There was also a significant reduction ($p<0.01$) in HLA-DR expression in classical monocytes in placenta with the onset of labour too. The reduced expression of HLA-DR in classical monocytes in early labour is somewhat difficult to explain, especially as there is a paucity of literature not only on the effect of early labour on immune cells peripherally but specifically within gestational tissues. HLA-DR is a MHC Class II cell surface receptor and its main role is to present antigenic peptides to the immune system to elicit or suppress T cell responses (365). Recently, much research has been undertaken in monocyte HLA-DR measurement as monocytes have been a key immune cell of clinical interest in management of patients with Covid-19 virus. Evidence in this area and other infection associated responses has shown that downregulation of HLA-DR is an indication of immunosuppression (366-368). It is therefore possible that to modulate the inflammation associated with physiological labour, monocytes may take a more immunomodulatory function. Having a significantly lower level of HLA-DR expression may also prevent hyperinflammation (369) which could be detrimental to the fetus and mother.

A statistically significant reduction in HLA-DR expression on non-classical ($CD14^{-}CD16^{+}$) monocytes was also seen in placenta with the onset of labour. Non-classical monocytes have weak phagocytic potential, however, can secrete high amount of pro-inflammatory cytokines such as TNF-α and IL-1β in response to viruses/infection (370). From my data in Chapter 3, TNF-α and IL-1β are not significantly increased in placenta in idiopathic PTL where there is an absence of infection whilst TNF-α and IL-1β are significantly raised in PTL associated with chorioamnionitis. This difference in these cytokines may be contributed to by the downregulation of HLA-DR on non-classical monocytes within placenta when infection is absent.

Non-classical monocytes are known on whole to promote resolution of inflammation (371); their activity being downregulated during early labour would make sense, as the onset of labour is well recognised to be a pro-inflammatory process (10, 145, 146).

4.7.3 T cells – $CD4^+T_{regs}$ and $CD8^+T_{regs}$ were significantly higher in DB in TNL

T cells are adaptive immune cells that have been studied over the last 30 years in the maternal-fetal interface, yet their role in the onset of labour and PTB is still not fully understood. My data showed that at term $CD4^+$ T cells were the predominant T cell group in all four compartments studied. It also showed the $CD4^+$ T cells proportions were similar throughout the third trimester in DB as there was no difference in proportion of $CD4^+$ T cells when comparing PTNL and TNL, which is supported by Williams and colleagues (53). At term $CD8^+$ T cells were significantly higher in DB than PB, placenta and cord blood (Figure 4.15) and although $CD8^+$ T cells are typically cytotoxic, decidual $CD8^+$ T cells have been shown to be less cytotoxic (55), and more tolerant (372), hence contributing to an immunotolerant maternal-fetal interface.

My data showed that on average 6% of $CD4^+$ T cells were identified as T_{regs} and these T_{regs} were significantly higher in both DB and placenta than PB at term. This could be because T_{regs} are known to peak in PB in the second trimester (373) and decrease continuously thereafter, reaching their lowest level in the postpartum period (138). T_{regs} are well evidenced to be powerful suppressors of inflammation associated immune responses (333) and they are critical to regulating maternal T cell activation against trophoblast. Their proportions being higher in gestational tissues at term gestation suggests they may play an important role in maintaining immune tolerance at the maternal-fetal interface. Conversely a lack of T_{regs}, both peripherally and within decidua have been associated with recurrent pregnancy loss (374) and pre-eclampsia (375).

$CD8^+$ T_{regs} cells are a $CD8^+$ subset that target activated T cells (376) and promote secretion of soluble factors, such as immunosuppressive cytokines (377). Their role has been looked at in various autoimmune conditions and has been shown to play a critical role in mucosal tolerance (378), promote allograft survival (379) and reduce disease activity (380). Although there were no significant differences noted in $CD8^+$ T_{reg} cells when comparing TNL to PTNL/TEL, $CD8^+$ T_{reg} cells were significantly raised in DB in TNL compared to the three other

compartments. This suggests that $CD8^+$ T_{reg} cells can facilitate immune tolerance in DB just like in other inflammatory conditions (381). The extent to which $CD8^+$ T_{reg} cells play a role in maintenance of immune tolerance is unclear, especially as there were no significant differences on comparison to TEL in DB, however they reportedly can display functional plasticity depending on their microenvironment (381). To understand this subset's role in pregnancy and within the DB, much more work is required.

When comparing PTNL to TNL, T_{reg} cells were significantly increased at term in all four compartments. This may suggest that there is a need for increased T_{regs} globally to maintain a healthy pregnancy to term gestation, but the exact role T_{regs} have in both gestational tissues and in PB, specifically at preterm gestation, needs further research. A recent systematic review of 34 studies revealed that lower levels of T_{regs} could only be associated with pre-eclampsia and that further fundamental studies would be required to truly understand their role in PTB (353). In addition to looking at proportions of T_{regs} in gestational tissues, a better understanding is required regarding their function to be able to better piece together the complex immune interactions that maintain pregnancy, whilst having the potential to trigger labour.

On comparison of TNL to TEL, there were no significant differences noted in any of the four compartments. T_{regs} did however seem to reduce in proportion following the onset of labour in DB and placenta. This depletion in resident T_{regs} within the gestational tissues may be a contributor to the loss of immune tolerance required for the inflammation of labour to occur. Further analysis of HLA-DR expression was undertaken (Figure 4.18); however, this did not show any significant differences to further our understanding.

Contrastingly T_{regs} in PB seem to be on an upward trend following the onset of labour. Our research group and others have investigated T_{regs} in PB and have noted that the onset of labour is associated with a targeted adaptation of Treg mediated immune tolerance (141, 382); there is a possibility that the increase in T_{regs} seen in PB are in fact an increase in naïve T_{regs} which are associated with a decline in T_{reg} cell function.

4.7.4 Neutrophils did not alter with the onset of labour in placenta, DB, PB or cord blood

Neutrophils are often thought of as the first line innate defence, which kills and traps pathogens, and, if in overdrive, can cause tissue damage (383). Neutrophilia is consistently associated with term labour (384), and it is likely neutrophils have a functional role in responding to sterile inflammation both locally and systemically (385). There is strong evidence that neutrophils have increased migratory capacity in labour (384), which suggests they are primed to migrate to the maternal-fetal interface during term labour. In PTB, as neutrophils may have a significant role in infection and inflammation; they may be involved in PTB associated with infection where they infiltrate, drive inflammation, and produce pro-inflammatory cytokines(215).

Our data unfortunately did not shed any new light into the role of neutrophils in TNL, PTNL or TEL. It did suggest that neutrophils were slightly higher (not significant) in number in the placenta followed closely by DB and PB and significantly higher in gestational tissues (placenta $p<0.01$, DB $p<0.05$) than cord blood. This could be explained by the fact that these term samples were taken from women who had no signs of infection, so although on the maternal side neutrophils are primed in the gestational tissue for sterile term labour, there was no trigger on the fetal side to increase the neutrophil count (386).

One observation that was made on analysis of the neutrophil data was that several of the samples essentially showed that 0-1% of granulocytes were neutrophils. Although all samples were collected and processed within 2hours, they were stained and fixed before storage at 4^{o}C in the fridge overnight before cell acquisition on the flow cytometer. This is specifically undertaken to maintain cell viability and this practice is based on published methods and not deemed to affect cell viability (387). However, there is always a possibility that some neutrophils were still too delicate to maintain viability. It has also been noted that delaying blood processing for an extended period (6-24 hours) leads to neutrophil contamination of PB preparation (388) which suggests that my timely processing and fixing of cells (<2hours), should provide accurate neutrophils counts.

4.7.5 Decidual NK^{bright} cells and T_{regs} work together to maintain uterine quiescence at term, which is lost with the onset of labour

The maintenance of immune tolerance to tolerate paternal antigens yet preserve the potential to protect the fetus from infection is one that requires many immune cells to work together and communicate. Based on this well-established understanding, I further analysed to see based on population proportions whether there is evidence of this possible interaction to maintain immune tolerance. The correlation analysis highlighted that there was a significant correlation between CD56^{bright} NK cells and T_{regs} in DB which was not seen in placenta, PB or cord blood (Figure 4.23).

This significant correlation in DB is lost with the onset of labour which suggests that these immune cells could help each other maintain immune equilibrium. The loss of their collaborative relationship may enable the inflammatory trigger that is required for labour to start. The crosstalk between these two cells groups may be direct or indirect. Tregs may be induced via interaction between NK cells and CD14^{+} cells in DB. These CD14^{+} cells release IDO which in turn induces T_{regs} (389). Alternatively, there may be direct induction of Tregs via Tim-3 which is a type I membrane protein T cell immunoglobulin and mucin containing protein 3 (390). NK cells, which are Tim-3^{+}, can also produce anti-inflammatory cytokines and induce T_{regs}.

Interestingly the imbalance of these cells has been associated with pregnancy complications such as pre-eclampsia where there is reduced decidual T_{regs} which leads to poor suppression of cytotoxic NK cells (391). I was able to demonstrate this relationship too in DB in TNL (Figure 4.24). In some term decidua, it is clear that higher proportions of cytotoxic NK cells did not trigger labour or cause pregnancy complications. This only supports the understanding that immune tolerance is not dependent on any one immune cell group but how several interact to maintain immune tolerance.

An alternative reason for why this inverse relationship of cytotoxic NK cells and T_{reg} cells is tolerated in TNL could be because these women were nearing spontaneous labour but did not have the opportunity to labour as their elective caesarean section was before. However,

it is clear that no such relationship is maintained between NK CD56+CD16+ cells and T_{regs} with the onset of labour.

Correlation analysis in placenta, PB and cord blood did not reveal any significant correlations, and this further emphasises that decidual immune cells, their adaptation as the pregnancy advances and their crosstalk are pivotal in maintenance and rejection of pregnancy i.e., labour.

4.8 Strengths and limitations

Although there was a good number of samples in the PTNL group, it was difficult to attain samples within this group without an associated medical indication for delivery. Most commonly the indication was fetal growth restriction so there is a small possibility this could have altered the immune cells quantified. Unfortunately, there was no other ethical way of obtaining preterm non labouring samples. This study could have been strengthened with recruitment of women who had a preterm labouring caesarean section to ascertain the immunological changes with the onset of preterm labour. Unfortunately, it was difficult to recruit these women, as many women who had spontaneous preterm labour, delivered vaginally. Those who did have preterm caesarean sections commonly had severe infection or abruption so were not suitable for recruitment. Another limitation was due to difficulties with achieving adequate live cells from DB, this study was limited to one flow cytometric panel, which was designed to be as extensive as it could be to identify presence of various immune cells. The study could have been strengthened by undertaking another flow panel looking more into what these cells expressed to better understand their function.

Another limitation is in the CD56-CD16+ NK cell gate, T cells, monocytes and neutrophils were excluded as potential contaminants. Other studies have also excluded B cells using CD19 which has not been done here. Nevertheless, there is no strong evidence describing a CD16 expressing B cell subset, so it is unlikely that this NK cells subset contains other cells.

The function and phenotype of NK cells in the decidua and peripheral blood are evidenced to be different (392). Decidual NK cells are poorly cytolytic and they release cytokines/chemokines that induce trophoblast invasion, tissue remodelling, embryonic

development (393). The decidual NK cells have low cytotoxicity and they show high expression of perforin, granzymes and several NK-activating receptors compared with peripheral blood $CD56^{bright}$ NK cells (394). Although this study assessed the difference in NK cell phenotype proportions in the four compartments, it cannot comment on the function of each NK cell subset in each compartment. This is a limitation; yet this creates direction for future work, in the form of functional assessment of matched decidual and peripheral blood NK cells in TNL, PTNL and PTNL.

Another limitation is that whilst we collected peripheral blood at time of delivery, the time of the day in which this was collected, naturally varied. All elective caesarean sections occurred in the daytime; however emergency preterm deliveries varied from day to late night depending on when the clinical emergency presented. Therefore, this could vary the immune cells present in peripheral blood as number of immune cells oscillates in the blood, peaking at the behavioural rest phase for humans i.e. night (395). Gestational variation in blood cells are controlled for as all samples collected were assigned to two groups based on term or preterm gestation.

This study has provided new insights into which immune cells are present in four matched samples in each woman in three different clinical states – TNL, PTNL and TEL. All samples were processed fresh and high standards were maintained. A reliable and reproducible methodology was optimised through this study which in the future means further flow cytometric work can be undertaken with confidence. The results have given insight into immune cell differences seen, especially in DB compared to neighbouring placenta and PB. These cells of interest (NK, monocytes, T cells) can be studied further with future work focusing immune cell function.

4.9 Summary

$CD56^{bright}$ NK cells did proportionately reduce in DB with the onset of labour, albeit not significantly but there was a clear trend. This trend may become statistically significant by recruiting more women into the TEL group. There were no significant differences noted in monocytes subsets in DB with either the onset of term labour or at preterm gestation. However, there was an interesting loss of HLA-DR expression on classical monocytes in the

placenta and PB with the onset of term labour. This loss of HLA-DR expression was not mirrored in DB. There was no significant difference in T_{reg} cells in DB with the onset of labour but there were significantly increased numbers of T_{regs} in all four compartments as the gestation advanced from preterm to term. $CD4^+$ T_{regs} and $CD8^+$ T_{regs} were significantly higher in the DB than neighbouring placenta and PB. Number of neutrophils quantified in each sample in each group had wide interquartile ranges and therefore no conclusion could really be made.

In conclusion DB is a unique gestational tissue in which immune cells that are known to promote immune tolerance were present, evidenced by high numbers of $CD56^{bright}$ NK cells, $CD4^+T_{regs}$, $CD8^+T_{regs}$ and higher proportion of intermediate monocytes. The shift in DB containing predominantly $CD56^{bright}$ NK cells at term to a predominant subset of $CD56^+CD16^+$ NK cells with onset of labour is particularly fascinating. In addition, the significant correlation seen only in the DB between $CD56^{bright}$ NK cells and T_{regs}, which is lost with the onset of labour highlights the importance DB has in maintaining maternal immune tolerance of the pregnancy. Although significant differences between the proportions of the immune cells studied were not attained when comparing TNL to TEL, future work should be targeted at how these immune cells may actually function differently.

5. Longitudinal study of urinary metabolites and the onset of labour

5.1 Introduction

Immunity and metabolism are interdependent and coordinated; they are core mechanisms by which the body maintains homeostasis (396). As a focal research field in recent years, immunometabolism reveals that the proliferation, differentiation, and function of immune cells can be directly or indirectly modulated by reprogramming the intrinsic metabolic pathways in immune cells (397). Although this area of research has been investigated in various fields such as cancer (398), cardiometabolic disease (399), inflammatory bowel disease (400) and diabetes (401), its potential in maternal-fetal medicine is very much lagging behind (396).

Understanding the crosstalk between immunological and metabolic processes at the maternal-fetal interface could give a better overall picture of what maintains maternal immune tolerance and whether a change in metabolic pathways has a role in the onset of labour. There is a possibility that the immunometabolism regulates the phenotype and function of immune cells, consequently regulating tolerance and the triggers of physiological labour, and even if it does not directly regulate these cells' function, changes in immunometabolism could be important biomarkers to predict pregnancy disrupted pregnancy tolerance. If changes in metabolism are detected, then investigating whether these are linked to changes in immune cell function that may be involved in the modulation of maternal tolerance might suggest a novel mechanism to explain the onset of labour. Particularly, whether changes in metabolism are associated with altered function of NK cells, T cells and monocytes/macrophages.

5.1.1 Metabolic pathways and pregnancy

Metabolomics has been used to investigate pregnancy related changes in a variety of biospecimens including serum (402), amniotic fluid (403) and cervical fluid (404, 405). It has

also been studied in urine (discussed in 5.1.2), however the studies focussing on metabolic pathways and the onset of labour are few in number.

A recent study by researchers in Bristol have specifically focussed on metabolite changes associated with the onset of labour, by comparison of intervillous maternal serum and cord blood (406). The study demonstrated an interplay between metabolites in the maternal and fetal circulations which point to the involvement of the endocannabinoid, sphingolipid, ceramide and steroid systems in the mechanism of active labour (406). Although a pilot study, the clear-cut difference seen in the metabolite levels between the two groups (women who laboured compared to women who did not labour) are clues to which pathways trigger spontaneous labour in women.

Endocannabinoids are described as non-classical neurotransmitters (407). There are two cannabinoid receptors, CB1 are present in areas of the brain, peripheral nerve terminals and extra neural sites including the uterus, whereas CB2 is largely limited to cells and organs of the immune system (408). N-arachidonoylethanolamine (AEA) binds to CB1 and CB2 and is broken down by the enzyme fatty acid amide hydrolase (408). The uterus can produce its own AEA (408) and it has been shown to have a concentration-dependent relaxation effect of AEA on human myometrial contraction in vitro (409). Fatty acid amide hydrolase can convert AEA to ethanolamine, releasing arachidonic acid for prostaglandin synthesis, providing a potential link between elevated AEA and labour (410).

Ceramide is a sphingolipid second messenger released rapidly in response to apoptosis and stress (411). Activation of CB1 can lead to an acute production of ceramide (408) and it is interesting that TNF-α can promote the activation of CB1 and CB2 (408, 411, 412). Ceramide has also been shown along with IL-6 to increase in placenta of labouring women suggesting that ceramide metabolism and signalling may be implicated in controlling important inflammatory mechanisms driving gestation (413). Both IL-6 and TNF-α were shown to be significantly raised in the choriodecidua in several types of PTB (Chapter 3, section 3.4/3.5) and this links the interaction between the inflammatory events that trigger labour and its metabolic consequences.

Labour is a stress induced physiological status; therefore, it is likely that metabolites of glucocorticoids will be increased in both maternal and fetal serum (414, 415). Birchenall and colleagues observed that cortisol and its major metabolite corticosterone were significantly increased in maternal serum of women who laboured spontaneously compared to women who did not labour (406).

Drawing from the changes seen in the work undertaken by Birchenall *et al*, it would be interesting to see if these metabolite changes relating these pathways to the onset of labour can be mirrored in urinary metabolome. Urine is an ideal biospecimen to screen antenatally as it is easy to collect, store and is pain free to the woman which is in contrast to peripheral maternal serum. Antenatal fetal serum as a biospecimen is obviously unsafe and unethical.

5.1.2 Urinary Metabolites and the onset of labour

Metabolomics today is recognized as a powerful approach in a prenatal research context, since it can provide detailed information during pregnancy and it may enable the identification of biomarkers with potential diagnostic or predictive value (416). Urine is a particularly useful metabolomic tool as it is both easy to collect and non-invasive. There are only two studies to my knowledge that have specifically looked at urinary metabolome in relation to labour; Caboni and colleagues have shown that alanine, glycine, glucose, lactic acid and creatine were down-regulated in urine of women in the active phase of labour and in contrast 3-hydroxybutyric acid, acetoacetic acid and acetone were up-regulated (185). This suggests that the synthesis and degradation of ketone bodies is the most relevant biochemical pathway (185). Alternatively, Gevi and colleagues' study revealed that the two metabolic pathways that played an important role in labour were steroid hormone biosynthesis and amino acid metabolism (416). Most other work considering associating urinary metabolites and pregnancy has been focused on prolonged rupture of membranes, hypertensive diseases, and fetal growth restriction (188, 417, 418).

A new study evaluated the profile of a normal human urinary metabolome across 348 children and 315 adults; they identified 1005 metabolites in urine for the first time (419). Interestingly this study showed that the expression level of many inflammatory-related functions in adults was higher than that in children, possibly indicating that adults have a higher content of metabolites involved in immune function, such as carnosine, creatine, cytidine (419).

Recently more research relating urinary metabolome to COVID-19 has been undertaken in the hope of creating a screening method in Covid positive patients to differentiate and better predict severity of this condition. One such study correlated 13 urinary metabolites with alteration of CD4+, CD3+ and CD8+ T cells, as well as cytokines – IFN-γ, IL-2 and IL-4. This suggests close interactions between these microbial metabolites and host immune dysregulation in COVID-19 (420).

Another study on COVID-19 showed that when comparing urine to serum, more cytokines and associated receptors were detected in urine than in serum (421). They correlated urinary CXCL14, IL-34 and CCL12 as potential biomarkers reflecting the lymphocyte counts of patients with COVID-19 (421). Such correlations highlight that urine can be a useful biospecimen to improve understanding and reflect dysregulated immune responses.

As previously discussed, there is a paucity of evidence in the use of urinary metabolome in pregnancy, specifically relating to the onset of labour. Further research needs to be undertaken to develop a better understanding of the urinary metabolome with the aspiration of one day identifying reliable and sensitive biomarkers of labour.

Although we appreciate that the trigger of labour is complex and multi-factorial, our understanding beyond that is limited. Onset of labour is still a retrospective diagnosis defined by clinical factors and assessment; the predictability of its onset is poor. By studying the urinary metabolome in a longitudinal study, it is feasible to further identify metabolomic pathways that may be crucial to labour. This on the background of the immune cells of interest identified from Chapter 4 could help develop a better understanding of the immunometabolism of term labour.

5.2 Hypotheses

1) Urinary metabolite profiles will be different between 36 weeks gestation and at delivery.
2) Metabolites associated with oxidative stress will increase in the urine as a pregnant woman approaches spontaneous onset of labour.
3) Metabolites associated with steroids will increase in the urine as a pregnant woman approaches spontaneous onset of labour.

4) Metabolites associated with ketone body synthesis will increase in the urine as a pregnant woman approaches spontaneous onset of labour.

5.3 Aims

In this chapter I aim to develop a better understanding of the urinary metabolome at term in low-risk pregnant women. I want to study this via a longitudinal study to see what shifts in metabolites and hence metabolic pathways may be associated with both quiescent uterine activity and active uterine activity.

The ultimate goal is to see if there are any urinary metabolites that are consistently associated with the onset of labour which could be studied in more depth in the future. Along with identifying a set of urinary metabolites, the aim is to see if they may reflect or can be associated with changes in immune cells that play a role in triggering labour, specifically at the maternal-fetal interface. This could lead onto future research in immunometabolism, with specific focus on term and preterm labour.

5.4 Sample Collection and Methodology

5.4.1 Recruitment of women to study

Women were recruited from low-risk antenatal clinics that were held in the birth centre at Chelsea and Westminster Hospital. This was to avoid any clinical factors which may alter urinary metabolites such as pre-existing medical conditions, pregnancy complications and regular medications. Women who developed any obstetric complications such as pre-eclampsia or preterm premature rupture of membranes following recruitment were excluded from the study.

Below is a figure that summarises recruitment of patients to this study (Figure 5.1).

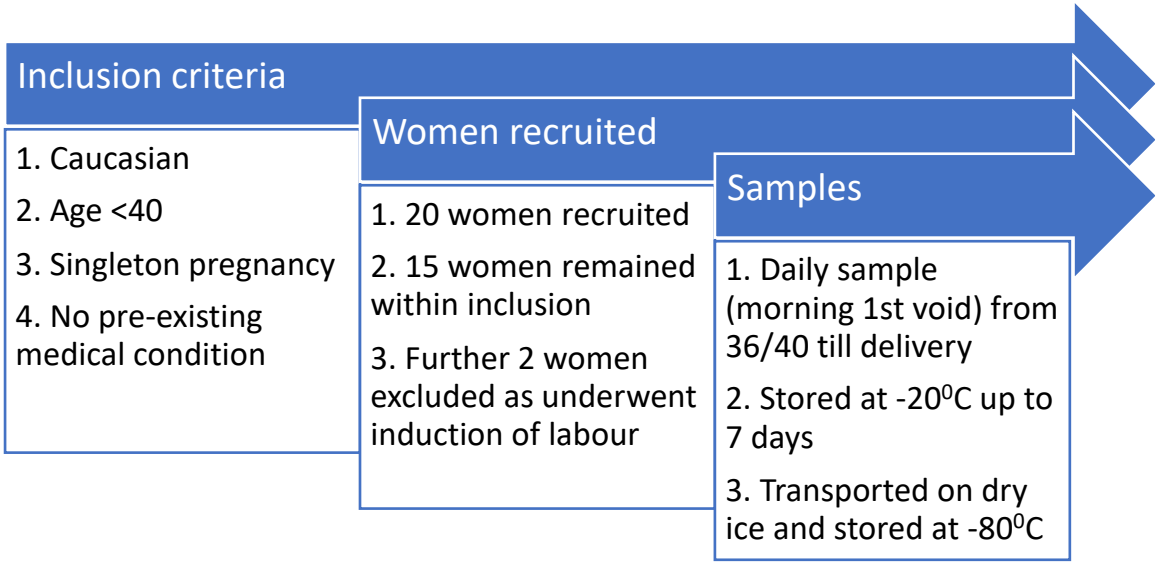


Figure 5. 1 Recruitment of patients to study

5.Urine Collection and Storage

Morning first void urine samples were collected daily in dry universal bottles by recruited patients from 36+0 weeks gestation till onset of labour. Samples were initially stored at -20^{0}C at the patients' homes for the first 7 days either in their personal home refrigerators or in refrigerators provided to the patients by our research team at their request. Once a week the samples were collected and transported on dry ice by me to Chelsea and Westminster Hospital to be stored at -80^{0}C till analysis.

5.4.2 Sample preparation

Prior to mass spectrometry, all 400 samples were aliquoted. 400 samples were processed in 4 batches of 100 samples to manage time effectively. 100 samples were thawed overnight for 13 hours at room temperature. Each sample was initially centrifuged at room temperature, at 10000 rpm for 10 minutes. 540 µl of urine supernatant from each sample was aliquoted to a 1.5 ml Eppendorf, to which 60 µl of urine buffer was added. Following vortexing, the sample was sat to mix well for 5 minutes and centrifuged at room temperature, at 13000 rpm for 10 minutes. Aliquoted prepped samples were stored at -80^{0}C and transferred to the MRC-NIHR National Phenome Centre, Imperial College London, on dry ice. 575 µl of prepped urine

supernatant was transferred into a 5mm NMR tube, carefully avoiding bubbles. A pooled sample from all urine extracts were created for quality control (QC) purposes. This is well summarised in Figure 5.4.

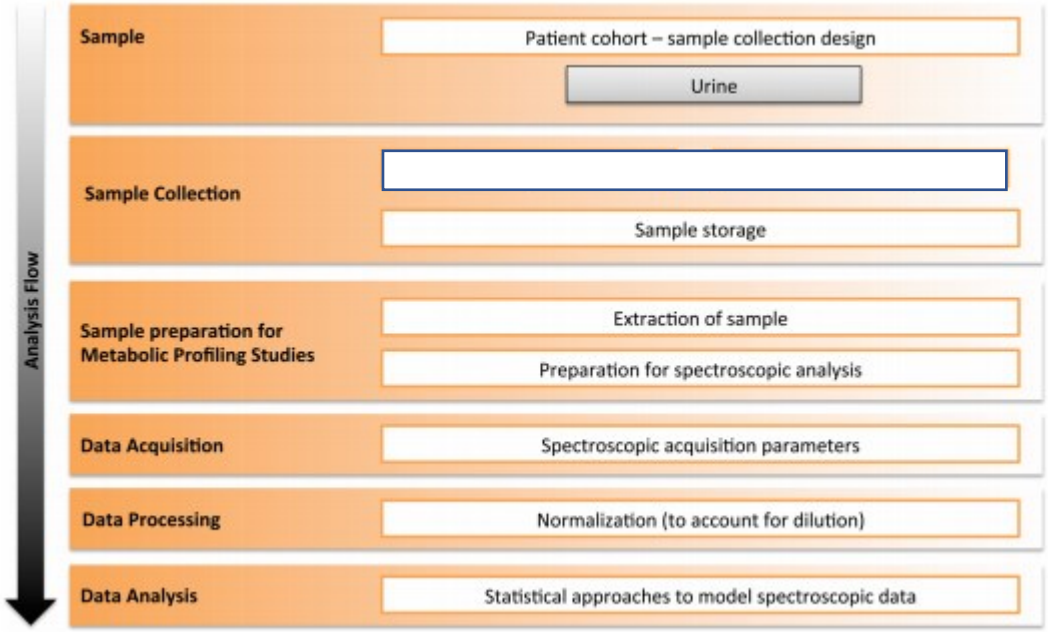


Figure 5. 2 A workflow describing the collection, preparation, and processing of urine samples.

5.4.3 Acquisition of ^{1}H NMR Spectral Profiles of Urine and Blank Samples

^{1}H NMR spectra were acquired on a Bruker DRX-600 spectrometer (Bruker Biospin, Karlsruhe, Germany) operating at 600.29 MHz for proton observation using a standard one-dimensional water pre-saturation pulse sequence [relaxation delay-90°-t_1-90°-t_m-90°-acquire free induction decay (FID)]. The relaxation delay was 4 seconds, with application of a 90° radio frequency pulse, t_1, referring to the interpulse delay, which was set to 3 µl, while t_m is the mixing time of 100 ms. The probe was matched and tuned automatically to the proton transmitter resonance frequency before acquisition for each sample and samples were run at a temperature of 27°C (300K). Processing of ^{1}H NMR spectra was carried out using TOPSPIN 3.1 software package (Bruker Biospin, Rheinstetten, Germany). The FIDs were transformed into a spectrum by Fourier transformation. The 400 spectra were manually phased, baseline corrected and calibrated to the TSP signal at δ 0.0 for the urine samples. This was undertaken by Dr Frances Jackson, a postdoctoral research fellow.

5.4.4 Assessment of Dilution Factor and Spectral Normalization on Human Urine Profiles

The spectral data were imported into Matlab software (version 2014a, the Mathworks Inc, MA, USA) and were transformed into 32K data points. Resonance of the water (δ4.7 -5.05) was also removed from each spectrum. Each 1H NMR spectrum was aligned using an in-house algorithm (195) and normalised using Probabilistic Quotient (196) Normalisation (PQN) in order to remove variation in metabolite concentrations linked to dilution (197).

5.4.5 Analyses/Statistical Analyses

PCA analysis is a qualitative chemometric technique that reduces the number of variables that need to be considered into principal components that are linear combinations of the original variables (443). This method is unsupervised, therefore no *a priori* knowledge of the sample classes is used and only the inherent variation in the data set is described. By using a few components, each sample can be represented by relatively few numbers instead of by values for thousands of variables (443). Samples can then be plotted, making it possible to visually assess similarities and differences between samples and determine whether samples can be grouped (422). The first principal component (PC1) is the direction along which the samples show the largest variation. The second principal component (PC2) is the direction uncorrelated to the first component along which the samples show the largest variation (422). PCA allows the reduced multivariate data to be projected into two or three dimensions. Principal component analysis (PCA) was carried out using mean-centred and unit variance scaled data. Statistical comparison was undertaken using an ANOVA test.

OPLS-DA analysis is a supervised multivariate data analysis. It can be used to identify discriminatory metabolites or features that are perturbed in the biological system. It is used to filter out unwanted variation within data and to obtain maximum separation between classes of data (423).

5.5 Results

5.5.1 Demographics of women recruited to this study

Table 5.1 summarises the demographics of the women who were recruited into this study. They are all categorised as "low risk" from an obstetric perspective and required only midwifery care antenatally.

Table 5. 1 Summary of demographics of women recruited to this study.

Patient Characteristics	Median	IQR
Age at booking (years)	32	31-35
White race, n (%)	13 (100)	
BMI at booking, kg/m^2,	22	21-24
Nulliparous, n (%)	9 (69.2)	
Para 1, n (%)	4 (30.8)	
Never smoked	13 (100)	
Gestation age at delivery (weeks)	40.86	38.86-41.43
Fetal weight (grams)	3600	3340-3830
Fetal sex		
Male, n (%)	11 (84.6)	
Female, n (%)	2 (15.4)	

5.5.2 Data quality

QC samples were used to evaluate the stability of the detection system and methods throughout the experiment. This PCA score plot shows the quality control samples cluster together and were close to the centre (Figure 5.3), indicating a good instrumental stability over the sample acquisition.

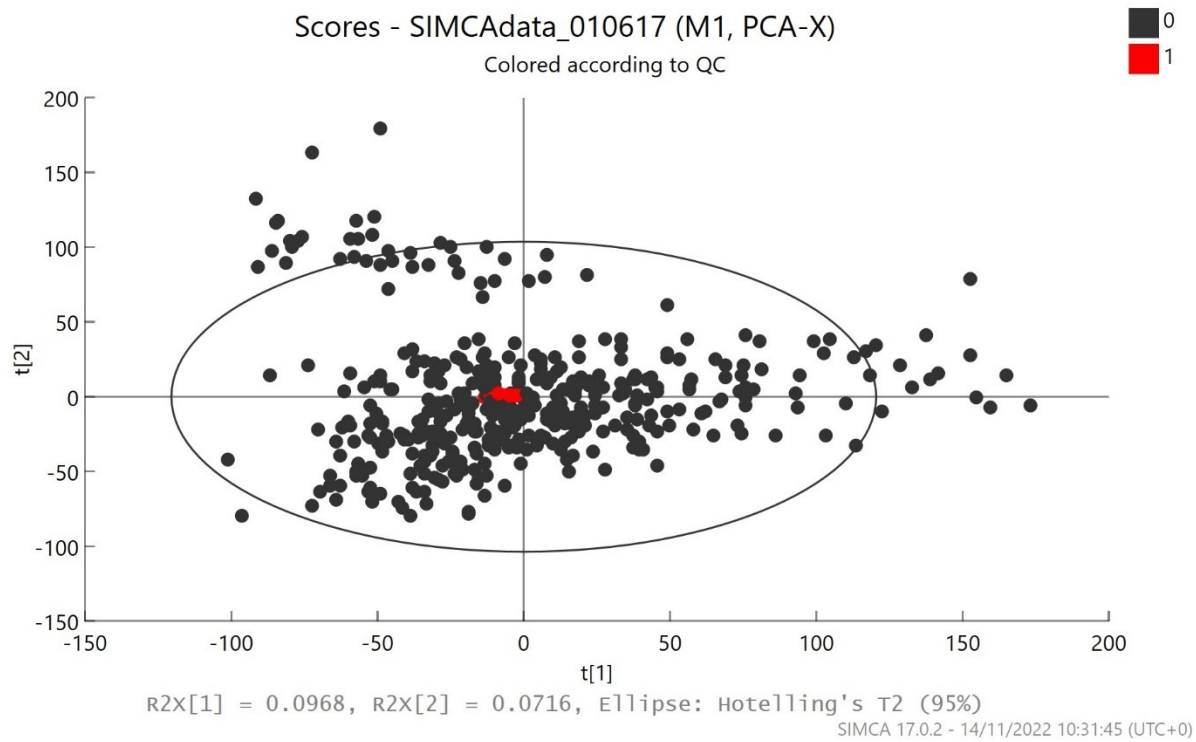


Figure 5. 3 PCA scores plot confirming data quality (black dots = experimental samples, red dots = quality control samples).

5.5.3 Overview of data

The PCA scores plot in Figure 5.4 show all 13 patients plotted. PC1 Patient AB separated clearly from the remaining patients along the first principal component (PC1). Samples from KJ separated from the rest along the second principal component (PC2).

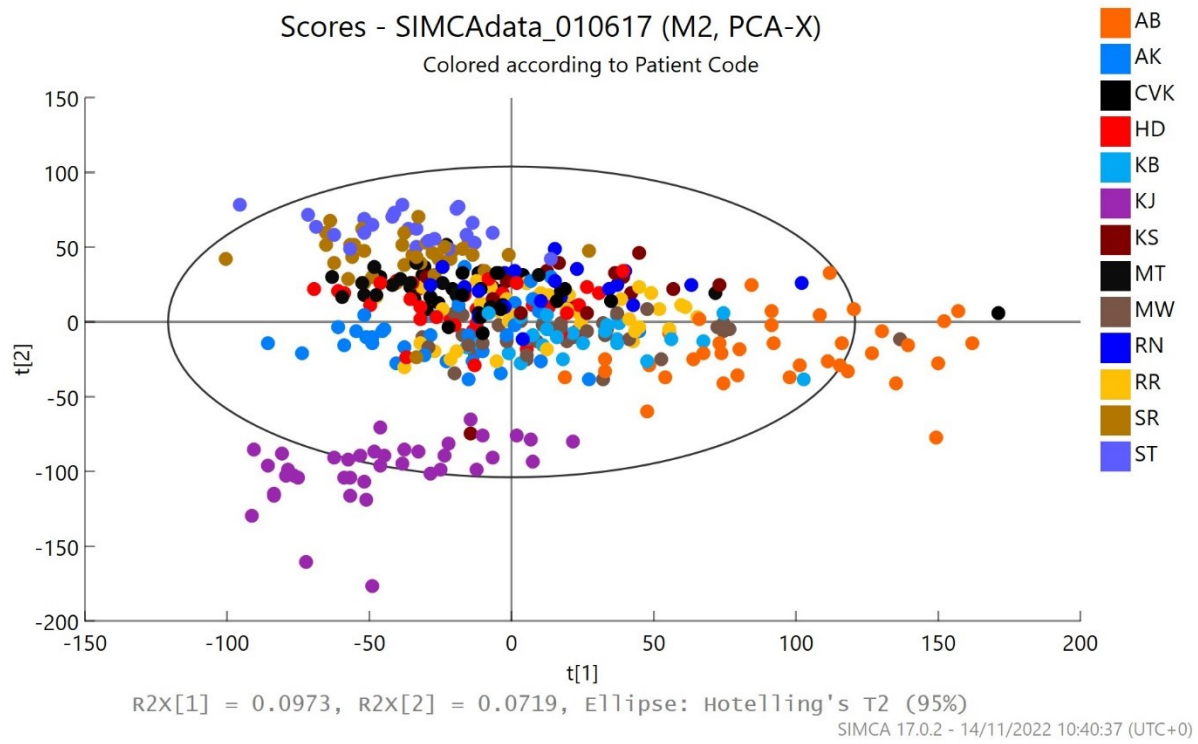


Figure 5. 4 PCA score plot without QC samples. PCA plots are colour coded based on different patients.

Mass spectrum of one patient recruited to this study is shown as an example below (Figure 5.5).

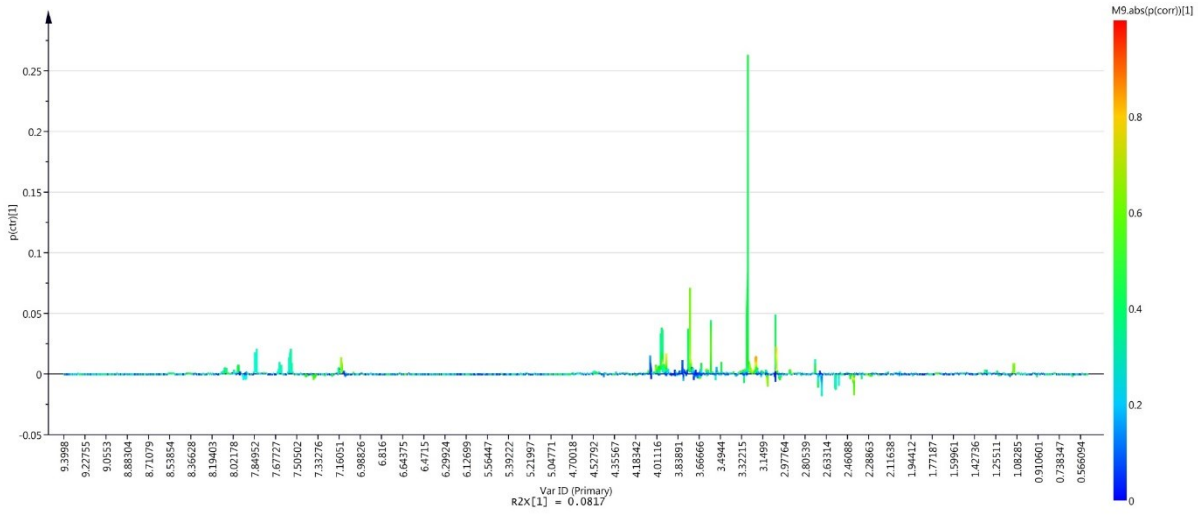


Figure 5. 5 Mass spectrum of patient KS

5.5.4 Overview of data based on ethnicity

Although all the women recruited to this study were Caucasian, one woman identified herself as White American and her sample separated very clearly from the rest (Figure 5.6).

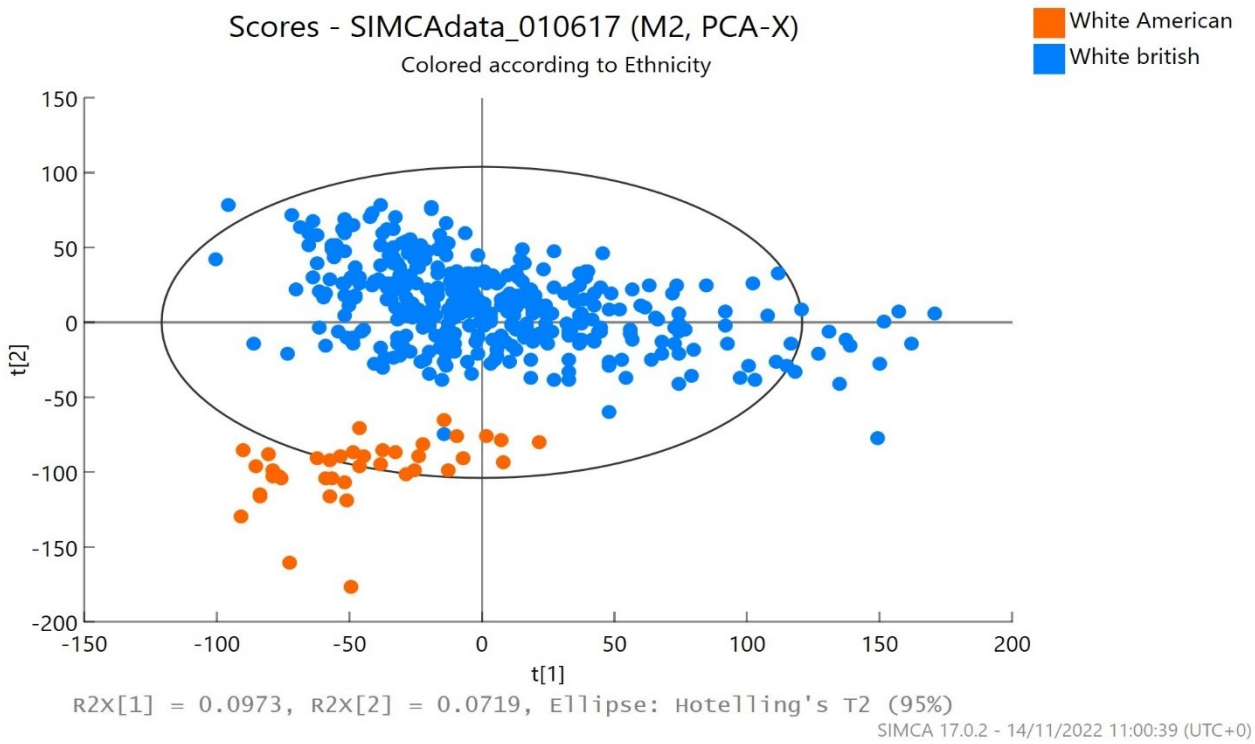


Figure 5. 6 PCA score plot exhibiting the results colour coded on ethnicity

5.5.5 Overview of the data excluding the outliers

The PCA plot below shows the overview of all the samples excluding the data of two patients – AB and KJ who were identified to be outliers on previous analysis (Figure 5.7).

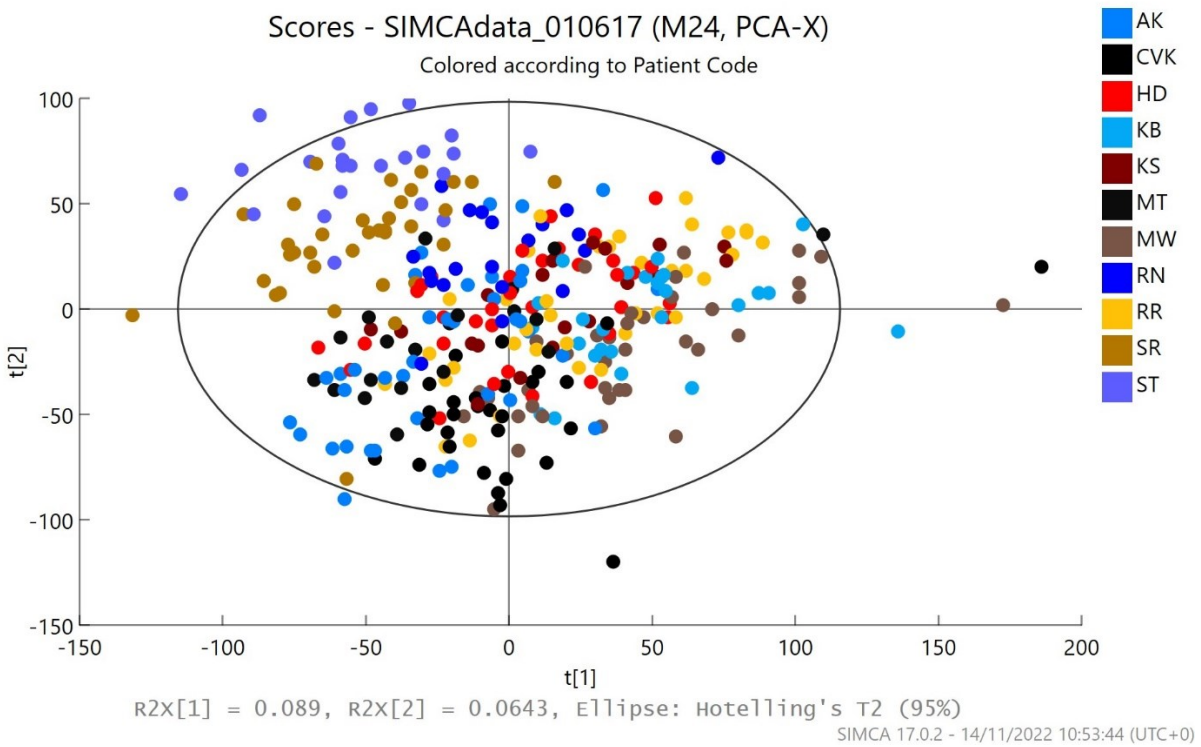


Figure 5. 7 PCA plot exhibiting the results excluding QC and two outliers (KJ and AB).

5.5.6. Time trajectory of the metabolic profiles

Figure 5.8 shows a time trajectory of the median value of all metabolites at each gestational age. Each colour indicates a different patient. The first graph indicates PC1 values and then second graph indicates PC2 values of the six women who went into labour before their expected date of delivery.

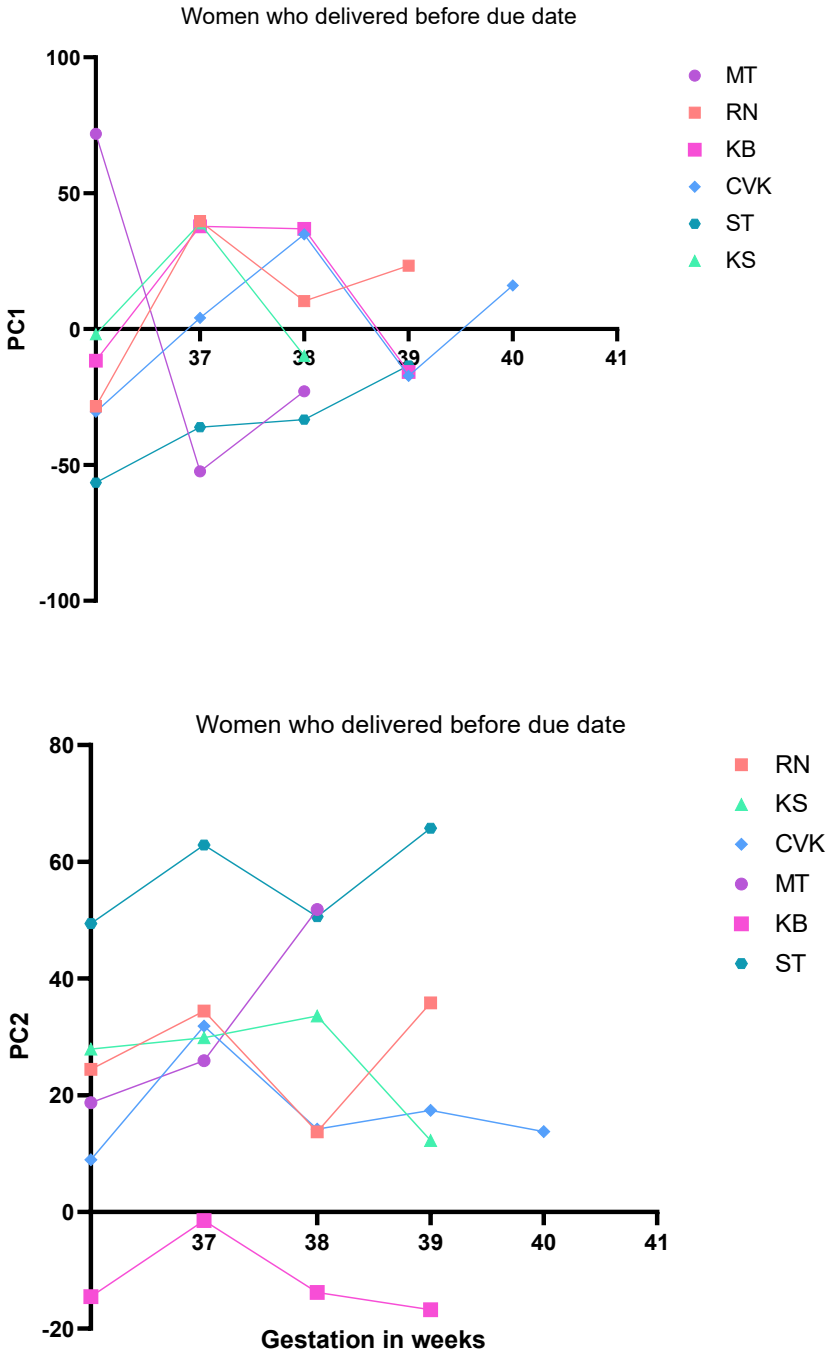


Figure 5. 8 Time trajectory of metabolic profiles of women (n=6) who delivered before their expected date of delivery. The weekly levels are calculated from each week's median value.

Figure 5.9 shows a time trajectory of the median value of all metabolites at each gestational age. Each colour indicates a different patient. The first graph indicates PC1 values and then second graph indicated PC2 values of the seven women who went into labour after their expected date of delivery.

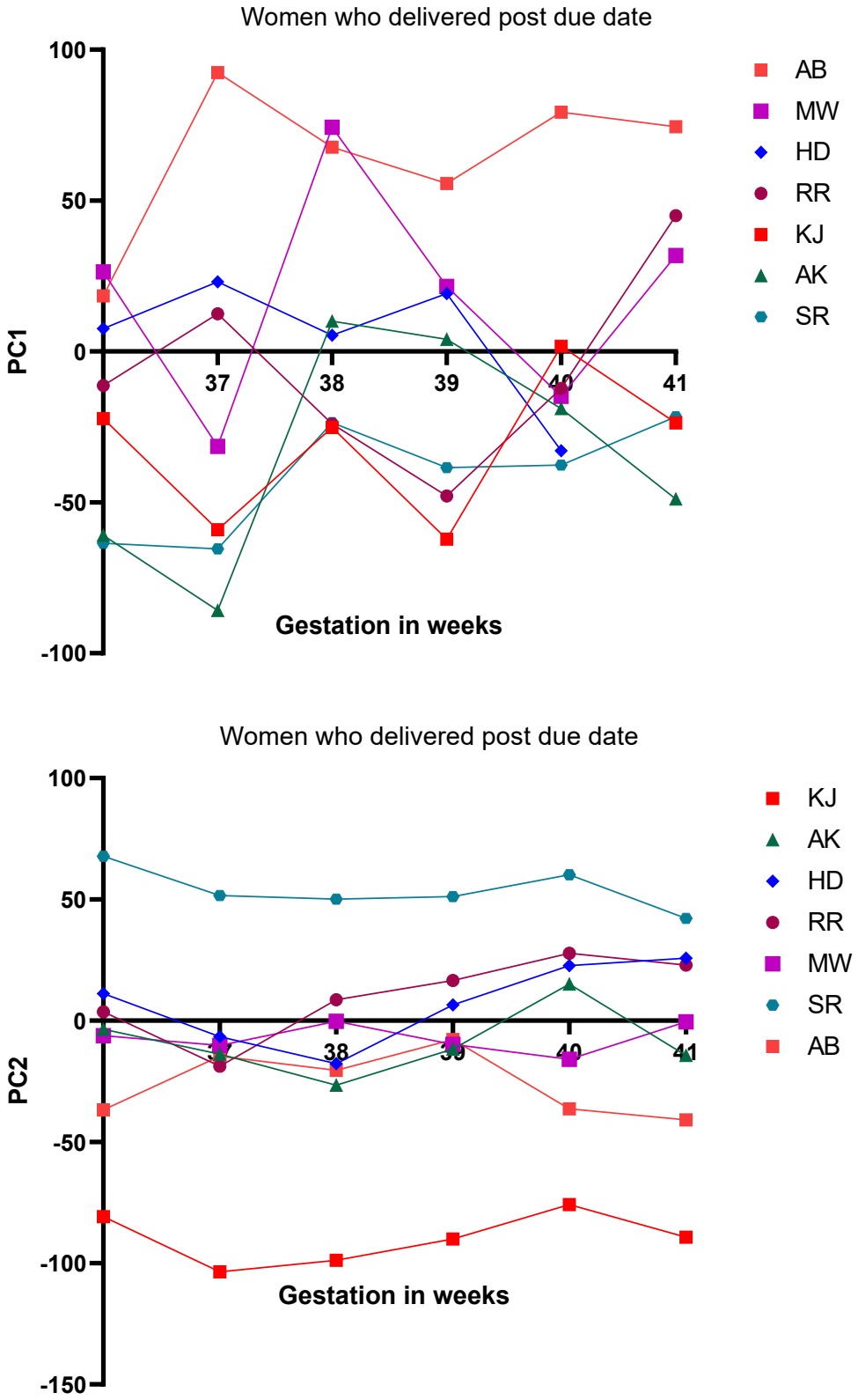


Figure 5. 9 Time trajectory of metabolic profiles of women (n=7) who delivered after their expected date of delivery. The weekly levels are calculated from each week's median value.

5.5.7 PCA analysis comparing 36 weeks with delivery

Figure 5.10 shows a PCA plot comparing 36 weeks to delivery. There is some overlap in scores, and it is evident that there was no clear separation in the two groups in PCA scores plot (Figure 5.11).

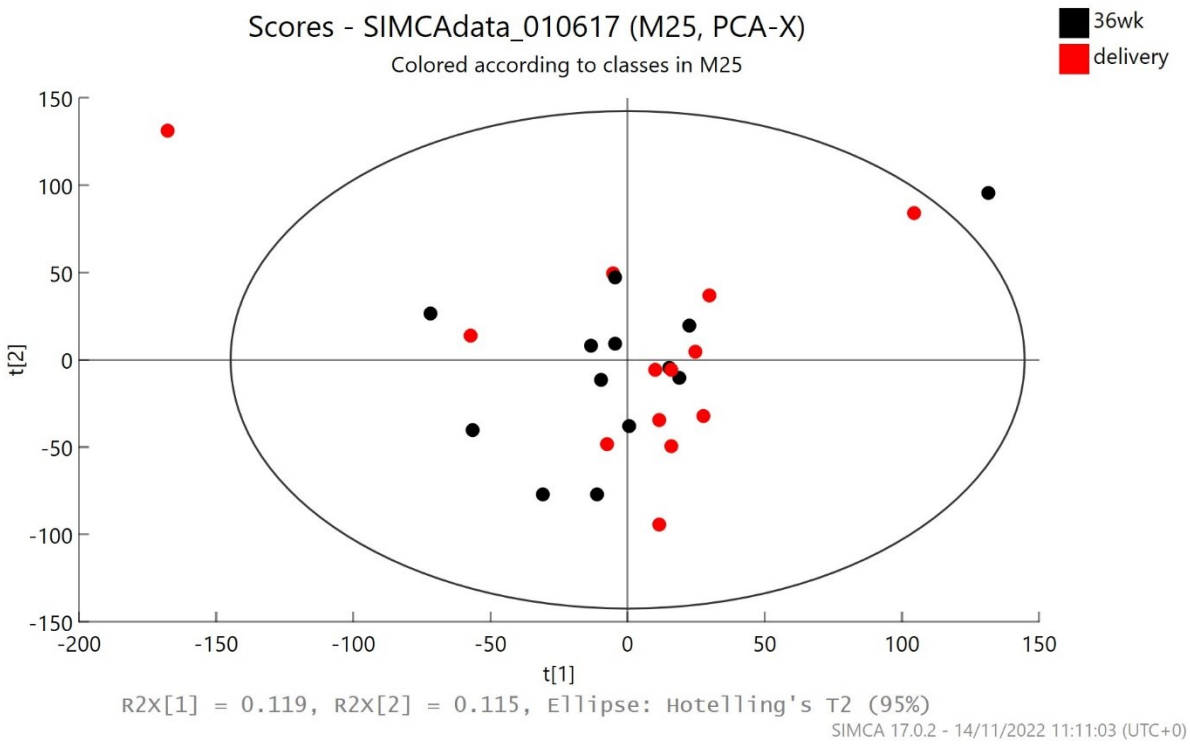


Figure 5. 10 PCA analysis of 36 weeks compared to delivery

The OPLS-DA analysis comparing 36 weeks to delivery is displayed in Figure 5.11. This is a supervised multivariate data analysis. There were no significant differences between the two groups (CV-ANOVA $p>0.05$. $Q^2Y<0$) (Figure 5.11).

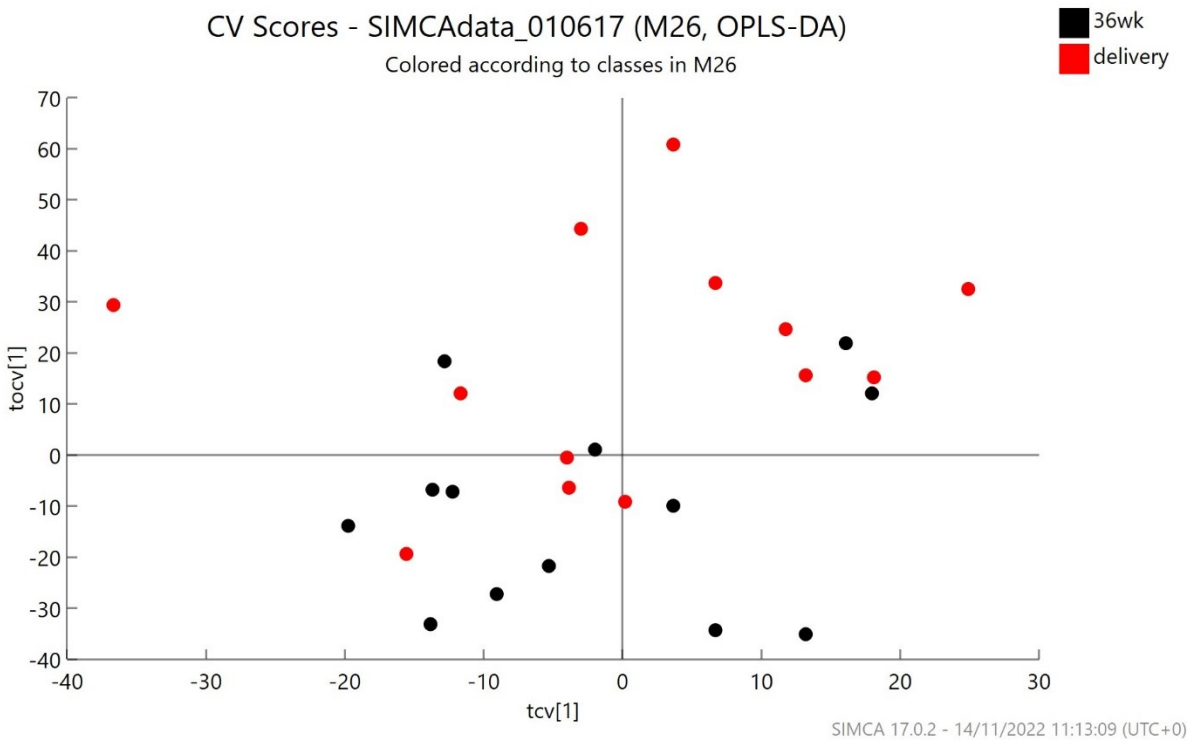


Figure 5. 11 OPLS-DA analysis (supervised method) of 36 weeks compared to delivery

Preliminary trends of metabolites which may be higher in later gestation (near time of spontaneous labour and delivery) is summarised in the figure below (Figure 5.12). In collaboration with Dr Frances Jackson the raw data was preliminarily assessed to identify metabolite peaks that showed higher levels with later gestation week. Of the peaks that were determined to be increased with gestation week, the most increased ones we identified were alanine, acetoacetate, acetyl carnitine, choline, and creatinine. Further analysis of individual metabolites is still in progress due to a technical issue with data storage and individual metabolite data extraction. When extracted, the changes in these metabolites will be confirmed using either linear regression analysis, or a mixed-methods non-parametric analysis, to assess longitudinal urine metabolite changes.

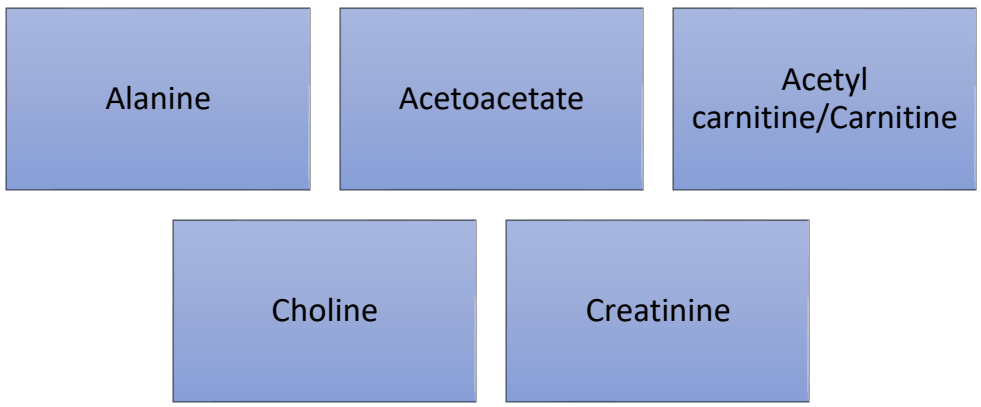


Figure 5. 12 Metabolites that seem to increase towards the end of pregnancy and nearer to the onset of labour

Further analysis of results is still in progress (delayed due to a technical issue with data storage) to confirm if these trends are relevant and/or statistically significant. With the help of Dr Jia Li (Reader in Biological Chemistry, Imperial College), I plan to further analyse the data. As the inter-personal variation was significant, more sophisticated analysis is required via subtraction of metabolites at 36 weeks from that at delivery, to identify how the metabolites change. This analysis may clarify if any metabolic pathways are consistently involved as the onset of labour approaches.

5.6 Discussion

This is the first study to the best of our knowledge where a longitudinal collection of urine has been performed daily from near 36 weeks gestation until the spontaneous onset of labour. A total of 400 samples were collected from 13 patients that were included in this study based on their pregnancies being classified as low risk. The aim of looking at only low risk women was to avoid confounding factors which may alter their metabolic profile such as pre-eclampsia or gestational diabetes. The aim was to limit all confounding factors to focus on how the changes seen may be related to pregnancy and the onset of labour. Even with recruiting low risk women, 5 women were excluded as they no longer fitted the inclusion criteria by 36 weeks due to complications such as premature preterm rupture of membranes

and change in mode of delivery (elective caesarean section). Two further women had to be excluded after 36 weeks, as they required induction of labour.

The overview of the data (Figure 5.4) showed that although most of the patients' urinary samples clustered together, AB and KJ separated along PC1 and PC2 clearly from the rest. AB and KJ were not distinctively different to the others in ethnicity, BMI, or age.

As part of the inclusion criteria, a single ethnicity was chosen as it is known that different ethnicities have different metabolic profiles (424) and to avoid this confounding factor, Caucasian ethnicity was chosen, especially as this ethnicity was the predominant ethnicity at Chelsea and Westminster Hospital where I recruited patients from. Even with such a tight inclusion criterion, it was very interesting to see that KJ separated clearly on the plot from the others (Figure 5.6). Although KJ was also Caucasian, she identified herself as White American whereas all the others were White British. This difference seen on the plot could be because of a different diet (425, 426) and/or lifestyle.

When the PCA plot was analysed following exclusion of the outliers (Figure 5.7) it was clear that ST and SR formed a cluster. It seems that metabolic profiles of pregnant women in the current study have larger inter-personal variation than intra-personal variation.

When focusing on the time trajectory of metabolites using median values per gestational week, there was no clear pattern seen in the women who delivered before their expected date of delivery in both PC1 and PC2 analysis. When looking at the women who delivered after the expected date of delivery, it was interesting that excluding AB and KJ, there was a similar clustering of the trajectory around 0-50 on the PC2 plot. This could suggest that as these women clustered together, there may have been a more similar metabolic profile as they went into labour.

On direct comparison on 36-week samples to pre-delivery samples, on both the supervised and unsupervised PCA analysis, no differences were seen. This is not entirely surprising as although there were a large number of urinary samples in total (n=400), this was a small number of patients. The inter-personal variation seen in previous analysis suggests that to get any reliable significant differences, much larger number of women will need to be recruited.

This is especially because there are many factors that could affect each woman's urinary metabolites.

One of the metabolites noted to increase towards the onset of labour was alanine (Figure 5.12 which was previously observed by Diaz et al (298, 427). Alanine is one of the 20 proteogenic amino acids (442) and is a non-essential amino acid that can easily be made from either conversion of pyruvate or the breakdown of the dipeptides carnosine and anersine (442). Alanine can also be synthesised from branched amino acids such as valine, leucine and isoleucine (442). Alanine is highly concentrated in muscle and is one of the most important amino acids released by muscle, functioning as an energy source (442). Alanine is also an important participant and regulator of glucose metabolism (Figure 5.13). It is possible that like in exercising skeletal muscle, with the onset of labour, anaerobic glycolysis predominates and produces pyruvate and consequently alanine in excess (428). The increase in urinary alanine may be a sign of increase in anaerobic glycolysis in general to meet the energy requirements to trigger labour.

It is also interesting that alanine is an important amino acid for lymphocyte production and in particular has been shown to be vital in T cell activation (429). This is contrasting the results achieved in Chapter 4 which showed T cell activation was not seen with the onset of physiological labour at term (Chapter 4.6.13, Figure 4.18). This could suggest the T cell activation although not seen in DB could be occurring elsewhere such as in the decidua parietalis as shown by Sindram-Trujillo and colleagues (430). Alternatively, it is likely that the metabolic mass of each subtype of immune cells are too small to truly be represented in an individual's urinary metabolic profile.

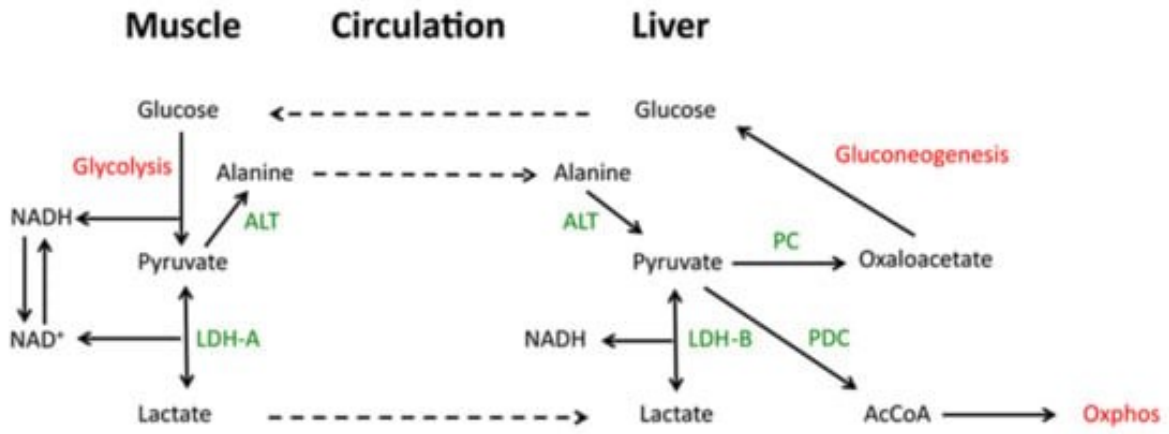


Figure 5. 13 How alanine is involved in glucose metabolism and production. ALT alanine transferase, LDH-A lactate dehydrogenase A, LDH-B lactate dehydrogrenase B, PC pyruvate carboxylase, PC pyruvate dehydrogenase complex NADH nicotinamide adenine dinucleotide hydrogen (Prochownik & Wang, 2021). © 2021 Prochownik & Wang

Acetoacetate was also noticed to be higher towards the end of pregnancy in preliminary analysis. Acetoacetate is a ketone body (431) and are important vectors of energy transport from the liver to extrahepatic tissues, especially during fasting when glucose supply is low (431). Acetoacetate is a product of fatty acid oxidation in the liver and in extra-hepatic organs it can feed into the TCA cycle (Figure 5.13) to release stored energy (432). Both TCA and fatty acid oxidation are metabolic pathways that are associated with quiescent immune cells and T_{reg} generation (161). Increased clearance of acetoacetate into urine could be a sign that metabolic pathways associated with quiescence is being withdrawn in order for labour to be triggered. This is supported by the fact that T_{regs} were decreasing (non significantly) in both placenta and decidua basalis with the onset of labour at term.

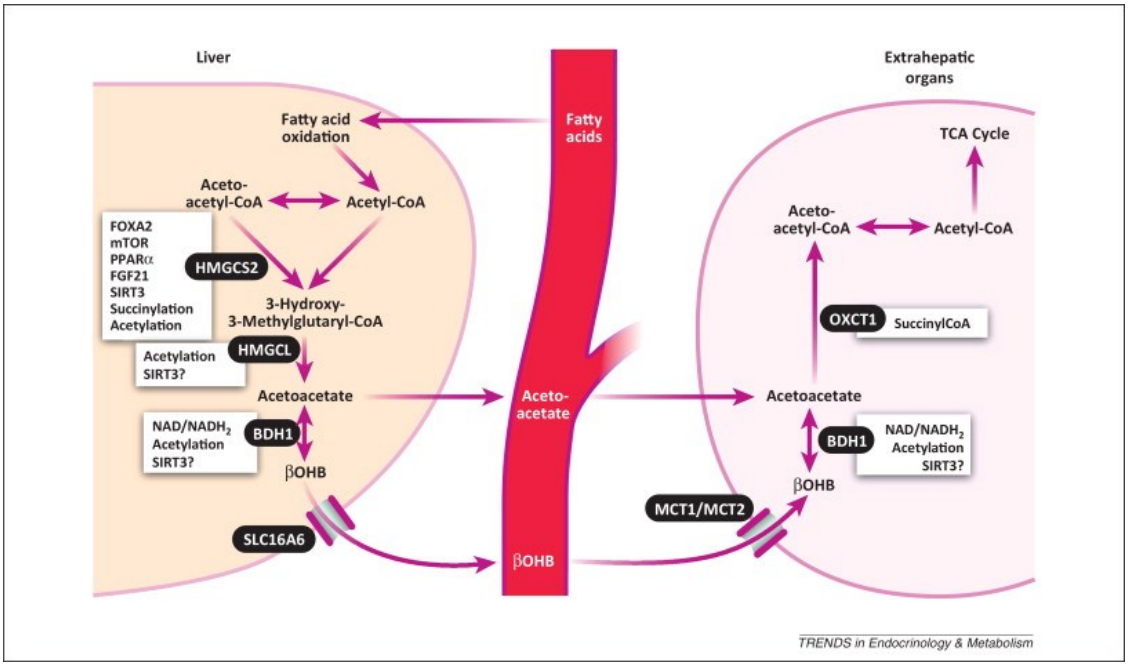


Figure 5. 14 Outline of ketone body metabolism and regulation. Abbreviations: BDH1, β-hydroxybutyrate dehydrogenase; FGF21, fibroblast growth factor 21; FOXA2, forkhead box A2; HMGCS2, 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase 2; HMGCL, HMG-CoA lyase; MCT1/2, monocarboxylic acid transporters 1/2; mTOR, mechanistic target of rapamycin; OXCT1, succinyl-CoA:3-ketoacid coenzyme A transferase; PPARα, peroxisome proliferator-activated receptor α; SIRT3, sirtuin 3; SLC16A6, solute carrier family 16 (monocarboxylic acid transporter), member 6; TCA cycle, tricarboxylic acid cycle. (432) ©2014 Newman & Verdin Image reproduced with permission from Elsevier and Copyright clearance.

Acetylcarnitine and carnitine also seemed to be increasing towards the end of pregnancy on preliminary analysis. Acetylcarnitine is the acetylated derivative of the amino acid L-carnitine whose function is generally correlated with regulation of energy metabolism within mitochondria (442). Both these metabolites play a role in fatty acid oxidation which as mentioned above, is key in promotion of T_{regs}. Increased excretion of these metabolites may suggest reduction in fuelling T_{regs} as labour is nearing. This is supported by the data in Chapter 4 which shows that T_{regs} likely play a significant role in maintaining immune equilibrium at term which is lost with the onset of labour (Chapter 4.6.17).

It is also possible that carnitine is actually derived from one's diet and its significance in urinary metabolites has to be considered in line with this. All the women who were recruited into this study were healthy women with normal body mass indices, which is associated with a healthy diet. The increased levels of acetylcarnitine and carinitine seen in the urine may be

associated with their dietary intake of a health diet consisting of of dairy products, fish and meat. However, this would not explain it's increase towards the latter end of pregnancy, as it is unlikely that one's diet would vastly differ from that at 36 weeks gestation to that at delivery.

Choline which is an essential vitamin was also noted to be increased towards delivery in this study. This has been noted once before by Diaz and colleagues (427) and it may actually be due to the intake of folic acid. There has been evidence that there is preferential activation of homocysteine remethylation in later gestation, which can occur in connection with the choline/demethylglycine pathway or demethylation of tetrahydrofolate (433). This increased urinary choline may suggest that tetrahydrofolate pathway is preferred which could be in associated with folic acid supplementation (427). Unfortunately, I had not formally collated information regarding pregnancy vitamins from the women recruited, however from regular conversations with them I know most of them were taking a well recognised and recommended multi-vitamin throughout their pregnancy.

Creatinine was also increased towards the end of pregnancy which is a physiologically normal and well recognised alteration in pregnancy. It is a waste product that muscles produce at a steady rate and is filtered out of the body through urine (434). It is clinically used as a measure of renal function and glomerular filtration rate increases by 50%, with subsequent decrease in serum creatinine in pregnancy (435). It is interesting there seems to be a difference noted between 36 weeks and nearer the onset of labour, as creatinine clearance is known to reduce closer to term (436). This increased rate may be suggestive of increased work by muscles, such as the myometrium as creatinine is a by product of muscle, however more work would need to be carried out to truly understand this.

5.7 Strengths and Limitations

This study is limited by the small number of women who remained within inclusion criteria to partake in the study. Recruitment to this study was not easy as it required a significant commitment to the process of taking daily urine samples from 36 weeks till delivery. In addition, the women had to be comfortable in storing their own urine samples in a freezer for 7 days till I collected, which was on weekly basis. I had offered them research freezers to

make it more comfortable for the patients to store the samples, but most women who agreed to be recruited declined this due to lack of space at home. Most of the women in this study stored their urine in enclosed boxes in their own freezer. The loss of recruited women also further limited the number as some women developed pregnancy complications or required induction of labour.

I chose one ethnicity to recruit patients from to limit variability in their metabolism due to ethnic differences, therefore the findings of this study are not generalisable. I was aware of this limitation but felt it was most important to limit confounding factors to attribute any metabolite changes to the labour process as opposed to other factors.

In retrospect, it would have been useful to have a food diary or a validated questionnaire which could help understand any dietary differences these women had, as this may have helped explain the inter-personal variability seen in this study.

The metabolic mass of each subtype of immune cells may have been too small to truly alter the metabolic urinary profile, although with further planned analysis we may be able to identify shifts in metabolic pathways which may reflect an overall shift from tolerant to active immune cell activity.

Birchenall *et al* were able to identify different metabolic pathways that could be key in the onset of labour from their pilot study (406) which was not replicated by my study. I think the key reason for this was that the localised changes seen between intervillous serum and maternal serum were lost when focusing on the urinary metabolome which reflects imbalances of all biochemical pathways in the body. I feel future work would benefit from correlating matched serum and urine for metabolomic assessment, along with cytometric analysis of decidua basalis/parietalis and maternal blood.

This study looking at urinary metabolites and its relation to the onset of labour longitudinally is the first of its kind. To my knowledge there are only two papers specifically looking at the onset of labour and urinary metabolites, neither of these are longitudinal in design. This study involved 400 urine samples and large amount of data has been generated from these samples. As is expected with untargeted data collection, further analysis is required, and this is still ongoing. This study has provided a starting point for urinary metabolites being a possible

biomarker in the future. More importantly and more relevant to my thesis is that it helps maybe understand a little more about immunometabolism.

5.8 Summary

There was no signature urinary metabolic profile seen with the onset of labour. There was quite clear inter-personal variation seen amongst these women even when controlled for ethnicity, age and body mass index. Preliminary analysis of the metabolites has shown that alanine, acetoacetate, acetyl carnitine/carnitine, choline and creatinine were increased closer to delivery. Alanine is an important amino acid released from muscle and its increase towards the onset of labour may be suggestive of its role as an energy source in preparation for labour. Increased urinary clearance of acetoacetate could be a sign that metabolic pathways associated with quiescent immune cells are being withdrawn for labour to be triggered. Anaerobic glucose metabolism and fatty acid oxidation may be key metabolic pathways in the preparation for labour. It is important to acknowledge that with a small number of women and other factors such as diet and lifestyle altering metabolic profiles, further studies are needed to consolidate this explorative study.

6. Final summary and discussion

Preterm birth is a global problem, with a global preterm birth rate of 11% (437) leading to both death and long-term morbidity. It is still the leading cause of death in children, with 1 million children dying due to preterm birth before the age of 5 (437). A recent study examining the trends of preterm birth rates found that the global preterm birth rate rose from 9.8% in 2000 to 10.6% in 2014 (5). Although there is much research targeted to understanding and preventing preterm birth, preterm birth rates have not improved, with 150 babies being born premature every day in the UK alone (www.tommys.org).

Our understanding of the triggers of labour whether at term or at a preterm gestation is still very limited. There are many factors that are known to play a part in preterm labour such as uterine overdistension, cervical disease, placental disorders, and infection amongst others (9). Labour is widely accepted as an inflammatory process which breaks down the maternal-fetal tolerance (146). Localised inflammation within the gestational tissues is of much interest, and most research has been focused on myometrium (145, 438), amnion (146, 439) and placenta (440). In comparison, decidua, which is regarded as the maternal-fetal interface, on the whole has been overlooked.

This thesis highlights the importance of the decidua and its potentially significant role in maintaining immune quiescence preceding both preterm birth and in term labour. It has compared the inflammation occurring in decidua, neighbouring placenta and peripheral blood, to gain a better understanding of how essential the decidua is in maintaining maternal-fetal tolerance.

6.1 Principal findings

The findings described in Chapter 3 showed that the choriodecidua is a more inflammatory gestational tissue when compared to placenta and amnion. Cytokines and chemokines on the whole were significantly increased in the choriodecidua, placenta, and amnion in the chorioamnionitis group, with 14/19 analytes being significantly increased in all 3 gestational tissues. IL-8 levels in the choriodecidua were significantly associated with worse neonatal

outcomes in the chorioamnionitis group, however this was not observed in relation to IL-6 or TNF-α, which may be due to the small number of patients studied within each neonatal outcome group (good outcome n=5, adverse outcome n=6).

In idiopathic PTL, along with pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α, immunoregulatory cytokines such as IL-2, IL-4 and IL-10 were significantly raised in the choriodecidua, with no change seen in matched placental samples. This highlights that idiopathic preterm birth is not a simple switch in immune tolerance and represents a far more complex process requiring a lot more research.

In preterm birth caused by placental abruption, there was very little cytokine/chemokine signalling. This may be because labour is driven by the thrombin present in the retroplacental clot and inflammation has no role.

Multiples pregnancies are known to be associated with preterm birth, some are iatrogenic, but others are spontaneous preterm birth commonly assumed to be associated with uterine overdistension. This overdistension has been associated with an inflammatory pulse (281) and this was further evidenced with 10 of 20 cytokines analysed being significantly raised both in choriodecidua and placenta in the twin labouring group.

The inflammation marker panel highlighted that the choriodecidua was more inflammatory than nearby amnion, with 23 out of the 37 biomarkers analysed being significantly higher in choriodecidua compared to amnion.

Based on the cytokines/chemokines and inflammatory markers analysed in this study, along with existing literature, NK cells, T cells, monocytes and neutrophils were identified as immune cells of interest and investigated further in Chapter 4.

The study in Chapter 4 looked at the forementioned immune cells in decidua basalis (DB), placenta, peripheral and cord blood in term non-labouring, preterm non-labouring and term early labouring women. $CD56^{bright}$ NK cells did decrease in DB with the onset of labour, albeit not significantly but there was a clear trend. The shift in DB containing predominantly $CD56^{bright}$ NK cells at term to a predominant subset of $CD56^{+}CD16^{+}$ NK cells with onset of labour is particularly fascinating. This may indicate a switch in the decidua from an immunotolerant environment to one that promotes inflammation. At term, peripheral blood

had significantly higher expression of HLA-DR than placenta, DB, and cord blood in all three subsets of NK cells. These HLA-DR$^+$ NK cells produce more IFN-γ (360) which is increased in the choriodecidua in idiopathic preterm labour, described in Chapter 3. It is possible that peripheral HLA-DR$^+$ NK cells may contribute to this by migrating into the gestational tissues.

There were no significant differences noted in the monocyte subsets in DB with either the onset of term labour or at preterm gestations. However, there was an interesting loss of HLA-DR expression on classical monocytes in the placenta and PB with the onset of term labour. This loss of HLA-DR expression was not mirrored in DB. There was no significant difference in T_{reg} cells in DB with the onset of labour but there were significantly increased numbers of T_{regs} in all four compartments as gestation advanced from preterm to term. CD4$^+$ T_{regs} and CD8$^+$ T_{regs} were significantly higher in the DB than in the placenta and PB. A significant correlation seen only in the DB between CD56^{bright} NK cells and T_{regs}, which is lost with the onset of labour. This highlights the potential importance of the DB in maintaining immune tolerance at the maternal-fetal interface. Number of neutrophils quantified in each sample in each group had wide interquartile ranges and therefore no conclusion could be made.

In conclusion DB is a unique gestational tissue in which immune cells that are known to promote immune tolerance were present, evidenced by high numbers of CD56^{bright} NK cells, CD4$^+$$T_{regs}$, CD8$^+$$T_{regs}$ and higher proportion of intermediate monocytes. Term labour is associated with disturbance of correlation between CD56^{bright} NK cells and T_{regs} in the DB.

To further address the loss of tolerance described above, I embarked on an investigation of the metabolic pathways that may be upregulated or downregulated with the onset of labour. The longitudinal study in Chapter 5 showed that there was no signature urinary metabolic profile seen with the onset of labour. Although ethnicity, body mass index and age were controlled for, there was still clear inter-personal variability noted in the women's metabolic profile. Metabolic pathways that may be upregulated closer to labour are anaerobic glucose metabolism suggested by increased urinary clearance of alanine. Fatty acid oxidation may be downregulated closer to labour, which may reflect withdrawal of metabolism typically associated with quiescent immune cells.

6.2 Limitations

This thesis has several limitations which have to be acknowledged. Chapter 3 was undertaken on frozen tissues (immediate transfer to a -80^{0}C freezer which were not snap frozen. The risks of direct freezing include damage to the cells by ice crystals and cell destruction due to direct mechanical action (441). This limited the research methods that could be undertaken on these tissues, for example the quality of the tissue was deemed unsuitable and unreliable to undertake immunohistochemistry (IHC). Ideally theses samples would have fared better if snap frozen and it would have enabled IHC which would have given further insight into tissue architecture and the location of the immune cells which may have been producing these cytokines/chemokine changes.

Chapter 4 was undertaken on fresh samples, processed within 2 hours to optimise live cell count, especially from decidua basalis and placenta. Although collection was ideal, it took nearly 6 months to identify a reliable methodology for tissue digestion which consistently produced 1-2million live cells from the decidua basalis. This therefore limited my work to a single flow cytometry panel, and I was unable to analyse the samples for other relevant immune cells such as dendritic cells and macrophages.

Chapter 4 was also limited as I was unable to collect adequate numbers of suitable preterm labouring samples to make a comparison to the PTNL group. This was mainly because many preterm patients who laboured delivered vaginally; these samples were excluded from my study as they could be contaminated during the delivery process. Additionally, the preterm labouring patients who did undergo a caesarean section had significant associated conditions such as placental abruption which could skew the inflammatory activity within the placenta and decidua.

Although the study in Chapter 5 was a longitudinal study with a large number of timepoints (400 samples in total), it was limited by the small number of patients recruited into the study. Metabolic profiles are clearly influenced by many factors including diet and lifestyle, hence interpretation of the metabolic changes and its relation to the onset of labour is also limited. Unfortunately, assessment of the maternal diet and lifestyle were not undertaken on the women who were recruited to this study. These data could have helped in the interpretation

of the findings, perhaps allowing consistent patterns to be detected, which were otherwise obscured by confounding variables such as different dietary patterns.

The main limitation of this thesis was that the samples in each chapter were taken from different women so results from each chapter cannot be directly correlated with results from another chapter. To be able to do this a further study with multiple research methods evaluating immune cells and metabolites in a single cohort of women would need to be undertaken.

6.3 Future work

To build on the findings from Chapters 3 and 4 further recruitment of idiopathic preterm labouring women would be ideal to investigate changes in immune cells proportions across the four compartments. It would help inform and establish whether there are any immunological similarities between term labour and preterm labour at a decidual level. To further understand the relationship between the immune cells identified using flow cytometry and cytokines, matched tissues could be snap frozen to undertake cytokine PCR complemented with immunohistochemistry. This would help tie together the findings of Chapters 3 and 4, and to truly associate which cells may be contributing to the cytokines production, effector function and/or immune-regulation at the maternal-fetal interface.

Developing a methodology that successfully isolated at least one million live cells from tissue, specifically from the DB required much time and perseverance. The number of live cells I was initially achieving from DB limited the number of immune cells I could analyse. Having optimised and established this methodology and knowing other colleagues have used it to undertake research on other tissue including skin and gestational tissues (Cocker et al, IN PRESS) is encouraging. Further flow panels can be designed to look for other immune cells of interest such as dendritic cells and macrophages. It would also be interesting to look at the immune cells in the decidua parietalis as this would be a fascinating comparison to the DB; especially as there is literature suggesting that decidua parietalis lymphocytes display a more activated phenotype than DB (332).

In addition, it would be particularly interesting to isolate NK cells and/or T_{regs} via cell sorting using a flow cytometer that would facilitate quantitative single-cell transcriptomics analysis. Once these decidual immune cells have been sorted, it would be interesting to stimulate these decidual immune cells with cytokines. The cells' cytotoxicity could be assessed via production of perforin or granzyme B using an enzyme-linked immune absorbent spot assay. This would give a better understanding of the function of these decidual cells and their cellular interactions within the decidua and how these change during the transition into established labour at term and preterm gestations.

To expand the work on urinary metabolites, it would be best to design a study where urine samples are collected bi-weekly from the gestation of viability, 24 weeks, until delivery. Alongside this, it would be useful to collect blood at timed intervals during the pregnancy and on admission in labour to undertake mass spectrometry. Recruited women should also complete a standardised questionnaire on diet to ascertain an understanding of their dietary intake and whether it changes from week to week. This information would help correlate metabolic profiles with individual diets. Within this study, the women who consequently deliver via a caesarean section should have flow cytometry analysis of decidua, placenta, and maternal blood to further correlate urinary metabolites to immune cell proportions.

The onset of labour and preterm birth is a complex, multifactorial phenomenon which is poorly understood and predicted. Preterm birth is a global problem and is the single biggest cause of cause of neonatal mortality and morbidity. The decidua seems to be the gestational tissue where immune tolerance is maintained to safely support and maintain the pregnancy through tolerant immune cells such as NK cells and T_{regs}. The trigger of labour, which is understood as inflammatory needs more understanding, to improve prediction possibly via biomarkers and importantly to identify preventative treatment. A longitudinal study as described above would not only better consolidate the findings of this thesis but also improve understanding of immunometabolism of labour and maybe even preterm labour.

7. Appendices

7.1 Protocol for Preparation of Decidual and Placental Leukocytes

Standard Operating Procedure (SOP) #14 ***Decidual Leukocyte Separation***	**Immunotherapy Group**	**Created by:** **SP Sivarajasingam, A Cocker, N Imami 2016**

1. Decidua Basalis

1. Dissect a piece of the basal plate from one cotyledon of the placenta

2. Place it on a sterile cutting board with the placental villi facing upward. The side showing the placental villi is usually bloody red with hairy tissue in appearance. The basal plate is smooth and pale red in colour.

3. Use sharp, fine-point scissors and forceps to remove the villous tissue and blood vessels. Keep the tissue soaked in 1x PBS (Ca2+- andMg2+- free) during the process. Collect 2 to 3 of these pieces and rinse them thoroughly with 1x PBS to remove the blood. Place in 50ml container of PBS at 4 deg cel.

2. Placental tissue

1. Dissect a small cotyledon of placental tissue from the central aspect of the placenta away from cord insertion.

2. Keep the tissue soaked in 1x PBS (Ca2+- and Mg2+- free) during the process. Collect 2 to 3 of these pieces and rinse them thoroughly with 1x PBS to remove the blood. Place in 50ml container of PBS at 4 deg cel.

Mechanical Disaggregation and Enzymatic Digestion

1. Wash the tissue dissected from the decidua basalis or the decidua parietalis with sterile 1x PBS in a 50 ml plastic tube. Collect tissue pellets by centrifugation at **300 x g for 5 min at RT**.
2. Aspirate the supernatant located above the tissue pellet carefully without disturbing the pellet. **Resuspend vigorously**.

NOTE: At this point, the pellets are very loose because they contain red blood cells. If the volume of the pellet is about or less than 3 ml, re-suspend the pellet in 6 ml of **cell detachment solution** pre-warmed to 37 °C. If the pellet is larger than 3 ml of volume, add more cell detachment solution (add about 2 times the volume of the tissue samples).

3. Transfer the homogenized tissues (decidua basalis + cell detachment solution or decidua parietalis + cell detachment solution) to a C tube.

4. Place the C tube in the automatic dissociator and run the corresponding program (1min, spleen setting).

5. Following the mechanical disaggregation of the tissues, digest the tissues with a commercially available cell detachment solution such as ***Accutase***; (a cocktail that contains proteolytic and collagenolytic enzymes) for **45 min at 37 °C with gentle agitation** (incubator used)

Leukocyte separation

1. Pass cells through a **70µm nylon cell strainer**.
2. Use a syringe plunge to gently push through cells whilst adding 15ml DWB at the end to aid cell straining.
3. In a sterile tube, over-lay the cells onto **Histopaque** at a ratio 1:1.
4. Centrifuge at **900 RCF/2000rpm** for **20 minutes** at room temperature with **NO brakes or acceleration**. Carry the samples over in the centrifuge bucket to prevent mechanical disturbance of leukocyte layer.
5. With a sterile Pasteur pipette aspirate the leukocyte layer/interface into a new tube and wash with 40ml DWB by centrifugation at **700 RCF/1800rpm** for **10 min** with brakes and acceleration high. Discard the supernatant and resuspend in 5ml DWB.

Assessment of Cell Number and Viability

6. Transfer 20µl aliquot into an Eppendorf tube.
7. Add 20µl of 0.4% Trypan blue solution to the 20µl of cells and mix by pipetting.
8. Place approximately 10µl on a KOVA Glastic® slide.
9. Place KOVA slide on an inverted microscope and count the PBMC using the 40x lens. (PBMC are relatively large, rounded and have a slight green fluorescence).
10. Count all PBMC in three of the 9 large squares, then count all of the viable (bright/light coloured) cells in three of the 9 large squares (do not count blue coloured cells).
11. If there are more than 200 cells in 1 square, count cells again, diluting the original cell suspension.
12. Cell number in suspension = cell count average x 2 x Volume of suspension ml x 10,000.
13. *If fresh cells required for other experiment i.e. ELISpot, Flow cytometry, take quantity of cells required then top up remaining sample to 50ml with Wash Buffer before the cryopreservation of sample. Cells taken for immediate use should be centrifuged with appropriate medium.*

7.2 Phenotyping of decidual/placental leukocytes

Protocol for Surface Staining of Decidual/Placental Leukocytes to Analyse Phenotype by Flow Cytometry

Standard Operating Procedure (SOP) ***Phenotyping of Decidual/Placental Leukocytes***	**Immunotherapy Group**	**Created by:** **S P Sivarajasingam, A Cocker, N Imami *et al.* 2016**

Stain cells

1. Label FACs tubes:
    a. CD
    b. Placenta
    c. Unstained cells
    d. Isotypes
    e. Compensation beads

2. Identify each Isotype and add Xµl as per panel design of each to isotypes FACs tube

3. Centrifuge samples at 1500rpm, 10mins, brake 9, acceleration 9

4. Add antibodies + brilliant stain buffer + fixable viability stain 510 (live/dead stain) to each sample FACs tube

5. Remove samples from centrifuge and discard supernatant

6. Add cells (1-2x10^6) to labelled FACs tubes (use placental cells for unstained cells and isotypes tubes)

7. Leave to incubate in the dark at room temperature for 20 minutes.

8. Make FACs fix – 1ml PBS + 20µl PFA (2%)

9. Add 3ml of PBS to each tube to wash cells. Spin at 1500 for 5mins and discard supernatant.

10. Resuspend cell pellet and add 200ul of PBS containing 2% PFA (FACS Fix).

11. Store tubes in the dark at 4^{o}C until acquisition.

12. Acquisition should be carried out as soon as possible on the BD LSRII cytometer with BD FACSDiva software, by fully trained staff. Prior to acquisition prepare and run compensation beads if necessary, SOP #12.

7.3 Consent form for collection of urine samples for chapter 5

Immunosuppression in Pregnancy
Study 1: Longitudinal Changes in Pregnancy

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have**.** We'd suggest this should take about 15-20
minutes. Please ask us if there is anything that is not clear.

What is the purpose of the study?

During pregnancy, the mother's immune system is suppressed to allow the baby to develop. The suppression of the mother's immune system means that pregnant women are more vulnerable to infection. We plan to study what happens to the mother's immune system during pregnancy and the role of hormones, such as progesterone, which are thought to be essential in this process. Specifically, we will investigate how the immune system changes during normal pregnancy and with the onset of term and preterm labour.

Why have I been invited?

You have been invited because you are currently pregnant.

Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive. If you withdraw from the study we will destroy all your identifiable samples but we will need to use the data collected up to your withdrawal.

What will happen to me if I take part?

Once you have read the information leaflet, your midwife or doctor will confirm your willingness to participate at your next routine antenatal appointment. This will take approximately 10 minutes and should not delay your appointment time.

Blood tests and salivary samples will be taken at booking, 28 weeks, 34 weeks, at delivery, 24hrs after delivery and six weeks after delivery. These samples will coincide with your routine blood test and visits in your pregnancy. In addition, patients will collect daily urine samples from 23 weeks gestation for patients at high risk of preterm delivery and 36 weeks for patients likely to deliver at term. If at any time in your pregnancy you are admitted in labour (either preterm or term) and require a caesarean section for obstetric reasons, we would take a small sample of the womb (0.5 by 2.0cm) from the edge of the incision already made after delivery of your baby.

Normally most women will see their GP 6 weeks after delivery for a postnatal check. As you agree to participate in our study we will see you here at Chelsea and Westminster for this postnatal check.

What are the possible risks of taking part?

Taking these samples will not put you at any increased risk.

What are the possible benefits of taking part?

It is unlikely that this study will help you but the information we get from this study will help us to understand in more detail how the immune system changes in normal pregnancy and during labour.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions.

Will my taking part in the study be kept confidential and what will happen to the samples taken?

All the information collected on you will be treated as confidential and only the study personnel will have access to your details. The samples taken will be given a study ID and analysed in the laboratory. The samples will not be stored for future use in other studies.

Your GP be informed of your participation in the study (with permission).

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by the London-Chelsea Ethics Committee

Further information and contact details

Patient ID number: Title of project: Immunosuppression in pregnancy

Name of Researcher:

Please initial box

1. I confirm that I have read and understand the information sheet dated 28th October, 2013 (version 4) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at anytime without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals 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 record. ☐

4. I agree to take part in the following study and for the blood and salivary samples to be collected at 28 weeks, 34 weeks, at delivery, 24 hours later and 6 weeks after delivery. ☐

5. I agree to collect daily urine samples from 23 weeks or 36 weeks. ☐

6. I agree to allow any stored plasma/serum samples to be used. ☐

7. I agree for Cord blood samples will be taken immediately after delivery. ☐

8. I agree to have a myometrial biopsy if I undergo a cesarean section. ☐

9. I am happy for my GP to be informed. ☐

10. I do not wish for my GP to be informed. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of person taking Consent	Date	Signature

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

7.4 Supplementary data

Choriodecidua data - comparison of the good neonatal outcomes versus adverse neonatal outcomes group in the chorioamnionitis group.

Cytokine/Chemokine	p value	Statistical significance
IFN-γ	0.32	NS
TNF-α	0.66	NS
IL-1β	0.42	NS
IL-2	0.79	NS
IL-4	0.97	NS
IL-6	0.08	NS
IL-8	0.03	Significant
IL-10	0.2	NS
IL-16	0.96	NS
CCL1	0.18	NS
MCP-1/CCL2	0.54	NS
RANTES/CCL5	0.66	NS
MCP-3/CCL7	0.15	NS
TARC/CCL17	0.25	NS
CCL20	0.43	NS
TECK/CCL25	0.5	NS
GROa/CXCL1	0.61	NS
GROb/CXCL2	0.86	NS
GCP-2/CXCL6	0.22	NS
CX3CL1	0.46	NS

7.5 Permissions summary table for third party copyright works

Page number	Type of work	Name of work	Source of work	Copyright holder and contact	Permission Requested on	Permission	Permission note
	Figure	1.1 *Estimated preterm birth rate and numbers of preterm births in 2014*	Global, regional, and national estimates of levels of preterm birth in 2014: a systematic review and modelling analysis Lancet Glob Health. 2019 Jan;7(1):e37-e46. doi: 10.1016/S2214-109X(18)30451-0. Epub 2018 Oct 30.	© Elsevier 2019 permissions @elsevier.com	20.10.22	Yes	Licensed with Elsevier & Copyright Clearance Center License number: 5413290990423
	Figure	1.2 *Clinical morbidities noted during birth hospitalization from pooled data for late preterm and term patients*	Late Preterm Infants Morbidities, Mortality, and Management Recommendations Pediatr Clin North Am (2019) 10.1016/j.pcl.2018.12.008	© Elsevier 2019 permissions @elsevier.com	20.10.22	Yes	Licensed with Elsevier & Copyright Clearance License number: 5413300428246
	Figure	*1.3 An integrated model for preterm birth which highlight that PTB is not a disease of single system acting in silo but contributed by systems working together to produce an outcome*	Vidal MS Jr, Lintao RCV, Severino MEL, Tantengco OAG, Menon R. Spontaneous preterm birth: Involvement of multiple feto-maternal tissues and organ systems, differing mechanisms, and pathways. Front Endocrinol (Lausanne). 2022 Oct 13;13:1015622. doi: 10.3389/fendo.2022.1015622. PMID: 36313741; PMCID: PMC9606232.		N/A	Yes	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	1.4	Gestational tissue transcriptomics in term and preterm	© 2015 Eidam et al	N/A	Yes	Open-access article distributed

		The tissues of pregnancy	human pregnancies: a systematic review and meta-analysis (2015) BMC Med Genomics. 2015 Jun 5;8:27. doi: 10.1186/s12920-015-0099-8. PMID: 26044726; PMCID: PMC4456776.				under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	1.5/3.7 *Immune cells: effector mechanisms and their role in the pregnant uterus*	Decidual immune cells: Guardians of human pregnancies Best Pract Res Clin Obstet Gynaecol. 2019 Oct;60:3-16. doi: 10.1016/j.bpobgyn.2019.05.009. Epub 2019 May 24. PMID: 31285174.	© Elsevier 2019 permissions @elsevier.com	20.10.22	Yes	Licensed with Elsevier & Copyright Clearance License number: 5413311129736
	Figure	1.6 *Characteristics of human NK cells in circulation and peripheral tissues*	Emerging insights into natural killer cells in human peripheral tissues. Nat Rev Immunol. 2016 Apr 28;16(5):310-20. doi: 10.1038/nri.2016.34. PMID: 27121652.	© Springer nature 2016	20.10.22	Yes	Licensed with Springer nature & Copyright Clearance Centre Licence number: 5413311495891
	Figure	1.8 *A possible pathway to term or preterm labour*	Immune cells in term and preterm labor. Cell Mol Immunol. 2014 Nov;11(6):571-81. doi: 10.1038/cmi.2014.46. Epub 2014 Jun 23. PMID: 24954221; PMCID: PMC4220837.	© 2014 Chinese Society of Immunology and The University of Science and Technology	20.10.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	1.9 *Proportion of immune cells in the decidua*	Dynamic Function and Composition Changes of Immune Cells During Normal and Pathological Pregnancy at the Maternal-Fetal Interface. Front Immunol. 2019 Oct 18;10:2317. doi:	© 2019 Yang Zheng and Jin	20.10.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction

			10.3389/fimmu.2019.02317. PMID: 31681264; PMCID: PMC6813251.				
	Figure	1.10 Overview of metabolic adaptations during pregnancy	Zeng Z, Liu F, Li S. Metabolic Adaptations in Pregnancy: A Review. Ann Nutr Metab. 2017;70(1):59-65. doi: 10.1159/000459633. Epub 2017 Mar 16. PMID: 28297696.	© 2017 S. Karger Basel	28.10.22	Yes	Licensed with Karger Publishers & Copyright Clearance Centre Licence number: 5417600630085
	Figure	1.11 *Overview of metabolic pathways described in distinct immune cells subsets, such as activated DCs, macrophages, neutrophils, NK cells, effector T cell subsets, naïve, and resting and regulatory T cells, as well as B cells*	Thiele K, Diao L, Arck PC. Immunometabolism, pregnancy, and nutrition. Semin Immunopathol. 2018 Feb;40(2):157-174. doi: 10.1007/s00281-017-0660-y. Epub 2017 Oct 25. PMID: 29071391.	Copyright © 2017, Springer-Verlag GmbH Germany	28.10.22	Yes	Licensed with Springer nature & Copyright Clearance Centre Licence number: 5417601118500
	Figure	2.8 *A workflow describing the collection, preparation and processing of urine samples for mass spectrometry.*	Development of a Pipeline for Exploratory Metabolic Profiling of Infant Urine. J Proteome Res. 2016 Sep 2;15(9):3432-40. doi: 10.1021/acs.jproteome.6b00234. Epub 2016 Aug 23. PMID: 27476583; PMCID: PMC5716460.	© J Proteome	20.10.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	3.4 *Conceptual model for ischaemic*	Ischemic placental disease: a unifying concept for preeclampsia, intrauterine	© Elsevier 2019 permissions@elsevier.com	04.08.22	Yes	Licensed with Elsevier & Copyright Clearance License

		placental disease	growth restriction, and placental abruption. Semin Perinatol. 2014 Apr;38(3):131-2. doi: 10.1053/j.semperi.2014.03.001. PMID: 24836823.				number: 5361820294797
	Figure	4.1 The anatomical position of the decidua parietalis and decidua basalis	Exhausted and Senescent T Cells at the Maternal-Fetal Interface in Preterm and Term Labor. J Immunol Res. 2019 May 23;2019:3128010. doi: 10.1155/2019/3128010. PMID: 31263712; PMCID: PMC6556261.	© 2019 Slutsky R, Romero R, Xu Y, Galaz J, Miller D, Done B, Tarca AL, Gregor S, Hassan SS, Leng Y, Gomez-Lopez N	7.11.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	4.2 Function of decidual NK cells in pregnancy	miRNAs in decidual NK cells: regulators worthy of attention during pregnancy Reprod Biol Endocrinol. 2021 Oct 2;19(1):150. doi: 10.1186/s12958-021-00812-2. PMID: 34600537; PMCID: PMC8486626.	© 2021 Li, L. Feng, T. Zhou, W. Liu, Y. Li, H.	7.11.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	5.1 The core metabolic pathways of immune cells	Metabolic Reprogramming of Immune Cells at the Maternal-Fetal Interface and the Development of Techniques for Immunometabolism. Front Immunol. 2021 Sep 9;12:717014. doi: 10.3389/fimmu.2021.717014. PMID: 34566973; PMCID: PMC8458575.		20.11.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	5.2 Venn diagram showing different	What Defines NK Cell Functional Fate: Phenotype or		20.11.22	N/A	Open-access article distributed under the

		metabolic processes that may underpin NK cell function	Metabolism? Front Immunol. 2019 Jun 19;10:1414. doi: 10.3389/fimmu.2019.01414. PMID: 31275330; PMCID: PMC6593107.				terms of the Creative Commons Attribution License, which permits reproduction
	Figure	5.4 A workflow describing the collection, preparation and processing of urine samples for mass spectrometry	Development of a Pipeline for Exploratory Metabolic Profiling of Infant Urine. J Proteome Res. 2016 Sep 2;15(9):3432-40. doi: 10.1021/acs.jproteome.6b00234. Epub 2016 Aug 23. PMID: 27476583; PMCID: PMC5716460.		20.11.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	5.14 How alanine is involved in glucose metabolism a and production	The Metabolic Fates of Pyruvate in Normal and Neoplastic Cells. Cells. 2021 Mar 30;10(4):762. doi: 10.3390/cells10040762. PMID: 33808495; PMCID: PMC8066905.	© 2021 Prochownik & Wang	20.11.22	N/A	Open-access article distributed under the terms of the Creative Commons Attribution License, which permits reproduction
	Figure	5.15 Outline of ketone body metabolism and regulation	Ketone bodies as signaling metabolites. Trends Endocrinol Metab. 2014 Jan;25(1):42-52. doi: 10.1016/j.tem.2013.09.002. Epub 2013 Oct 18. PMID: 24140022; PMCID: PMC4176946.	©2014 Newman & Verdin	20.11.22	N/A	*Image reproduced with permission from Elsevier and Copyright clearance.*

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