# A SPRINGBOARD FELLOWSHIP: INVESTIGATING THE T CELL REPERTOIRE IN MALPLACENTATION DISORDERS AND DEVELOPING A NOVEL CLASSIFICATION SYSTEM FOR ASSIGNING CAUSE OF DEATH IN TWIN PREGNANCIES

By

**DR NIDHI GULATI BMedSci BMBS MRCOG**

A thesis submitted to the University of Birmingham for the degree of

**MSc By Research**

**Word Count:** 20,425

**Institute of Metabolism and Systems Research**

**College of Medical and Dental Sciences**

**University of Birmingham**

**January 2022**

## DEDICATION

To my best friend and my biggest supporter, my husband Gurinder. I love you.

To the light of my life and my reason for being, my baby boy, Aaryan.

## ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Mark Kilby for his unwavering support, both in this project, and in helping me to achieve my career aspirations. Thank you for seeing potential in me.

Thank you to my supervisor Professor Paul Moss for your consistent enthusiasm and optimism in the development of the lab-based project. You made me believe that I was a real scientist.

I would like to extend my gratitude to Richard Powell, whose PhD thesis on the T cell repertoire in normal pregnancy is the foundation upon which I conducted my project on pathological pregnancies. Thank you for teaching me vital lab techniques and helping me to set up experiments in the early days before you left the UK.

The team of research midwives at Birmingham Women's Hospital were integral to the recruitment of patients for the malplacentation study and for helping me to navigate the logistical challenges of collecting and storing blood and placenta samples required for the project. Their support and commitment to the project was invaluable.

Thank you to Fiona Mackie, Dr Phil Cox, Dr Tamas Marton, Professor Alex Heazell and Professor Katie Morris for the collaborative work that led to the completion and publication of CoDiT in the BJOG. I am extremely proud of this paper and honoured to have worked with you all as co-authors to complete the project.

I am grateful to the Birmingham Health Partners starter fellowship programme, through which this Masters was funded which has provided me with a career-defining insight in to translational research and empowered me with the skills and knowledge to incorporate research in to my clinical role.

To my Mum and Dad, you have done everything and more to help me realise my dreams and for that “thank you” is not enough. I am where I am today because of your sacrifice and your commitment to helping me achieve my full potential. You have set an incredible example for me and Gary to follow as new parents.

## CONTENTS LISITNG

1. Table of Contents
2. List of Tables and Figures
3. List of Abbreviations

## TABLE OF CONTENTS

# LIST OF TABLES AND FIGURES

## PROJECT 1 TABLES



## PROJECT 1 FIGURES

**PROJECT 2 TABLES**



**PROJECT 2 FIGURES**

# LIST OF ABBREVIATIONS

## PROJECT 1

| | |
|---|---|
| AC | Abdominal circumference |
| AEDF | Absent end diastolic flow |
| ANOVA | Analysis of Variance |
| APC | Antigen presenting cell |
| BMI | Body mass index |
| BP | Blood pressure |
| BW | Birth weight |
| CM | Central Memory |
| CMV | Cytomegalovirus |
| CPR | Cerebroplacental ratio |
| CS | Caesarean section |
| CXCL 10 | C-X-C motif chemokine ligand 10 |
| DAVID | Database for Annotation, Visualisation & Integrated Discovery |
| DMSO | Dimethyl Sulfoxide |
| EFW | Estimated fetal weight |
| ELISA | Enzyme-linked immunosorbent assay |
| EM | Effector Memory |

| | |
|---|---|
| EMRA | Effector memory re-expressing CD45RA |
| EVT | Extravillous trophoblast |
| FCS | Fetal Calf Serum |
| FGR | Fetal growth restriction |
| FoxP3 | Forkhead box P3 |
| hCG | Human Chorionic Gonadotropin |
| HLA | Human Leukocyte Antigen |
| ICM | Inner cell mass |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| iTreg | Induced regulatory T cells |
| IVF | In vitro fertilisation |
| MAFR | Maternal anti-fetal rejection |
| MAP | Mean arterial pressure |
| MCA | Middle cerebral artery |
| MHC | Major histocompatibility complex |
| NICE | National Institute for Health and Care Excellence |
| NK cells | Natural Killer cells |

| | |
|---|---|
| nTreg | Natural regulatory T cells |
| PAPP-A | Pregnancy-associated plasma protein–A |
| PBMCs | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered Saline |
| PCR | Protein:creatinine ratio |
| PD-1 | Programmed Cell Death-1 |
| PE | Pre-eclampsia |
| PI | Propium iodide |
| PI | Pulsatility Index |
| PPI | Patient and Public Involvement |
| RBC | Red blood cell |
| REC | Research Ethics Committee |
| RNA-seq | RNA sequencing |
| RPMI | Roswell Park Memorial Institute |
| RUPP | Reduced uterine perfusion pressure |
| SFH | Symphysial fundal height |
| SGA | Small-for-gestational-age |
| TCR | T cell receptors |
| TGF | Transforming Growth Factor |

| | |
|---|---|
| Th cells | Helper T cells |
| TNF | Tumour Necrosis Factor |
| Treg | Regulatory T cells |
| UA | Umbilical artery |
| uNK | Uterine Natural Killer cell |
| UtA | Uterine artery |
| VUE | Villitis of unknown aetiology |

**PROJECT 2**

| | |
|---|---|
| BMI | Body Mass Index |
| Codac | Cause of death and Associated Conditions |
| CoDiT | Cause of Death in Twins |
| CS | Caesarean section |
| DC | Dichorionic |
| DCDA | Dichorionic diamniotic |
| EDF | End diastolic flow |
| FLA | Fetoscopic laser ablation |
| ICD-10 | International Classification of Disease (10$^{th}$ Revision) |
| MBRRACE | Mothers and Babies: Reducing Risk through Audits and Confidential Enquiries |

| | |
|---|---|
| MC | Monochorionic |
| MCA-PSV | Middle cerebral artery-peak systolic velocity |
| MCDA | Monochorionic diamniotic |
| MCMA | Monochorionic monoamniotic |
| MoM | Multiples of the mean |
| NND | Neonatal death |
| PPROM | Preterm prelabour rupture of membranes |
| RCOG | Royal College of Obstetricians and Gynaecologists |
| ReCoDe | Relevant Condition at Death |
| sGR | Selective growth restriction |
| TAPS | Twin anaemia-polycythaemia sequence |
| TRAP | Twin reversed arterial perfusion syndrome |
| TTTS | Twin-to-twin transfusion syndrome |
| WHO | World Health Organisation |

# THESIS OVERVIEW

In 2018, I was successfully awarded a competitive 12-month research fellowship by Birmingham Health Partners, which is designed to provide clinicians with the opportunity to take the first steps towards a career in research.

The aim of this fellowship was to explore postgraduate medical research in two settings: firstly through a laboratory-based basic medical sciences project, and secondly in the context of a clinical project, thus drawing on my previous experience of working in a lab during my undergraduate BMedSci degree and my pre-existing knowledge as an Obstetrics and Gynaecology registrar.

Conducting these different research projects in parallel, with differing sets of skills, methodologies and analysis, has given me a breadth of research experience in a limited time frame. Not only have I gained a deeper understanding of basic science laboratory techniques, running experiments and interpreting flow cytometry results relating to cellular immunology, I have also acquired skills in systematic literature search, critical appraisal of literature, design of a retrospective cohort study and statistical analysis.

In the first project I examined ***the peripheral and decidual T cell populations in pre-eclampsia and fetal growth restriction*** by collecting samples of maternal blood and placenta from postpartum women whose pregnancies were affected by these placentally-mediated disorders, herein known as malplacentation disorders. After isolating white cells from these different samples, antibody staining was performed to enable comparison of the T cell repertoire in maternal blood and placental tissue in healthy pregnancies and those complicated by malplacentation.

For the second project I worked with a multi-disciplinary panel of obstetricians and perinatal pathologists supervised by Professors Kilby (University of Birmingham) and Heazell (University of Manchester) to develop a ***novel classification system for assigning the cause of fetal and neonatal death in twin pregnancies*** using post-mortem reports. This new classification system was then tested using a retrospective cohort sample of 256 twin pregnancies with single or double fetal death. I was able to determine inter-rater, intra-rater and inter-disciplinary agreement. Successful peer reviewed publication of this work in the *British Journal of Obstetrics and Gynaecology* (*BJOG*) establishes this project as an important piece of original work in the field of fetal and maternal medicine. The *BJOG*, an international journal of Obstetrics and Gynaecology, now has an impact factor of 5.19, and is ranked 6th out of 83 obstetrics and gynaecology journals in the Journal Citation Reports rankings.

In both projects I have also explored the potential for further research and how I might take each project forward with more time and funding.

I proudly present this thesis as evidence of my hard work and commitment to research as a clinician for the degree of MSc by Research.

# PROJECT 1

## Investigating the T-cell repertoire in malplacentation disorders

## ABSTRACT

Objective: to determine the feasibility of collecting and isolating T cells from women whose pregnancies were complicated by pre-eclampsia or fetal growth restriction, so that future studies can be planned to investigate T cell function and possible dysregulation in malplacentation disorders.

Design: Laboratory-based study

Population: Pregnant women delivering at Birmingham Women's Hospital, West Midlands.

Participant inclusion eligibility criteria: over 18 years old, understands and speaks English, with either a diagnosis of pre-eclampsia (PE) defined as BP ≥140/90 and urine PCR ≥30mg/mmol over 20 weeks' gestation, or fetal growth restriction (FGR), defined as either an EFW or AC plotting below the 3$^{rd}$ centile or plotting below the 10$^{th}$ centile with evidence of placental dysfunction (abnormal uterine artery Doppler between 20-24 weeks' or abnormal umbilical artery Doppler) in a non-smoker and not secondary to an underlying congenital anomaly.

Methods: Of the 69 participants recruited to the study, triplet samples of placenta, maternal and cord blood were collected from 75% of participants (52/69); of which 48% (25/52) had PE, 37% had FGR (19/52) and 15% (8/52) were healthy controls. White cells were isolated from blood samples using standard density gradient centrifugation techniques. A novel method for isolating white cells from placental tissue was applied by dissecting out and culturing 2cm samples of the decidua basalis layer overnight, followed by gentle tissue dissociation to obtain a single cell suspension, from which white cells were isolated by density gradient centrifugation. A subset of 39/52 maternal

blood and decidual samples were stained with fluorescent antibodies prior to flow cytometric analysis to determine T cell composition and subtypes.

Results: In all 3 cohorts there was a significantly greater naïve T cell phenotype in blood compared with decidual tissue and a conversely dominant effector memory T cell population in tissue compared with blood. All cohorts demonstrated significantly increased expression of the checkpoint protein Programmed-Death-1 (PD-1) in decidual effector memory (EM) T cells compared with matched peripheral blood. There was a significantly lower percentage of peripheral blood naïve T cells in FGR compared to control. Differences were seen between cohorts, including greater PD-1 expression by decidual EM T cells in PE than control and reduced decidual EM T cell PD-1 expression by decidua from FGR compared with control. However these findings were not statistically significant.

Conclusions: The data confirms findings from this research group's previous work on healthy pregnancies and demonstrates reliability in the novel method used to isolate T cells from decidua to provide reliable and reproducible results. This work also suggests new findings in pregnancies affected by malplacentation that warrant further investigation. Having successfully demonstrated the feasibility of collecting and sampling placental tissue from cohorts affected by malplacentation, the preliminary data was used to support grant applications for further study.

Details of ethical approval: Granted by the West Midlands Research Ethics committee (REC reference 14/WM/1146).

# INTRODUCTION

The placenta is a vital extracorporeal organ that supports fetal life. The placenta is the maternal-fetal interface enabling exchange of gases, nutrients and waste products as well of transfer of immunoglobulins and metabolites and secretion of hormones required for fetal growth and development and maintenance of pregnancy. The human placenta is described as villous haemomonochorial, in that the placenta barrier consists of only a single layer of syncytiotrophoblast dividing the maternal blood space from the blood in the fetal capillaries, whilst other species such as rodents and sheep have different placental types[1,2]. This is an important consideration when reviewing studies using animal models of human placentation. The human placenta appears macroscopically as a disc-shaped structure, on average measuring 22cm in diameter, is 2.5cm thick at the centre and weighs approximately 500g by term[3]. It has two surfaces, one that faces the fetus to which the umbilical cord inserts and is known as the chorionic plate, whilst the basal plate of the placenta is attached to the lining of the uterus, which in pregnancy is known as the decidua. The umbilical cord typically comprises of one large vein, carrying oxygenated blood and nutrients towards the fetus, and two arteries carrying deoxygenated blood away from the fetus towards the maternal circulation. The fetally-derived placental membranes comprise of two fused layers: the amnion and chorion. The amnion, a single cell epithelial layer derived from extraembryonic somatic mesoderm, is innermost and in direct contact with the fetus, umbilical cord and amniotic fluid, whilst the chorion, which is outermost layer formed from extraembryonic mesoderm and trophoblast, is in direct contact with the decidua and forms the feto-maternal interface[4,5]. The two layers are connected through a collagen-rich extracellular matrix containing mesenchymal cells from both layers. The

two layers start their development at the time of implantation and fuse in the late 1st or early 2nd trimester to form a single unit that lines the decidua. The fetal membranes maintain the fluid environment around the fetus, which is crucial for survival in utero and provide mechanical, immune, endocrine, transport and antimicrobial support to the pregnancy.

Placental Formation

Placentation begins early in pregnancy with implantation of the blastocyst (Figure 1). The blastocyst is comprised of the outer trophoectoderm and the inner cell mass (ICM). The trophoectoderm is the undifferentiated progenitor tissue of the entire outer epithelial component of the placenta, known as trophoblast, providing the functional bridge between the fetus and the mother and performing the majority of the absorptive, immunoprotective and endocrinological functions of the placenta[6]. After attaching to the uterine surface epithelium day 6-7 post fertilisation, the trophoectoderm fuses to form a primary multinucleated cell layer known as the syncytiotrophoblast, which eventually secretes Human Chorionic Gonadotropin (hCG) among other hormones and growth factors, and after about 10 weeks' gestation, is in direct contact with the maternal blood, and thus the main site of gas and nutrient exchange. After implantation the primary syncytium invades deeper into the endometrium, which transforms into specialised tissue in pregnancy known as the decidua, and also erodes in to decidual glands to allow direct contact with secretions[7]. The blastocyst becomes completely embedded in the decidua by two weeks post fertilisation. Lacunae also begin to form within the syncytium and merge, creating a meshwork of trabeculae[7].

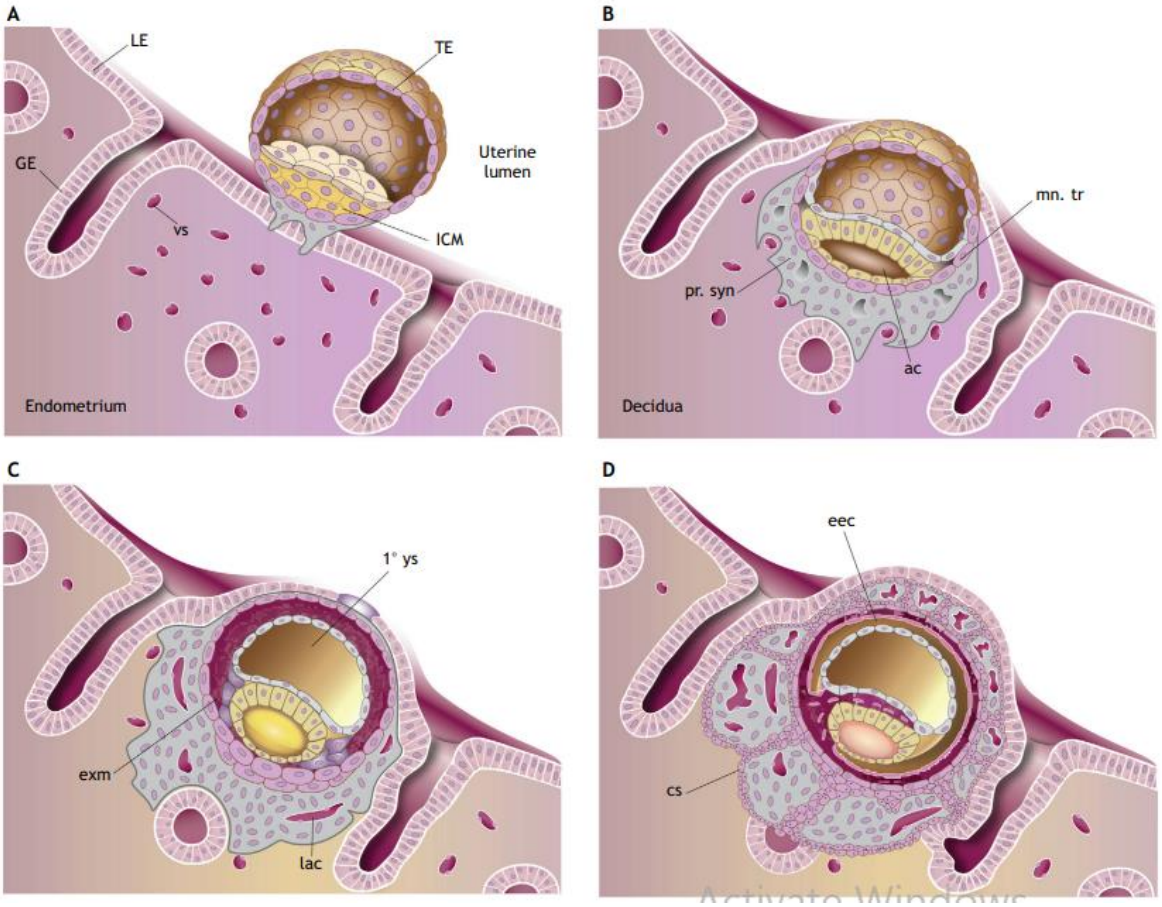



**Figure 1[7]:** The early stages of human placental development begins with implantation of the blastocyst. (A,B) The prelacunar stages: After attaching to the uterine surface epithelium day 6-7 post fertilisation, the trophoectoderm fuses to form the syncytiotrophoblast. GE, glandular epithelium; LE, luminal epithelium; vs, blood vessels; TE, trophectoderm; ICM, inner cell mass; pr. syn, primary syncytium; ac, amniotic cavity; mn. tr, mononuclear trophoblast (C) The lacunar stage: The primary syncytium invades deeper into the endometrium, which transforms into the decidua. Lacunae begin to form within the syncytium. exm, extra-embryonic mesoderm; 1° ys, primary yolk sac; lac, lacunae (D) The primary villous stage: trophoblast cells beneath the syncytial layer form cytotrophoblast and rapidly proliferate to form finger-like projections that push through the primary syncytium to form the primary villi. cs, cytotrophoblastic shell;; eec, extra-embryonic coelom.

The trophoblast cells beneath the syncytial layer form the cytotrophoblast and rapidly proliferate to form finger-like projections that push through the primary syncytium to form the primary villi, with a cytotrophoblast core and an outer syncytiotrophoblast layer. These villi are the main structural and functional unit of the placenta[8]. Further branching and proliferation of cytotrophoblast together with

expansion of stromal cells forms the villous tree with intervillous spaces. The cytotrophoblasts then push out beyond the syncytiotrophoblast layer and spread laterally to envelope the entire pregnancy in a shell that separates the villi from the decidua. This is the maternal-fetal interface (Figure 2)[9]. Some of these cytotrophoblasts migrate from this interface deeper into the decidua, becoming the extravillous trophoblasts (EVTs). EVT invasion into and around the uterine spiral arteries that supply the endometrium enables remodelling of these vessels to increase their diameter and lose their elasticity thus transforming them into low resistance vessels to facilitate increased blood flow to the fetus. The process is thought to be complete by 20-22 weeks' gestation. Uterine Natural Killer (uNK) cells are believed to play a critical role in spiral artery remodelling and regulating invading trophoblast cells in early pregnancy. These innate lymphoid cells are the predominant immune cell in the uterine environment in the first trimester and express receptors for the EVT[7]. This is further discussed in the literature review below. Decidual macrophages have also been reported to play a vital role in decidual angiogenesis and spiral artery remodelling[10].

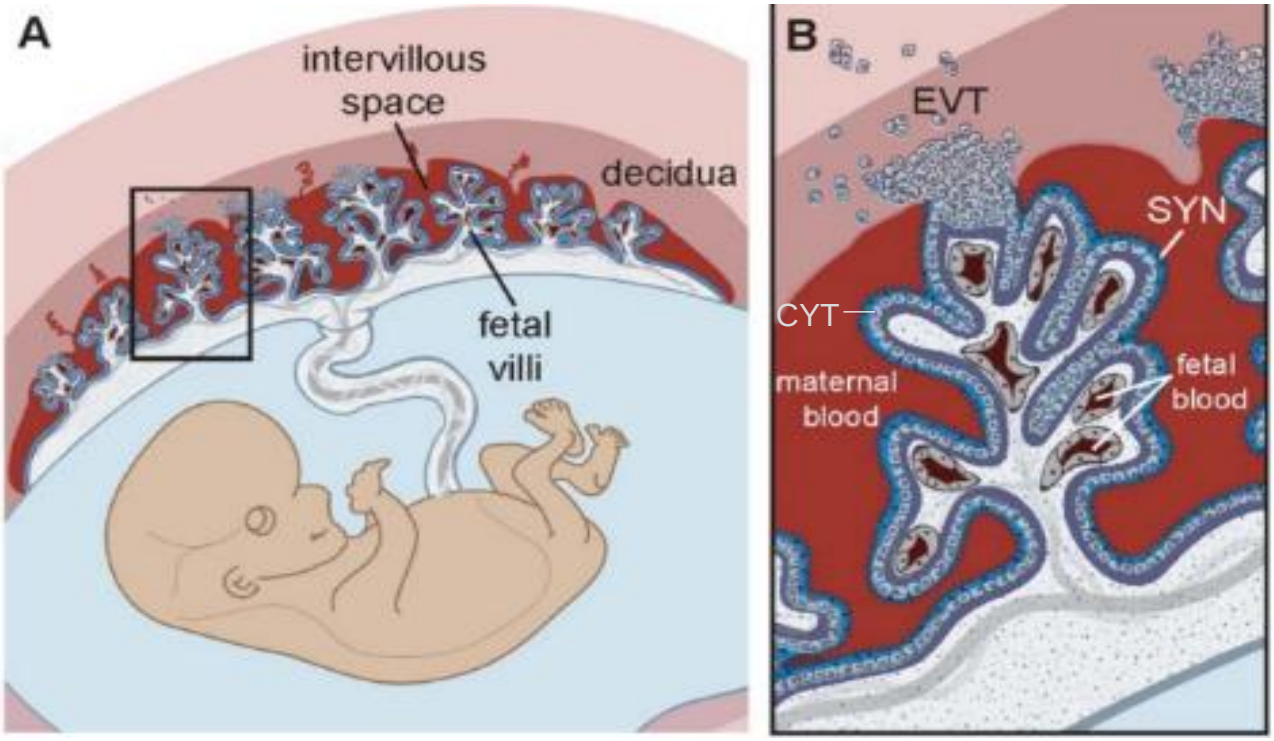



**Figure 2[9]:** The maternal fetal interface. A) Decidual spiral arterioles perfuse the chorionic villi which line the intervillous space. B) A primary chorionic villus; the main structural and functional unit of the placenta, with a cytotrophoblast (CYT) core and an outer syncytiotrophoblast (SYN) layer. Some of the cytotrophoblast cells invade deep in to decidua and become Extravillous trophoblast (EVT) which are critical for spiral artery remodelling.

The epithelium of the syncytiotrophoblast is covered with microvilli which increase the surface area by up to 7-fold and contain active transport proteins for glucose and amino acids across the membrane and receptors for Immunoglobulin G (IgG) antibodies to provide the fetus with passive immunity. This layer is devoid of human leukocyte antigen (HLA) proteins which prevents maternal immune cells from recognising the placenta as allogenic or “non-self” and therefore acts as a protective barrier against the maternal immune system.

Clinical disorders of malplacentation

Defective placental development, or malplacentation, is a common underlying association in a number of pregnancy-related diseases, such as pre-eclampsia (PE), fetal growth restriction (FGR), recurrent miscarriage and stillbirth. The exact clinical outcome depends on the nature of underlying placental pathology; the most common phenotype seen in early-onset PE, FGR and stillbirth is maternal vascular malperfusion[11]. It is thought that whilst PE results from the release of products from a poorly perfused and stressed placenta triggering a systemic endothelial disorder, superficial trophoblast invasion with inadequate spiral artery remodelling deep in to the myometrium hampers the growth of the placenta, in turn reducing the surface area of the maternal-fetal interface for gas and nutrient exchange, causing FGR[7,12,13]. However malplacentation alone does not adequately explain late-onset disease. There is emerging evidence to suggest that maternal cardiovascular dysfunction secondary to increasing haemodynamic demands with advancing pregnancy leads to placental hypoperfusion and subsequent placental dysfunction manifesting as hypertensive disorders of pregnancy or FGR[14].

PE, clinically defined as new-onset hypertension (≥140/90) with a urine protein:creatinine ratio (PCR) >30mg/mmol after 20 weeks' gestation, affects 3-5% of pregnancies[15]. It is a major cause of worldwide maternal morbidity including the potential of multi-organ failure and intensive care admission. There is up to a 55% risk of recurrence, and an increased lifetime risk of cardiovascular and cerebrovascular disease tracking into older age[15]. In addition, PE is associated with 5% of stillbirths and 8–10% of preterm births (many iatrogenic) in the UK[15]. Clinically, this syndrome is characterised by a systemic inflammatory response in the maternal endothelium, with

common clinical symptoms such as headache and visual disturbance, which reflect cerebral irritation, right upper quadrant pain as a sign of liver capsule oedema and sudden onset of non-dependent peripheral oedema. Clinical examination findings to support this inflammatory response include papilloedema, hyperreflexia and clonus. Delivery of the placenta results in rapid improvement of symptoms and blood pressure and allows recovery of end-organ damage; although a full recovery can take several weeks. Timing of delivery depends on a combination of the severity of the hypertension and derangement of biochemical and haematological markers, and fetal wellbeing. It is a major cause of iatrogenic preterm delivery, with short- and long-term implications for the neonate such as feeding problems, hypoglycaemia, retinopathy of prematurity, and developmental delay.

The clinical presentation of PE can be divided in to early-onset disease, developing before 34 weeks' gestation, or late-onset disease developing at or after 34 weeks' gestation[16]. Whilst the former is more likely to be associated with placental dysfunction, growth restriction, abnormal uterine and umbilical artery Dopplers on ultrasound scan, and poorer maternal and neonatal outcomes, as described above, the latter is more likely to be associated with normal fetal growth, Dopplers and better outcomes for mother and baby[16].

FGR is a pathological condition in which the fetus fails to reach its full biological growth potential[17,18]. There is an increased risk of perinatal morbidity and mortality, being present in up to 30% of cases of stillbirth[19]. Low birthweight neonates have a four-fold increased risk of perinatal death and as infants and children are at higher risk of impaired cognitive development[18]. The effects of fetal growth restriction are thought

to extend into adulthood with an increased prevalence of cardiovascular disease, diabetes and dyslipidaemia[19].

Defining FGR has been the focus of much clinical research[18]. Traditionally, in clinical practice, a small-for-gestational-age (SGA) fetus is defined as having an estimated fetal weight (EFW) or Abdominal Circumference (AC) below the 10$^{th}$ centile on a growth chart customised for maternal characteristics (age, weight, height, parity and ethnicity)[17]. However 50-70% of SGA fetuses will be constitutionally small with no underlying pathology and show linear growth, although an EFW plotting below 3$^{rd}$ centile is more likely to be associated with FGR[17]. In addition not all fetuses that are growth restricted will have an EFW below the 10$^{th}$ centile, as tailing or static growth above the 10$^{th}$ centile indicates a failure to reach their growth potential. FGR can be divided in to early-onset FGR (<32 weeks) and late-onset FGR (≥32 weeks)[18]. Whilst both may be due to placental dysfunction, early-onset FGR is more likely to be associated with congenital anomalies, chromosomal disorders and congenital infection (cytomegalovirus [CMV] or toxoplasmosis) than late-onset FGR, which is typically a result of placental dysfunction[17-19].

A Delphi Consensus study to define early-onset and late-onset FGR in the absence of congenital anomalies used ultrasound parameters to establish a consensus for defining FGR in these two groups (Table 1)[18].

| | **Early onset FGR** | **Late onset FGR** |
|---|---|---|
| **Gestation at onset** | < 32 weeks | ≥32 weeks |
| **Definition (as per Delphi consensus study[17])** | AC/EFW<3rd centile or UA-AEDF<br>*Or*<br>*1.* AC/EFW <10th centile *combined with*<br>*2.* UtA-PI > 95th centile *and/or*<br>3. UA-PI >95th centile | AC/EFW <3rd centile<br>*Or at least two out of three of the following:*<br>1. AC/EFW <10th centile<br>2. AC/EFW crossing centiles >2 quartiles on growth centiles (non-customised)<br>3. CPR<5th centile *or* UA-PI >95th centile |

**Table 1:** Definition of early-onset FGR (<32 week's gestation) and late-onset FGR (≥32 week's gestation) according to a Delphi Consensus study[18].

This Delphi consensus based definition has yet to prove useful in improving outcomes for affected pregnancies. Meanwhile, version 2 of the Saving Babies' Lives Care Bundle published by NHS England in 2019 aims to reduce perinatal mortality across England[20]. It provides clinicians with guidance on detection and surveillance of a number of aspects of maternity care, including managing FGR. The document defines FGR in a current pregnancy as either of the following:

- EFW or AC <3rd centile
- EFW or AC <10th centile with evidence of placental dysfunction (either):

- Abnormal uterine artery Doppler (mean pulsatility index (PI) >95$^{th}$ centile) between 20-24 weeks *and/or*
- Abnormal umbilical artery Doppler (absent or reversed end diastolic flow (EDF) or PI >95$^{th}$ centile)[20].

Most clinicians have adopted this definition for FGR to inform their management of affected pregnancies, and so for the purposes of this study, FGR has been defined in line with the Saving Babies' Lives Care Bundle definition outlined above.

Risk factors for fetal growth restriction can be identified as early as the first trimester (Table 2)[17]. Such women are offered serial ultrasound measurements from 26 weeks' gestation. Pregnant women at a lower risk of FGR should be offered serial symphysial fundal height (SFH) measurements every 4 weeks from 24 weeks' gestation and referred for ultrasound assessment if the SFH fails to follow a linear trend or plots below 10$^{th}$ centile[17].

| **Minor risk factors** | **Major risk factors** |
|---|---|
| Maternal age ≥35 years | Maternal age >40 years |
| IVF singleton pregnancy | Smoker ≥11 cigarettes/day |
| Nulliparity | Paternal SGA |
| BMI (Body Mass Index) <20 | Maternal SGA |
| BMI 25-34.9 | Cocaine use |
| Smoker 1-10 cigarettes/day | Daily vigorous exercise |
| Low fruit intake pre-pregnancy | Previous SGA baby |
| Previous pre-eclampsia | Previous stillbirth |

| | |
|---|---|
| Pregnancy interval <6 months | Vaginal bleeding |
| Pregnancy interval ≥60 months | Pregnancy associated plasma protein–A (PAPP-A) <0.4 |
| | Maternal medical conditions (chronic hypertension, diabetes with vascular disease, renal impairment, antiphospholipid syndrome) |

**Table 2:** Minor and major risk factors for development of FGR[17].

Biometric measurements through serial ultrasound scans every 2-4 weeks for patients at high risk of FGR help to determine whether the trend in growth is declining or becoming static. The 3 parameters for biometry are head circumference, abdominal circumference and femur length, which are computed into a formula (most commonly the Hadlock formula) to generate an estimated fetal weight. Other ultrasound markers for growth restriction include abnormal Doppler waveforms. Abnormal fetal middle cerebral artery (MCA) Doppler velocimetry reflects redistribution of blood to the cerebral circulation, known as the "brain-sparing effect" of growth restriction, and abnormal Ductus Venosus Doppler waveforms indicate abnormal fetal cardiac function[19]. Abnormal umbilical artery Doppler velocimetry reflects abnormal fetoplacental impedance associated with placental dysfunction and may be associated with a significant risk of perinatal mortality if delivery is not expedited[19].

It has been postulated that development of these disorders of placentation may be the result of inadequate prior involvement of the maternal adaptive immune system

(see literature review) from insufficient exposure to antigens in seminal fluid or donor gametes, and this may account for some of the known risk factors for PE, such as nulliparity, a pregnancy with a new partner, shorter sexual relationships prior to conception, use of barrier contraception and conception with a donor embryo[21-23]. As the clinical signs and symptoms of PE typically develop in the late second and third trimester, when T lymphocytes comprise 50-60% of the decidual leukocyte population, it is vital to consider the role of these leukocytes in the aetiology of these diseases[24]. By uncovering the possible immunopathological mechanisms of these diseases, potential targets for earlier detection and treatments for these diseases could be developed to positively alter the outcome of these pregnancies.

# LITERATURE REVIEW: DECIDUAL T CELL IMMUNOLOGY

## Introduction

In healthy human pregnancy the immune system adapts to tolerate the semi-allogenic fetus and emerging evidence suggests that dysfunction of this immune tolerance may be implicated in complications of pregnancy such as recurrent miscarriage, PE and FGR. Certainly the maternal decidua contains a wide variety of immune cells and must maintain immune tolerance whilst in close juxtaposition to the haemochorial placenta. Cell-mediated immune tolerance in normal pregnancy has been studied over recent years and exciting discoveries about the characteristics of T cells in human pregnancy have been made. It is known that T cells at the maternal-fetal interface recognise fetal cells and have specificity for fetal peptides. Extrinsic control of T cells mediated by T regulatory cells and intrinsic control by checkpoint proteins such as Programmed Death-1 (PD-1) are thought to prevent a destructive maternally-derived immune response towards the fetoplacental unit. This work has led researchers to postulate whether impaired control of the immune response by T cells may be implicated in the pathophysiology of PE, a pregnancy disorder characterised by a systemic inflammatory response to endothelial cells in the mother and the fetoplacental circulation. Both animal and human studies have implicated a T cell cytokine bias and defective intrinsic and extrinsic regulation in the pathogenesis of PE. As such, more human studies that follow a standardised approach for isolating T cells from the maternal-fetal interface are needed in order to determine the extent to which, if at all, T cells play a role in the aetiology of PE. In doing so, a targeted curative or attenuating immunotherapy may be developed for a currently incurable and irreversible disease that affects the lives of hundreds of thousands of mothers and babies worldwide.

Overview of the innate and the adaptive immune system[25-28]

The human immune response uses both innate and adaptive mechanisms to protect the host from pathogenic microbes and eliminate toxins and allergens that enter through mucosal surfaces, whilst being able to distinguish “self” from “non-self”[25]. This distinction is made by detecting structural features on the surface of invading pathogens that are different from host cells, known as antigens. The innate immune response can be broadly described as an in-built host response that recognises common patterns shared by many pathogens and is the initial immune response to an invading pathogen. In contrast, the adaptive immune system, which is formed from a small number of cells that can assemble highly specific antigen-binding molecules unique to a particular pathogen, toxin or allergen, is the secondary response, since it requires time for the highly specific cells to proliferate to mount an effective response. The cells produced by the adaptive immune response can persist in the host many years after the initial immune response has occurred, lying dormant until further exposure of the same pathogen, to which a more rapid response can be initiated. This immune memory function allows a more effective response on subsequent exposure following the initial sensitising event.

The innate and adaptive immune responses are entirely synergistic, which is crucial to a complete and fully effective immune response. The innate immune system activates the antigen-specific adaptive immune response, which in turn recruits innate immune mechanisms to amplify the response to the invading pathogen.

Mechanisms of the innate immune response include physical barriers such as mucociliary layers lining internal organ epithelia, which are always active, or those that

are activated upon recognition of surface antigens on invading microbes, such as complement proteins and cytokines. The cellular component of the innate response comprises key leukocytes in the human peripheral circulation, namely neutrophils and monocytes (which mature to macrophages in tissues), basophils, mast cells and eosinophils. The role of neutrophils and monocytes is to first secrete destructive chemicals and enzymes to digest pathogen-related proteins, and then engulf the resulting products in a process termed phagocytosis. Monocytes and macrophages are part of a key group of cells known as antigen-presenting cells (APC), the function of which is to present phagocytosed microbial antigens to cells of the adaptive immune system, thereby activating this arm of the immune response. Antigen presentation occurs via class I and class II major histocompatibility complex (MHC) molecules, cell surface glycoproteins expressed by APCs. Class I MHC molecules present peptides synthesised within the APC from microbial proteins, whilst Class II MHC molecules present microbial peptides ingested and proteolysed by the APC.

The cellular response of the adaptive immune system is led by key lymphocytes: T cells and B cells. These cells arise from differentiation of common lymphoid progenitor cells. Other mature lymphocytes that derive from this lineage are natural killer (NK) cells and NK-T cells. B cells are defined by their production of antibodies, otherwise known as immunoglobulins (Ig), which bind antigens and initiate downstream signalling in the immune system to neutralise the infectious trigger. There are five main Ig isotypes, all with different structures and functions: IgM, IgG, IgE, IgA and IgD. Notably IgG, which is a monomer structure made of 2 heavy and 2 light amino acid chains, is actively transported across the placenta to provide passive fetal and neonatal immunity.

T cells, whose role in disorders of placentation is the focus of this thesis, are defined by their selective expression of CD4 or CD8 surface molecules. Initially, during their development in the thymus, T cells are devoid of CD4 and CD8. They then subsequently express both (CD4+CD8+), before being positively selected to express either CD4 or CD8. CD4+ T cells make up 60-70% of the T cell population in blood and are known as “helper T (Th) cells” since they activate a B cell-mediated antibody (humoral) response as well as cellular responses. In contrast, CD8+ T cells, also known as cytotoxic T cells, account for the remaining 30-40% of circulating T cells and are recruited to destroy intracellular viruses and bacteria as well as acting against tumour cells. During a primary response to a foreign antigen, CD8+ and CD4+ T cells are activated through class I and class II MHC molecules respectively, which present antigens to the T cells and interact via T cell surface receptors (TCR). This results in T cells differentiating in to functionally distinct subsets. Upon stimulation via MHC Class II molecules, naïve CD4+ Th cells differentiate in to Th1, Th2 or Th17 cells depending on cytokines expressed at the site of activation. Interleukin (IL)-12 produced by macrophages and NK cells induces Th cells to differentiate in to Th1 which support cell-mediated immune responses. IL-4 from basophils, mast cells and NK-T cells triggers Th2 differentiation, which support humoral and allergic responses. IL-6 and Transforming Growth Factor (TGF)-β induce Th17, which recruit neutrophils in response to extracellular bacteria. Each subset is characterised by the transcription factors they express, the cytokines they produce and their subsequent function. An important subset of CD4+ T cells, known as regulatory T cells (Treg), downregulate the immune response and divide in to two groups. The first group, which develop in the thymus, are natural Treg cells (nTreg) and are characterised by cell surface

expression of CD4 and CD25 and nuclear expression of the forkhead box P3 (FoxP3) transcription factor. They secrete immunomodulatory cytokines TGF-β and IL-10. The second group develop in the periphery in response to antigen stimulation and are thus called induced Tregs (iTreg).

Upon antigen recognition and activation via MHC Class I, CD8+ T cells target infected or malignant cells through three main mechanisms. The first mechanism is through release of anti-viral and anti-tumour cytokines, namely Tumour Necrosis Factor (TNF)-α and interferon (IFN)-γ. The second is through release of cytotoxic granules called perforin and granzymes. Perforins perforate the target cell membrane, allowing grazymes to penetrate the cell and inducing cell apoptosis. Thirdly, CD8+ T cells express cell surface FasL molecules, which bind to Fas receptors on targets cells and activate the caspase cascade, ultimately causing apoptosis.

The maternal immune system in normal pregnancy

In humans, MHC class I molecules can be subdivided in to the classical class I antigens, HLA-A, -B, -C, which are highly polymorphic and mainly interact with T cells, and the non-classical class I antigens, HLA-E,-F, -G, which are predominantly monomorphic and mostly interact with NK cells[29.] In pregnancy, the invasive EVT of the placental villi, which migrate through the uterine decidua to remodel the endometrial spiral arteries that support increased blood flow in pregnancy, are thought to express HLA-C, -E, -F and -G[29]. Notably, EVT is devoid of HLA-A and -B, the antigens associated with graft rejection[29]. The syncytiotrophoblast, which forms the outermost layer of the placental villi and is in direct contact with maternal blood, does not express any surface membrane HLA class I or class II molecules and hence is not

involved in a fetal-specific immune response. As a result, in normal pregnancies, interaction of the placenta with the maternal immune system is restricted to (1) the EVT, (2) trafficking of paternally-derived fetal cells peripherally in the maternal circulation which can persist for many years, known as microchimerism, and (3) the potential breakdown of the syncytiotrophoblast barrier to expose underlying fetal-derived structures[22,24,29]. Microchimerism has been shown to induce a memory T cell response, which may have a role in suppressing the degree of microchimerism and inducing tolerance to these antigens on further exposure in subsequent pregnancies[22,24,30-32].

A cellular adaptive immune response to pregnancy

In early human pregnancy, uNK cells and macrophages dominate the immune cell type within the decidua and are essential for implantation and facilitating cytotrophoblast invasion to initiate spiral artery remodelling[10]. Indeed pregnant murine models depleted of uNK cells show immature and oedematous decidua and a lack of spiral artery remodelling[33-35]. The EVT interact via HLA -E, -G and –C with corresponding receptors on the surface of uNK cells to promote angiogenesis, stimulate production of inflammatory cytokines, inhibit NK-mediated killing of the trophoblast and influence trophoblast invasion[29,36]. As the pregnancy advances beyond the first trimester, the number of NK cells at the maternal-fetal interface plateaus, whilst the number of T cells increases. This temporal shift in leukocyte composition suggests that a dynamic change in cellular adaptive immune response takes place in the decidual environment and there is evidence that decidual T cells can recognise fetal tissue and are fetal-specific[24,30,37]. Indeed, work published by Moss and Kilby has demonstrated that HY-specific CD8+ T cells exist both peripherally and in decidual tissue following a male

pregnancy, with a higher number of these fetal-specific T cells located in the decidua than peripheral blood, and a further increase in HY-specific T cells is seen if there are subsequent male pregnancies[37]. The group has also shown significantly stronger proliferation of decidual CD4+ and CD8+ T cells in response to fetal antigen exposure[35]. There are also decidual T cells with specificity for cytomegalovirus and Epstein-Barr virus mediated through HLA-A and –B; an inherent adaptation to protect the pregnancy from infective pathogens[30].

In healthy human pregnancies, decidual T cells exhibit a highly differentiated effector memory (EM) phenotype, characterised by lack of CD45RA and CCR7 expression, compared to those in peripheral blood[22,37]; the proportion of CD4+ and CD8+ EM cells in the decidua is two- to three-fold higher than their respective populations in peripheral blood[24,37]. The traditional role of memory T cells is to enable the immune system to effectively target a recognised foreign antigen and enable a more efficient clearance of pathogens on secondary exposure. However, the same response in pregnancy would be devastating. Studies have demonstrated that EM T cells are likely to have altered functions, proliferation profiles and migratory abilities in pregnancy which help to generate immune tolerance towards paternal-fetal antigens in pregnancy[22,24,27,37].

Importantly, evidence has demonstrated these adaptive and functional T cells are under extrinsic and intrinsic control to protect the pregnancy. Treg cells comprise approximately 20% of the decidual CD4+ T cell repertoire at term and provide extrinsic regulation. Their evolution is thought to be the hallmark for successful eutherian pregnancy[24]. *In vitro* depletion of human decidual Treg cells leads to increased fetal-specific proliferation of decidual T cells on co-culture with cord blood lymphocytes[37]. Programmed death-1 (PD-1) is considered a key intrinsic inhibitory checkpoint

molecule associated with preventing immune-mediated tissue damage and facilitating peripheral tolerance by enabling Th2 expansion and exerting inhibitory effects upon TCR activation by antigen stimulation[38]. PD-1, which is expressed by different lymphocyte subsets including B cells and NK cells as well as T cells, and its ligands PD-L1 and PD-L2 are involved in transplant tolerance[39]. Our group, and others, have demonstrated that there is increased expression of PD-1 by decidual effector memory CD4+ and CD8+ T cells compared with peripheral blood T cells in healthy pregnancy, which is thought to represent an "exhausted state" that reflects the cells that have undergone repeated stimulation with an antigen[24,37]. PD-L1 is expressed by villous syncytiotrophoblast and cytotrophoblasts at the maternal-fetal interface, possibly reflecting the role of PD-1 in local regulation of the immune response to pregnancy[40].

Increased understanding of the physiological profile of circulating maternal, fetal and decidual T cells in healthy pregnancy and the mechanisms that underpin maternal immune tolerance in healthy pregnancy has led to a range of hypotheses which aim to explain mechanisms by which dysregulation of this immune tolerance may lead to the development of pregnancy-related disorders.

Animal studies in PE

Human placentation is a unique process distinct from any other animal, and whilst there is no perfect animal model for human placentation, cellular tolerance in the fetoplacental interface is ideally studied in haemomonochorial placental models such as non-human primates and Guinea pigs. However ethical considerations and costs increase with increasing animal size, limiting the use of larger animals such as primates in research[1]. Established mouse models and other rodents have the advantage of

shorter gestations, reduced housing costs and readily available embryonic stem cells to facilitate gene targeting studies. However there are marked differences between murine and human placentation, including relatively shallow trophoblast invasion of uterine arteries and vascular remodelling, which limits the use of mice as models, particularly in the context of PE and FGR[1].

The animal studies investigating the role of T cells in PE described below are summarised in Table 3.

*A Th1/Th2 imbalance*

One of the earliest models for immune regulation in pregnancy indicated that normal pregnancy favoured an anti-inflammatory Th2 profile, driven by progesterone and interleukin (IL)-4 secretion by the placenta, and suppressed an inflammatory Th1 phenotype[41]. Thus, it was commonly postulated that an increased Th1:Th2 ratio favoured an inflammatory response and could lead to the development of malplacentation. Indeed several animal models have demonstrated that PE-like symptoms can be mimicked from a Th1-mediated immune response[42]. Adoptive transfer of activated Th1-like cells into previously normotensive pregnant mice was shown to rapidly induce hypertension and proteinuria, a response not seen when non-pregnant control mice were injected with the same cells[43]. In another murine model, CD4+ cells isolated from the maternal surface of placentas from pre-eclamptic women were injected into pregnant rats. These rats demonstrated a significant rise in mean arterial pressure (MAP) and a significant increase in circulating inflammatory cytokines, TNF-α and IL-17, again a response that was not seen in rats injected with placental CD4+ T cells from healthy human pregnancies[44]. The authors had previously shown

that TNF-α causes hypertension associated with oxidative stress and IL-17 stimulates reactive oxygen species (ROS) in association with hypertension in pregnancy, and blocking TNF-α or IL-17 lowers blood pressure and vasoactive pathways in PE rat models[45-48]. Therefore, they suggested that CD4+ T cells and the associated inflammatory cytokines had a role in development of PE symptoms through a pro-inflammatory process[44].

*Defective extrinsic and intrinsic regulation of the cell-mediated immune response in PE*

The potential lack of extrinsic and intrinsic regulation of T cells in pregnancy leading to PE has also been investigated. In terms of extrinsic control, reduced uterine perfusion pressure (RUPP) pregnant rat models infused with IL-10, a cytokine vital for enhancing differentiation of Treg cells and regulating production of inflammatory cytokines, resulted in an increase in circulating Treg numbers to a level similar to normal pregnancy rats, as well as an improvement PE-like symptoms, and reduced numbers of circulating CD4+ T cells and pro-inflammatory cytokines such as TNF-α and IL-6[49,50]. In another study using RUPP rat models, anti-CD28 superagonistic antibody was used to stimulate endogenous Treg cell proliferation[51]. CD28 is a costimulatory receptor that is essential for T cell activation and differentiation, in addition to the primary antigen-mediated T cell activation that occurs via TCR. In vitro CD28 antibodies mimic the costimulatory signalling in combination with TCR-mediated stimulation, usually via TCR antibodies[52]. In contrast, CD28 superagonists can activate human, rat and mouse T cells upon CD28 binding, without the need for TCR activation[52]. The group found that the intervention significantly increased circulating Tregs in the RUPP rats as well as reducing vasoactive factors and improving hypertension[51]. This response was not seen with normal pregnancy rats injected with the superagonist antibody. Additionally

there was a significant increase in fetal weight, suggesting that induction of endogenous Tregs may positively affect maternal and fetal outcomes in response to placental ischaemia. Studies in mouse models also show that paternal antigen-specific Treg cells expand peripherally and locally during pregnancy, primed by seminal plasma, however, paternal antigen-specific Treg cells have not been directly identified in human pregnancy[53,54].

Regarding intrinsic control, murine models have shown that PD-L1 deficient-mice have significantly reduced fetal survival rates[55]. PD-L1 is an inhibitory T cell costimulatory molecule. In this model, fetal rejection was T cell-dependent because PD-L1 blockade caused fetal rejection in B cell-deficient mice but not T cell-deficient mice. Furthermore, a higher frequency of peripheral IFN-γ-producing cells was detected in the experimental group, as well as a higher expression of IFN-γ locally at the placental level. This suggests that PD-L1 may contribute to feto-maternal tolerance by limiting expansion of alloreactive Th1 cells that would otherwise promote fetal rejection. The same group subsequently investigated the interactions between Tregs and PD-L1, since these cells also express the ligand, and found that depletion of Tregs in pregnant mice negated the effect of subsequent PD-L1 blockade, suggesting that the effect of PD-L1 in facilitating immune tolerance may be mediated by Tregs[56]. Additionally, transfer of Tregs in to PD-L1-deficient mice significantly improved fetal survival. Thus, there is much evidence from animal studies that the PD-1/PD-L1 pathway is actively involved in promoting immune tolerance in pregnancy, and interruption of this pathway results in fetal rejection. Pre-eclamptic rat models treated with PDL1-Fc, an activator of the PD-1 pathway, demonstrated a significant and sustained reduction in systolic blood pressure and proteinuria, reduced fetal resorption, and higher fetal and placental

weights, suggesting that treatment improved PE symptoms, restored renal function in this model and reversed fetal growth restriction[57]. Administering PDL1-FC was also associated an increase in Treg cell numbers. Harnessing this pathway to treat PE by augmenting PD-1 levels would not only support induced Treg function but also suppress Th1 cell activation and is an exciting prospective therapeutic target that requires further investigation.

*Limitations of animal studies*

It is crucial to remember the limitations of animal models in their applicability to human pregnancy[42]. In normal human pregnancy both pro- and anti-inflammatory immune responses are important; implantation, spiral artery remodelling, protection against infection and parturition are all Th1-mediated[42]. Furthermore, unlike humans, mouse syncytiotrophoblast expresses paternally-derived MHC class I molecules and would therefore be susceptible to a T-cell mediated immune response if not regulated by mechanisms such as Treg cells and checkpoint proteins[29]. Thus, good quality human studies are crucial to understanding the role of T cells in the pathophysiology of malplacentation disorders.

| First Author | Year | Study design and methods | Results | Reference |
|---|---|---|---|---|
| Zenclussen et al | 2004 | Normotensive pregnant mice injected with activated Th1-like cells | Rapidly induced hypertension and proteinuria | 43 |
| Harmon et al | 2019 | Placental CD4+ T cells from pre-eclamptic women injected into pregnant rats | Significant rise in mean arterial pressure, inflammatory cytokines | 44 |
| Nevers et al<br>Harmon et al | 2011<br>2015 | RUPP pregnant rats infused with IL-10 (enhances Treg differentiation and regulates inflammatory cytokines) | Increased circulating Tregs, decreased circulating inflammatory cytokines, improved-PE like symptoms | 49<br>50 |
| Ibrahim et al | 2017 | RUPP rat models injected with anti-CD28 superagonist antibody (activate T cells) | Increased circulating Tregs, improved hypertension and fetal weight | 51 |
| Guleria et al | 2005 | Pregnant mice underwent PDL1 blockade (inhibitory T cell costimulatory molecule) | Increased T cell-dependent fetal rejection and higher peripheral and placental IFN-γ | 55 |
| Tian et al | 2016 | Pre-eclamptic rat models treated with PDL1-Fc (activates PD-1 pathway) | Significantly reduced BP and proteinuria, less fetal resorption, higher fetal and placental weights, increased Tregs | 57 |

**Table 3:** Summary of animal models demonstrating the role of T cells in PE.

Human studies

Historically, restricted access to human placental tissue has limited studies investigating the decidual T cell repertoire in normal and pathological pregnancies. Nevertheless attempts are now being made to uncover the composition and function of T cells in both the peripheral maternal blood and the maternal-fetal interface in human pregnancies.

The human studies investigating the role of T cells in PE described below are summarised in Table 4.

*PE as an inflammatory autoimmune response*

Immunohistochemistry of placental bed biopsies has shown that whilst the total number of decidual CD45+ leukocytes is similar in pre-eclamptic and normal pregnancies, the proportion of CD3+ T cells is significantly higher in PE[58]. Of these CD3+ cells, a higher proportion were CD8+ T cells in women with PE, indicating that inflammation is a key component of the pathogenesis of the disease[58]. Th17 lymphocytes are a subset of CD4+ T lymphocytes that produce IL-17. It is suggested that Th17 upregulation may contribute to the development of chronic inflammatory diseases, autoimmune diseases and transplant rejection[57]. A study investigating the prevalence of Th17 lymphocytes in PE found that intracellular expression of IL-17 in peripheral blood CD4+ T cells was significantly higher in PE compared to healthy pregnant controls[59]. Moreover the ratio of Th17 cells to Treg cells was higher in PE and there was a positive correlation between the percentage of IL-17-producing CD4+ T cells and the percentage of IL-2 and IFN-γ producing CD4+ T cells[59]. IL-2 and IFN-γ are Th1 cytokines, typically expressed as part of an inflammatory response. The

authors of this study suggested that in PE, upregulation of Th-17 immunity acts through the activation of these Th1 cytokines.

*A potentially altered and functionally impaired T cell repertoire in PE*

A study in which T lymphocytes were isolated from the peripheral blood and the maternal-fetal interface at Caesarean section (CS) showed that a skewed population towards a more differentiated memory phenotype exists in the peripheral blood of pre-eclamptic patients, with reduced naïve CD4+ T cell expression and concomitant increase central memory (CM) T cell expression[60]. A similar trend was seen in the maternal-fetal interface, though this was not statistically significant. The proportion of decidual T cell lymphocyte subpopulations has varied considerably in studies. In contrast to the study mentioned in the previous section, which showed higher CD8+ proportions in placental bed biopsies from women with PE, Rieger et al. found that the number of CD8+ T cells was reduced in the decidua of women with PE compared to control patients[58,61]. The authors of this study hypothesised that since an inflammatory environment is key for tumour cell invasion, and invasive trophoblasts share many properties of tumours, that reduced trophoblast invasion associated with PE development may be a consequence of the lower inflammatory cell population in the placental bed[61].

Nguyen et al showed that both CD4+ and CD8+ naïve T cells in the peripheral blood of pre-eclamptic patients may be functionally impaired, as measured by reduced production of IL-2, and peripheral blood memory CD8+T cells displayed a reduced capacity for IFN-γ production in PE, though production of other cytokines such as Granzyme A, Perforin A and TNF-α seemed to be unaffected[60]. This observation was

not seen in the decidua. This is an unexpected finding which seems to contrast the aforementioned human and mouse studies identifying PE as an inflammatory condition likely to be associated with an increase in IFN- γ. IL-2 is essential for thymic induction and peripheral maintenance of Treg functionality and so reduced levels of this cytokine may relate to reduced immune tolerance of the pregnancy by contributing to Treg dysfunction. This functional impairment was not reflected in the classical markers of T cell exhaustion, as CD27 and CD28 cell surface expression was similar in PE and healthy pregnancies[60].

*Peripheral and decidual Treg numbers and functionality in PE*

Whilst paternal antigen-specific Treg cells have not been directly identified in human pregnancy, there has been work to show that human decidual Treg cells have fetal-specificity. Tilburgs et al. demonstrated that decidual Treg cells exhibited higher suppression towards self-fetal cord blood than non-self cord blood, which was not seen with peripheral Treg cells, suggesting that fetal-specific Treg cells may exist at the human maternal-fetal interface[37,62]. Some studies have shown that peripheral and decidual CD4+CD25$^{bright}$ Treg cell pools decrease in PE, although it is unclear whether the reported reduction is a decrease in the total number of Tregs or in the number of fetal-specific Treg cells[63]. However, there is evidence that clonal expansion of decidual CD4+CD45RA−FoxP3$^{high}$ effector Treg cells is reduced in both early and late-onset PE compared with normal pregnancies in the third trimester, suggesting fetal-specific tolerance may be impaired[54,64]. In one study, intracellular expression of anti-apoptotic protein Bcl-2 in CD4+CD25+FoxP3+ Treg cells in the peripheral blood of pre-eclamptic women was significantly lower than healthy pregnant controls, suggesting that the lower number of Treg cells seen in PE may be associated with increased apoptosis

signalling[65]. In another study, whilst an unexpected increase in circulating Tregs was found in the PE cohort, these cells were functionally impaired as depletion of Tregs from peripheral blood in PE did not enhance the proliferative response of T cells on exposure to irradiated cord blood[60]. Decidual CD14+DC-SIGN+ APCs seem to play an important role in local iTreg cell induction in healthy pregnancies. In PE, CD14+DC-SIGN+ APCs have been shown to have reduced expression of tolerogenic molecules such as HLA-G and the immunosuppressive protein Immunoglobulin-like Transcript-4 (ILT4), associated with poor iTreg induction in vitro and likely compensatory nTreg cell induction to the inflamed decidua to control the local alloresponse[66].

*The PD-1/PD-L1 pathway in PE*

A recent study into the role of PD-1 in human severe early-onset PE (before 34 week's gestation) found PD-1 expression by peripheral CD8+ and CD4+ T cells, NKT-like cells and Treg cells was significantly upregulated in women with the disease, whilst the ligand PD-L1 was only significantly upregulated in NKT-like cells[67]. Additionally, cytotoxicity of PD-1+CD8+ T cells in early-onset PE was greater than in normal pregnancies. The authors hypothesised that this unexpected increase in PD-1 expression may represent cell activation rather than cell exhaustion and may be associated with the failure of the PD-1/PD-L1 pathway to downregulate Th1 responses, thus contributing to immune activation and the clinical inflammatory picture associated with early-onset PE. Furthermore, it was suggested that if the Treg population is decreased in PE, as discussed previously, peripheral induction of further Tregs from naïve T cells through the PD-L1/PD-1 pathway could be reduced, and thus contribute to the reduced immune tolerance in PE. Whilst this work is of interest, it is imperative

that human studies to investigate the role of PD-1 locally at the maternal-fetal interface are undertaken, rather than focus solely on human peripheral blood experiments.

*A potential infective trigger?*

Flow cytometric analysis on the peripheral blood of pre-eclamptic women demonstrated a significantly higher percentage of CD4+ CD45RO+ memory T cells and a significantly lower percentage of naïve CD45RA+ T cells compared with healthy pregnant controls[68]. These findings did not alter when pre-eclamptic patients were compared on parameters such as parity or gestation. It has been suggested that this leukocyte activation in PE may be associated with infection in pregnancy predisposing women to PE. This has been shown in animal models where transfusion of bacterial endotoxins in pregnant rats mid-gestation caused a significant increase in blood pressure and urine albumin excretion[69]. Another study which identified pregnant women at high risk of developing PE, found that 11.5% had bacteriuria and 14% had vaginal infections, all of which were treated with antibiotics. They found that the incidence of PE was reduced by almost 53% and hypothesised that chronic subclinical infections may increase maternal cytokine levels and affect vascular endothelial function, potentially lowering the threshold to develop PE symtoms[70,71].

*Aberrant HLA molecule expression may affect T cell activity*

The unique expression of non-classical HLA molecules by the EVT and lack of expression of HLA by the syncytiotrophoblast is thought to be a crucial mechanism for protecting the pregnancy against maternal allo-reactive T cells[72]. Therefore, there has been much focus on these molecules to determine whether these may be linked with pregnancy complications. Although it is well-established that the syncytiotrophoblast

does not express MHC class I or class II antigens, it has been reported that a soluble form of HLA-G may be secreted by the syncytiotrophoblast, though this remains controversial[72,73]. Membrane-bound and soluble HLA-G are expressed by the EVT and expression is thought to downregulate CD4+ and CD8+ T cells in order to protect the pregnancy from a cell-mediated immune response. It has been suggested that reduced soluble HLA-G expression by the trophoblast may lead to inadequate immune tolerance at the maternal-fetal interface and result in PE. Additionally, aberrant expression of HLA-DR, a MHC class II molecule, has been detected on the apical membrane of the syncytiotrophoblast cells of a significant number of pre-eclamptic women and none of the control samples, suggesting that HLA-DR expression may lead to potential downstream pathways leading to enhanced T cell responses in PE[74].

| First Author | Year | Study design and methods | Results | Reference |
|---|---|---|---|---|
| Milosevic-Stevanovic et al | 2019 | Immunohistochemistry of placental bed biopsies | Higher proportion of CD8+ T cells in women with PE | 58 |
| Darmochwal-Kolarz et al | 2012 | Th17 and Treg levels were measured in the peripheral blood of pregnant women with PE | Significantly higher 1L-17 levels (produced by Th17) in PE and higher Th17:Treg cell ratio. Positive correlation between IL-17 and Th1 cytokines in PE | 59 |
| Nguyen et al | 2017 | T cells isolated from peripheral blood and maternal-fetal interface at CS | More differentiated memory phenotype in PE (only significantly different in blood). Reduced IL-2 and IFN-γ production in naïve peripheral blood T cells | 60 |

| | | | | |
|---|---|---|---|---|
| | | | (suggesting they are functionally impaired). | |
| Rieger et al | 2009 | FACS analysis of decidual leukocyte subpopulations in decidual tissue of pregnant women with PE | Reduced decidual CD8+ T cell population in PE | 61 |
| Tilburgs et al | 2008 | Matched sampled of decidua basalis, peripheral blood and cord blood obtained from healthy term pregnancies. | Higher suppression towards self-fetal cord blood vs. non-self cord blood by decidual Tregs, suggesting Tregs have fetal specificity | 62 |
| Sasaki et al | 2007 | Flow cytometric analysis of peripheral blood Tregs and immunohistochemical staining of placental bed biopsies in PE and control | Reduced peripheral and decidual Tregs in PE | 63 |
| Tsuda et al | 2018 | Paired samples of peripheral blood and decidua from terminations of pregnancy and miscarriage, as well as 3rd trimester samples from PE patients | Higher frequency of clonally expanded Tregs in 3rd trimester vs. 1st trimester. Tregs rarely seen in peripheral blood. Reduced decidual populations of clonally expanded effector Tregs in pre-eclampsia compared with normal 3rd trimester samples. | 64 |
| Darmochwal-Kolarz et al | 2012 | Flow cytometric analysis of Treg cells | Significantly reduced intracellular | 65 |

| | | | | |
|---|---|---|---|---|
| | | from the peripheral blood of pre-eclamptic patients | expression of anti-apoptotic protein Bcl-2 in Tregs in PE | |
| Hsu et al | 2012 | Peripheral blood and decidua basalis samples collected from healthy 3rd trimester pregnancies and those affected by PE | CD14+ DC-SIGN+ APCs failed to induce iTregs in decidua of PE samples. Impaired peripheral iTreg expansion in PE vs. healthy pregnancy | 66 |
| Meggyes et al | 2019 | Flow cytometric analysis of blood collected from women with early-onset (34 weeks) PE | Significant upregulation of PD1 by CD4+, CD8+ and Treg cells in early onset PE | 67 |
| **Table 4:** Summary of human studies demonstrating the role of T cells in PE. | | | | |

Human Studies in FGR

Villitis of unknown aetiology (VUE) is a common placental lesion associated with histopathological examination of placentas from pregnancies affected by FGR and stillbirth[75]. It is an inflammatory condition of the placenta, not associated with an infective cause, and although seen in up to 15% of placentas from healthy pregnancies at term, it is present in almost 29% of FGR infants[76]. Studies to characterise the composition of immune cells in the placenta affected by VUE have shown that fetal macrophages and maternal CD4+ T cells were the most prevalent immune cells, with maternal CD8+ T cells still elevated but present to a lesser extent[77]. Examination of cytokines in VUE lesions has also shown a pro-inflammatory Th1-mediated cytokine environment, with increased IL-2 and IL-12[77]. These findings suggest that VUE is a Th1-mediated pan-placental inflammatory immune response, as opposed to affecting discrete areas in the placenta as suggested in earlier studies[77]. The presence of

cytotoxic CD8+ cells have been suggested to contribute to placental vasculopathy and intravillous cell apoptosis[77]. Studies have also found increased FOXP3+ Treg expression in VUE, suggesting a response by the maternal immune system to "rescue" the failing placenta from the immune response similar to that seen in graft rejection[78].

*Limitations of current human studies*

When interpreting these results it is important to consider the wide variation in methods for obtaining and preparing tissue samples. Whilst some studies have used placental bed biopsies, which may be contaminated by myometrial tissue and peripheral blood cells, other studies have obtained decidua either by endometrial curettage during CS or dissected decidual samples from the basal plate of the placenta following vaginal delivery[58,61]. Similarly, quantitative analysis is not standardised between studies, with some using immunohistochemistry and others using FACS-based analysis. Such inconsistencies make direct comparisons between results of studies extremely difficult and more work is needed to optimise a sampling and analysis method that can help to build definitive evidence for the role of T cells in pregnancy disorders.

<u>The possibility of immunotherapy for malplacentation disorder</u>

Parallels between tumour immunity and immune tolerance of pregnancy have led to Tregs being implicated as a potential target for treatment of PE and FGR. Treg depletion is associated with tumour immunity in rodents and tumours are found to be rich in Foxp3+ Tregs, so it is possible that nTregs that promote self-tolerance may act to impede immune surveillance against cancers in normal individuals whilst suppressing responsiveness to tumours in cancer patients[79,80]. Thus, Tregs have been proposed as a target of cancer immunotherapy[80]. In the same way, Tregs could be

targeted for boosting immune tolerance in pregnancy as natural Tregs retain their suppressive function and proliferative capacity after expansion in vivo and in vitro, so potential clonal expansion of antigen-specific natural Tregs could help augment feto-maternal tolerance. This principle would also apply to preventing transplant rejection[80].

## Conclusion of literature review

This review has summarised the current understanding of the role of T lymphocytes in normal pregnancy and discussed existing animal and human studies investigating the potential role of T cells in the pathophysiology of PE. Whilst there is consensus that T cells exhibit specificity towards fetal antigens and have a unique profile in the decidua, much work is needed to determine whether T cells are implicated in PE, either directly through a T cell-mediated inflammatory response that may underlie the development of the maternal syndrome of PE, or indirectly through impaired regulation. Indeed, it has been proposed that PE should be considered as a two-stage disease; with the first stage characterised by defective spiral artery remodelling leading to abnormal placental development, and the second hallmarked by the maternal signs and symptoms associated with a systemic inflammatory response with endothelial dysfunction and release of vasoactive substances[29]. It may be that T cells have a role in this second stage as a downstream consequence of NK cell activity, rather than being at the epicentre of the disease process.

## PROJECT AIM

As mentioned above, our group demonstrated previously that a maternal cellular immune response against the fetus is present in healthy pregnancies but is regulated by expression of inhibitory checkpoint proteins and T regulatory cells within the maternal decidua[15]. The aim of this project was to determine the feasibility of collecting and isolating T cells from women whose pregnancies were complicated by PE or FGR, so that future studies can be planned to investigate T cell function and possible dysregulation in these disorders.

## HYPOTHESIS

We hypothesised that there would be significantly higher decidual CD4+ and CD8+ EM T cell populations compared to matched blood samples in cohorts from PE and FGR, and that peripheral blood CD4+ and CD8+ T cell levels would be higher in these cohorts compared to healthy control. In addition we hypothesised that there would be significantly higher decidual CD4+ and CD8+ T cell populations in pregnancies affected by PE and FGR compared with healthy control pregnancies.

# METHODS

## Patient recruitment

The eligibility criteria for inclusion in the study were as follows:

- Over 18 years old
- Understands and speaks English
- PE as defined by the National Institute for Health and Care Excellence (NICE): BP ≥140/90 and urine PCR ≥30mg/mmol >20 weeks gestation)[6]
- No sepsis or chorioamnionitis in labour
- FGR, defined as either an EFW or AC plotting below the 3rd centile or plotting below the 10th centile with evidence of placental dysfunction (abnormal uterine artery Doppler between 20-24 weeks' or abnormal umbilical artery Doppler) in a non-smoker and not secondary to an underlying congenital anomaly[20].

Eligible patients for recruitment were identified from antenatal clinic, the antenatal inpatient ward and the induction suite. Healthy pregnant women were also recruited as control participants. Ethical approval for the study was granted by the West Midlands Research Ethics Committee (REC reference 14/WM/1146).

Potential participants were offered written and verbal information about the study and all participants provided written consent for recruitment in to the study. Maternal blood (20ml) was collected during venous cannulation in labour or prior to elective CS. Once delivered, cord blood (10ml) was aspirated from the umbilical cord. All blood samples were collected in in lithium-heparin bottles to prevent clotting. Blood samples along with the placenta were collected for T cell isolation. If immediate collection of samples

was not possible, the placenta was stored in a refrigerator and blood bottles stored at room temperature with a delay in collection of no more than 12 hours.

White blood cell isolation from whole blood

Blood samples were diluted in RPMI 1640 medium with 5ml penicillin G-streptomycin mix to prevent bacterial contamination in a 1:1 ratio of blood to medium. This diluted blood was then layered on to Lymphoprep$^{TM}$, a density gradient medium, before centrifugation at 2000 revolutions per minute (rpm) for 30 minutes to isolate peripheral blood mononuclear cells (PBMCs). Following isolation the PBMCs were suspended again in RPMI 1640 medium up to 30ml and spun at 1500rpm for 10 minutes in order to wash off any Lymphoprep. Following this, the supernatant was poured off and the cell pellet was suspended in 5ml of red blood cell (RBC) lysis and left for 10 minutes to ensure the remaining sample was devoid of any red blood cells. After this, the suspension was washed in a further 30ml of RPMI 1640 and spun down at 1500rpm for 10 minutes. After pouring off this supernatant PBMCs were suspended in 20ml of RPMI1640 and 10µL of the suspension was used to count the number of cells in the final sample using a haemocytometer.

If the sample was not being immediately stained for flow cytometry, the final sample of isolated PBMCs was suspended in a freezing medium of 10% Dimethyl Sulfoxide (DMSO) mixed with Fetal Calf Serum (FCS) and frozen down at -70°C in 1ml cryovials with approximately $5x10^6$ cells in each cryovial .

White blood cell isolation from the placenta

Macroscopic 2x2cm samples of decidua basalis were obtained from across the entire surface of the placenta using sterile forceps and a scalpel to dissect the smooth

decidua basalis layer from the underlying spongy placental tissue (Figure 3). Dissected samples were washed in Phosphate-buffered Saline (PBS) before being divided into smaller pieces (approximately 5mm$^3$ size) on a sterile agar plate to provide a greater surface area for cells to be released from the tissue in to the culture medium. Decidua basalis samples were cultured in RPMI 1640 medium supplemented with FCS and GlutaMAX to optimise the environment for cell culture. After culturing the cells in a 12-well plate at 37°C for 24 hours, the suspended cells were pipetted out of the plate in to 50ml tubes.

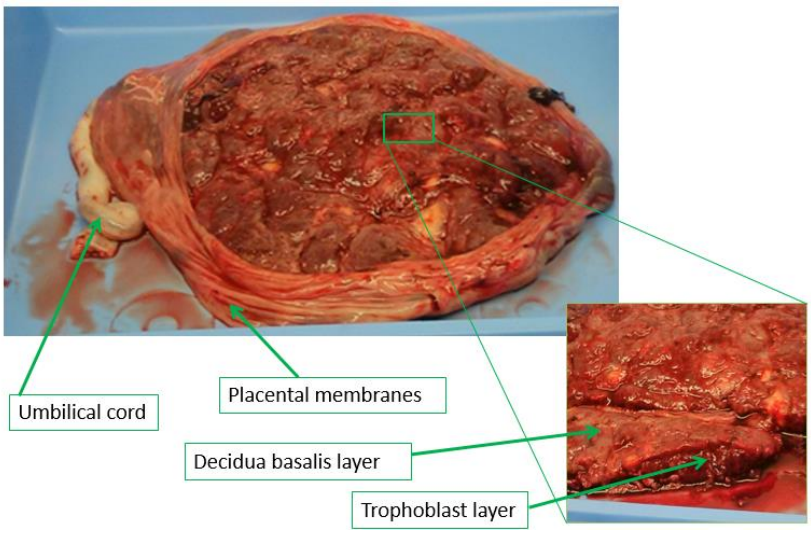



**Figure 3:** A full term placenta complete with cord and membranes. Zoomed image shows demarcation of the decidua basalis layer from the underlying villous placental tissue.

The decidua basalis tissue was collected from the wells and placed in to gentleMACS$^{TM}$ C-Tubes to gently dissociate the tissue using the “mouse brain” program setting on the Miltenyi Biotec gentleMACS$^{TM}$ dissociator to obtain a single-cell suspension. After this the suspension was passed through a 70µM cell strainer to allow cells from the suspension to be separated from the larger particles. This cell suspension was then added to the initial suspension of cells obtained from the 12- well

plate and the final suspension was subsequently washed in RPMI 1640 medium and centrifuged at 1500rpm for 10 minutes, after which the cell pellet was resuspended in 5ml of RBC lysis buffer for 10 minutes at room temperature. Following lysis the suspension was washed in 20ml of RPMI 1640 medium and spun at 1000rpm for 10 minutes. The remaining pellet was assumed to be white blood cells, which were resuspended in RPMI supplemented with FCS and had been pre-warmed in a water bath for 10 minutes. The cells from a 10µL sample of the suspended cells were counted in a haemocytometer for an estimation of final cell quantity from the sample. As with the PBMCs, if not being immediately stained and analysed, the cells were frozen down in freezing medium (10% DMSO+FCS) at -70°C in 1ml cryovials.

Antibody staining and flow cytometry

If using frozen samples, the cryovials were thawed in a water bath at 37°C for 60 seconds and then samples were transferred out of the cryovials and in to pre-warmed RPMI 1640 media with FCS. The thawed cell suspension was spun at 1000rpm for 8 minutes, after which the supernatant was poured off the pellet was then resuspended in 3ml of MACS buffer to facilitate cell separation. The suspension was then passed through a 30µM pre-separation filter in to a FACS tube to remove any cell aggregates or large particles in the suspension and thus optimise the flow of the sample through the flow cytometer. The filtered suspension was spun at 1500rpm for 5 minutes in the centrifuge and the resulting supernatant poured off. The cell pellet was resuspended in 20µL of FCR block, in order to block fluorescent antibodies binding to the Fc receptors on other white cells such as B cells, monocytes and macrophages, thus increasing the specificity of antibody binding to T cells.

Fluorescent antibodies were then added to each FACs tube as outlined in Table 5 and stained for 30 minutes in the fridge.

| **Antibody** | **Fluorophore** | **Volume (µL)** |
|---|---|---|
| CD3 | APC/Cy7 | 5 |
| CD4 | Brilliant Violet 510 | 4 |
| CD8 | PerCP55 | 4 |
| CD45RA | AF700 | 1 |
| CCR7 | FiTC | 5 |
| CD27 | PE | 5 |
| CD28 | APC | 3 |
| CD57 | Pacific blue | 5 |
| PD-1 | PE-Cy7 | 5 |
| (Dump antibodies)<br>CD19 (B cells)<br>CD14 (monocytes)<br>CD56 (natural killer cells) | <br>ECD<br>ECD<br>ECD | <br>3<br>3<br>3 |

**Table 5:** Composition and volume of fluorescent antibodies used for cell-staining.

After 30 minutes, the samples were washed in 3ml MACS buffer and spun at 1500rpm for 5 minutes. After pouring off the supernatant the remaining antibody-stained cell pellet was resuspended in 2 drops of MACS buffer ready for analysis in the flow cytometer. Immediately prior to running the samples on the Beckman Coulter Gallios Flow Cytometer, 1µL of propium iodide (PI) was added to each sample to identify and exclude dead cell artefact from the suspension during analysis.

Gating strategy for flow cytometric cell counting

The gating strategy used is summarised in Figure 4. Singlet cells were isolated on forward scatter-area vs. forward scatter-height plots. From this, lymphocytes were gated on forward scatter vs. side scatter. From this, T cells, which express CD3 were then isolated from all other white cells through a “dump channel” that included monocytes, which express CD14, B cells, which express CD19 and natural killer cells, which express CD56, as well as dead cells, identified through PI staining. CD3+ T cells were then gated for CD4+ and CD8+ expression, after which each type of T cell was gated based on CD45RA and CCR7 expression to determine the composition of memory T cell subtype in the blood and in the decidual tissue of women affected by PE and FGR, compared with control (normal healthy pregnancy). These memory subtypes were naïve T cells (CD45RA+CCR7+), central memory T cells (CD45RA-CCR7+), effector memory T cells (CD45RA-CCR7-) and effector memory re-expressing CD45RA (EMRA) T cells (CD45RA+CCR7-). In addition CD4+ and CD8+ T cell effector memory (EM) subtypes were gated based on CD28 and CD27 expression; EM1 (CD28+CD27+), EM2 (CD28-CD27+), EM3 (CD28-CD27-) and EM4 (CD28+CD27-). Gating for EM PD-1 expression was also done since the group’s previous work showed markedly increased PD-1 expression in decidua compared with peripheral blood in healthy pregnancy.

Statistical analysis

GraphPad Prism 9.2.0 was used for statistical analysis. Wilcoxon matched-pairs signed rank test was used to compare matched blood and decidual samples within a cohort. Mann-Whitney U test was used to compare samples from different cohorts.

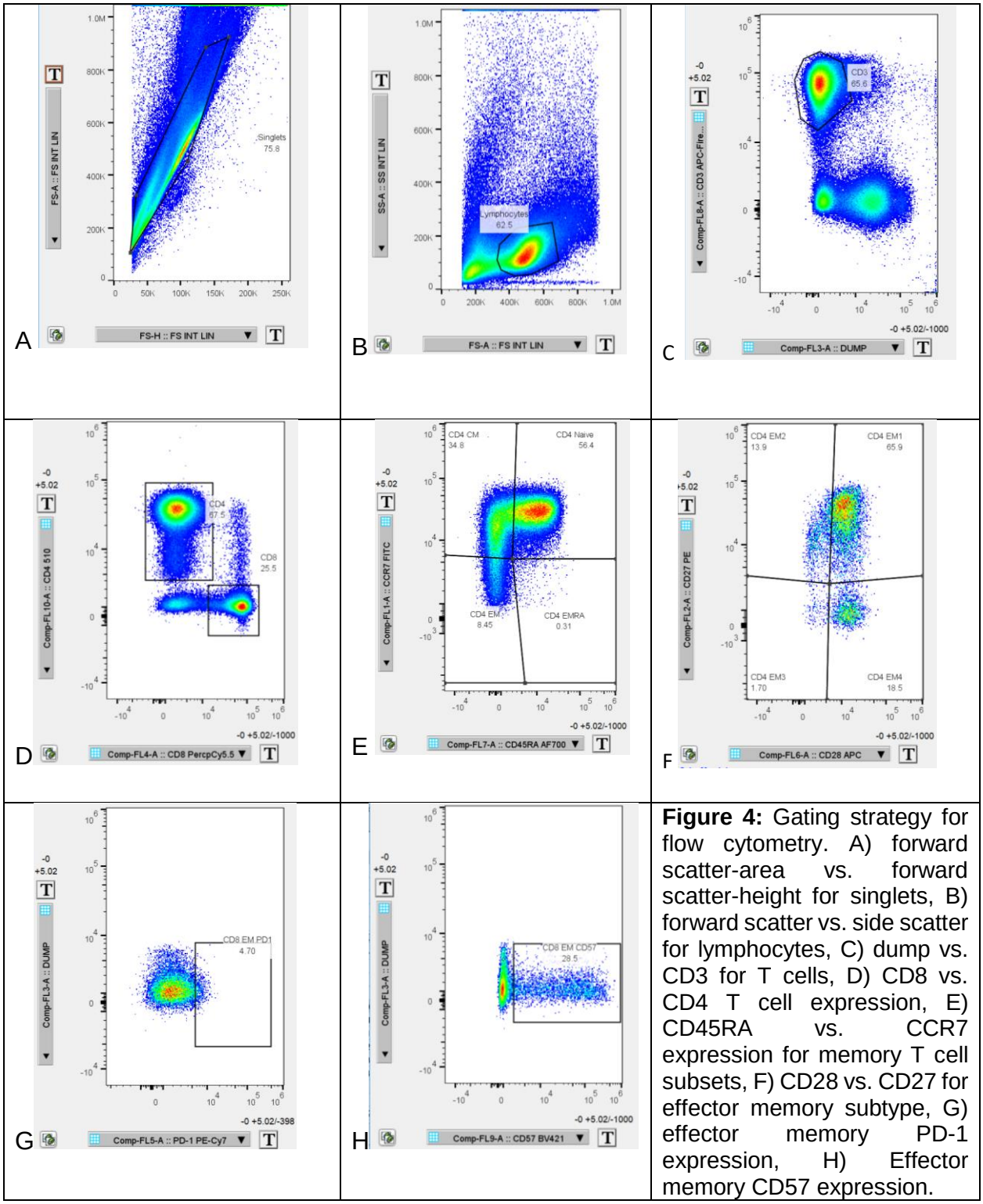



**Figure 4:** Gating strategy for flow cytometry. A) forward scatter-area vs. forward scatter-height for singlets, B) forward scatter vs. side scatter for lymphocytes, C) dump vs. CD3 for T cells, D) CD8 vs. CD4 T cell expression, E) CD45RA vs. CCR7 expression for memory T cell subsets, F) CD28 vs. CD27 for effector memory subtype, G) effector memory PD-1 expression, H) Effector memory CD57 expression.

# RESULTS

## Participant demographics and delivery data

69 patients were considered eligible and successfully recruited to the study. Of those, triplet samples of placenta, maternal and cord blood were collected from 75% of recruited participants (52/69); 48% of samples (25/52) were from participants with PE, 37% of samples were from pregnancies affected by FGR (19/52) and 15% (8/52) were controls samples from healthy pregnancies. Participant demographics and information about timing and mode of delivery are summarised in Table 6. Amongst the PE cohort, mean PCR was 225 mg/mmol (range 30-1495 mg/mmol), and mean creatinine was 69 (range 47-171). The earliest delivery for PE was at 31 weeks and 6 days gestation. In the FGR cohort, all of which were delivered due to suspected growth restriction on antenatal ultrasound scans, the median birthweight (BW) was 2705g (range 2070 – 3405g), and when BW was plotted against the participant's customised growth chart, 53% of these babies were born with a BW below the 10$^{th}$ centile for gestation. The earliest gestation for delivery in the FGR cohort was at 28 weeks and 3 days gestation and there was absent end diastolic flow (AEDF) in the umbilical cord on Doppler ultrasound measurement.

| | Pre-eclampsia | Fetal growth restriction | Healthy controls |
|---|---|---|---|
| **% primigravid** | 10/25 (40%) | 10/19 (53%) | 2/8 (25%) |
| **% assisted conception** | 2/25 (8%) | 2/19 (11%) | 0/8 (0%) |
| **Mean age at booking** | 32 years (range 24-42 years) | 27 years (range 22-40 years) | 34 years (range 28-39 years) |
| **Mean booking BMI** | 31.5 (range 21.1 – 51.6) | 28.7 (range 21.6 – 38.2) | 21.7 (range 18.5-26.6) |
| **Median gestational age at delivery** | $37^{+2}$ weeks (range $31^{+6}$ – $42^{+0}$) | $38^{+4}$ weeks (range $28^{+3}$ – $40^{+2}$) | $39^{+2}$ weeks (range $39^{+0}$ – $39^{+3}$) |
| **% elective CS delivery** | 4/25 (16%) | 0/19 (0%) | 8/8 (100%) |
| **% emergency CS delivery** | 18/25 (72%) | 5/19 (26%) | 0/8 (0%) |
| **% operative vaginal delivery** | 2/25 (8%) | 2/19 (11%) | 0/8 (0%) |
| **% normal vaginal delivery** | 1/25 (4%) | 12/19 (63%) | 0/8 (0%) |

**Table 6:** Summary of participant demographics and timing and mode of delivery**.**

There were significantly more primigravid women in the pathological group of pregnancies (combined PE and FGR) compared with the control group (45% vs 25%; p=0003). Although the mean age of the women in the control group was higher than the combined mean age in the PE and FGR groups, this was not statistically significant (p=0.4). Mean booking BMI of the pathological group was significantly higher than the control group (BMI 29.6 vs 21.9; p=0.005; 95% CI 2.4-13). Median gestational age at delivery in the combined pathological group of pregnancies was significantly lower than the control group (37 weeks' vs $39^{+2}$ weeks'; p=0.04; 95% CI -4.2 – -0.1). Significantly more women were delivered by emergency CS in the combined PE and FGR group (n=23) compared with control (n=0; p=0.0001). Significantly more women in the FGR

group achieved a vaginal birth, either spontaneously or operatively (n=14) compared with the PE group (n=3; p=0.0001). Significantly more women with PE were delivered by emergency CS compared with FGR (p=0.009).

Flow cytometry

To determine whether the method of T cell isolation from placental tissue was adequate for further studies, a subset of 39 out of the total 52 samples (75%) collected underwent flow cytometry, with the remaining samples frozen down for later study. Flow cytometry of this initial subset compared total (i.e. CD4+ and CD8+) T cell memory subset distribution and total EM PD-1 populations between samples from matched maternal blood and decidua in each cohort (Figure 5) as well as CD4+ and CD8+ subpopulations between matched samples in each cohort.

*Healthy control cohort*

There was a significantly greater total (CD4+ and CD8+) naïve and CM T cell population in peripheral blood compared with matched decidual samples, with total naïve T cells comprising 52% of the total T cell population in blood compared with 25% of the T cell population in matched decidual samples (p=0.007). CM T cells comprised 24% of the total peripheral T cell population compared with only 15% of the decidual T cell population in matched samples (p=0.005). In the decidua there was a significantly greater population of total EM T cells compared with matched peripheral blood (49% vs 16%, p=0.0005) and a non-significantly greater total EMRA population in decidua from healthy pregnancies compared with matched blood (11% vs. 8%, p=0.33) (Figure 5a). Total EM PD-1 was significantly higher in control decidua vs. matched blood (13.7% vs. 3.5%, p=0.0005) (Figure 6).

Individual CD4+ and CD8+ naïve T cell subpopulations were greater in control blood than decidua (53.7% vs. 26.6%, p=0.003 and 47.2% vs 23.8%, p=0.0093 respectively). EM T cell subpopulations were significantly greater in control decidua for both CD4+ (52.4% vs 10.7%) and CD8+ (54.1% vs. 20.7%) subtypes compared with control blood (p=0.0005 for both). CD4+ and CD8+ EM PD-1 populations were higher in decidua vs. matched control blood (20.5% vs. 5.5%, p=0.0005 and 19.4% vs. 4.8%, p=0.0024 respectively).

*PE cohort*

As with the control cohort, there was a significantly greater total naïve T cell population in maternal blood compared with decidua (50% vs. 23%, p<0.0001). A significantly greater total EM and EMRA T cell population was found in PE decidua compared with blood (44% vs. 18%, p=0.0002 and 13% vs. 9%, p=0.0214 respectively) (Figure 5b). There was no significant difference in total CM T cell population between matched decidua and blood in PE (23% vs. 20%, p=0.78). Total EM PD-1 was significantly higher in PE decidua vs. matched blood (17% vs. 5.7%, p=0.0008) (Figure4). On comparing CD4+ and CD8+ subpopulations, there were greater CD4+ and CD8+ naïve T cell populations in PE blood vs. matched decidua (49.4% vs. 25.1%, p=0.0012 and 50.1% vs. 20.2%, p=0.0006 respectively), greater decidual CD4+ and CD8+ EM subpopulations vs. matched blood (48.4% vs. 12%, p=0.0001 and 52.9% vs. 17.4%, p<0.0001 respectively), and for CD8+ only, a significantly greater EMRA population in decidua vs. blood (23.5% vs. 14.6%, p=0.034). Both CD4+ and CD8+ EM PD-1 populations were greater in decidua than blood (19.8% vs. 5%, p<0.0001 and 16.4% vs. 8%, p=0.048 respectively). CD4+ and CD8+ EM1 populations were higher in PE

blood vs. decidua (65.6% vs. 43.4%, p=0.002 and 51.5% vs. 27.3%, p=0.0026 respectively).

*FGR cohort*

In this cohort, a significantly higher CM population was found in blood compared with decidua (26% vs. 13%, p=0.0195) and a significantly higher EM population in decidua than blood (61% vs. 30%, p=0.0117) (Figure 5c). Unlike the control and PE cohorts, the total EMRA population was significantly higher in blood compared with decidua from pregnancies affected by FGR (9% vs. 6%p=0.0117) and this was only significantly different in the CD8+, but not CD4+ EMRA subpopulation (18% vs. 11.6%, p= 0.0195). The naïve T cell count in blood was not significantly greater than matched decidua, unlike other cohorts. Total EM PD-1 was significantly higher in FGR decidua vs matched blood (11.1% vs. 4.5%, p=0.0078) (Figure 6). Subpopulation comparison in the FGR cohort showed both in CD4+ and CD8+, the EM population was higher in decidua than in blood (66% vs. 19.6%, p=0.0078 and 62.7% vs. 28.8%, p=0.0195 respectively).

*Comparison between cohorts*

When comparing PE and FGR samples with control, there was no significant difference in total memory T cell subset composition in either blood or decidua between samples from diseased pregnancies and those from normal healthy pregnancies.

There were significantly greater CD4+ and CD8+ naïve T cell population in the control blood compared with blood from FGR samples (53.7% vs. 37.9%, p=0.0227 and 47.2% vs. 33.1%, p=0.0491 respectively) (Figure 7) but no significant difference in total naïve

T cell value in blood, nor any significant difference when comparing decidua from these 2 cohorts.

*Effect of mode of delivery on T cell populations*

There was no significant difference in either peripheral blood or decidua CD4+ or CD8+ T cell populations between those participants who had delivered vaginally vs. those who were delivered by elective CS without rupture of membranes.

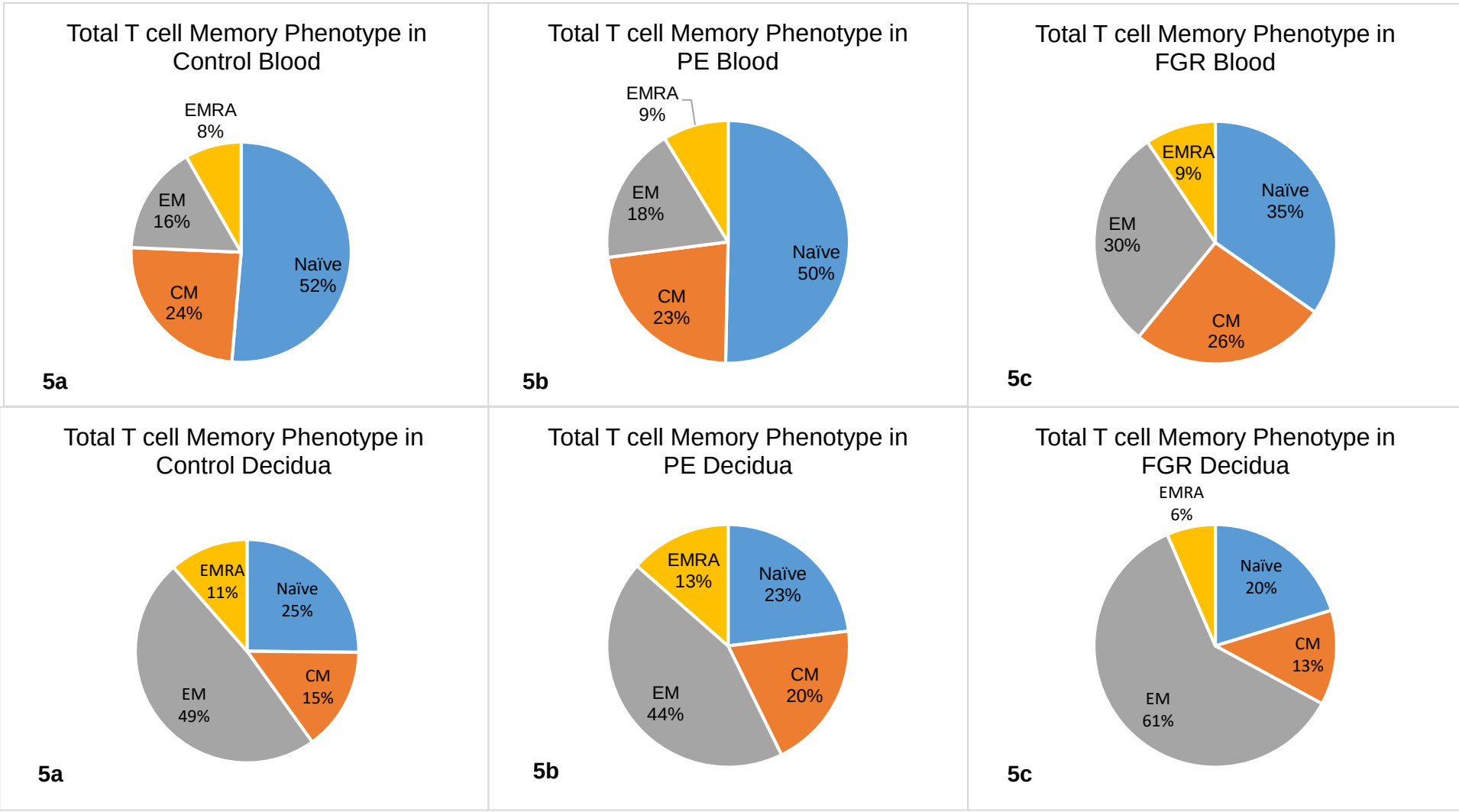



**Figure 5:** Total (i.e.CD4 & CD8) T cell memory phenotype in the maternal blood and decidua of control pregnancies, PE and FGR. Freshly isolated lymphocytes from all samples are gated on live CD3+, CD4+ or CD8+ and examined for memory status using CD45RA and CCR7 expression via flow cytometry (n=39). Memory status is classified into 4 categories: Naïve: CD45RA+ CCR7+; Central Memory (CM): CD45RA- CCR7+; Effector Memory (EM): CD45RA- CCR7-; Effector Memory RA (EMRA): CD45RA+ CCR7-. For control and PE cohorts, a significantly greater naïve population is seen in blood compared with matched decidual samples ($p<0.05$) and a significantly greater EM population is seen in the decidua of all cohorts compared with matched blood samples ($p<0.05$).

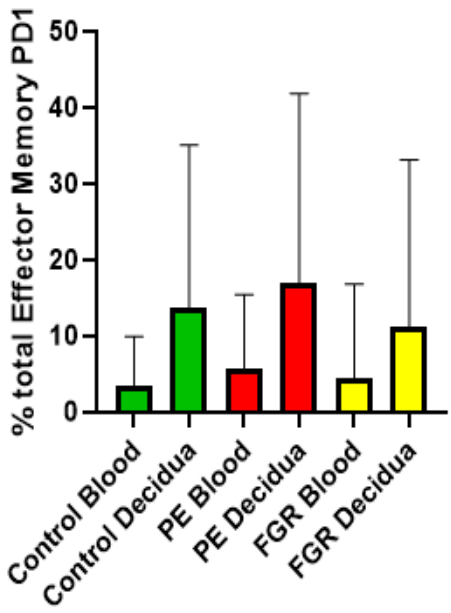
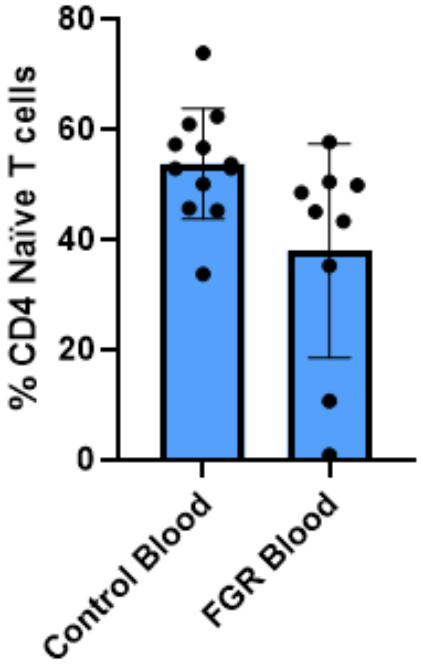
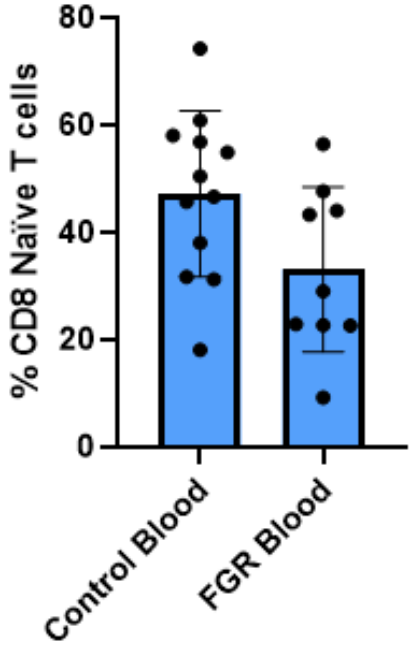



**Figure 6:** Percentage of total (CD4+ and CD8+) Effector Memory T cell PD-1 expression in all samples (blood and decidua) of all cohorts; healthy pregnancy (control), pre-eclampsia (PE), and fetal growth restriction (FGR). Total EM PD-1 was significantly higher in decidua vs. matched blood in all cohorts (control 13.7% vs. 3.5%, p=0.0005; PE 17% vs. 5.7%, p=0.0008, FGR 11.1% vs. 4.5%, p=0.0078).




7a




7b

**Figure 7:** Percentage of peripheral blood naïve CD4+ and CD8+ T cells in healthy pregnancies (control) and fetal growth restriction (FGR). A significantly lower percentage of a) CD4+ naïve T cells and b) CD8 naïve T cells are seen in FGR compared with control ($p<0.05$).

# DISCUSSION

## Main findings

The data confirms findings from the group's previous work in healthy pregnancies, which demonstrated a significantly greater naïve T cell phenotype in blood compared with decidual tissue and a conversely dominant effector memory population in tissue compared with blood[37]. For the first time, we have shown that this distinction in memory T cell composition between blood and decidual tissue is also seen in pregnancies affected by PE or FGR. This might represent accumulation of EM T cells in tissue, or that naïve T cells do not accumulate in tissue[22]. We have also confirmed an increased expression of PD-1 in decidua compared with peripheral blood, which is seen in all cohorts, and a non-significantly higher amount in PE decidua than control and a non-significant reduction in FGR decidua than control.

This preliminary work suggests new findings in pregnancies affected by malplacentation that warrant further investigation, with a significantly lower peripheral naïve T cell phenotype in FGR compared with healthy pregnancies, and early insight into T cell characteristics in pregnancy disorders.

## Interpretation

Whilst much work has been done to characterise and compare peripheral T cell lymphocyte populations in pre-eclampsia with either healthy pregnancy or non-pregnant controls, very little work has been done to characterise T cell subpopulations in human placental tissue, and even less with FGR. The unpredictability of pregnancies affected by pre-eclampsia and fetal growth restriction can mean that collecting samples can be very challenging, especially when a decision to deliver for clinical need is made

very quickly. Attempts have been made to study the placenta in vitro through production of organoids, though these models are limited to early pregnancy[81]. It is crucial to recognise that pregnancy is a dynamic physiological process, and so as well as studying the placenta in the first trimester when it is established, it is important to study the placenta in the third trimester, at the time when patients suffer the symptoms of pre-eclampsia and that the time of delivery. It is reasonable to assume that immunological changes in the placenta will take place as the systemic inflammation associated with the disease takes hold and blood pressure worsens. Similarly, in FGR, growth of the fetus may be small but linear throughout the third trimester, or may become static at any point, and in the extreme state for some fetuses, particularly before 32 week's gestation, altered blood flow in the Ductus Venosus warrants urgent delivery[82]. Experiments involving 3rd trimester placentas are therefore vital. As a clinician I have been uniquely placed to be able to intercept the many pathways through which patients are seen in hospital and follow their pregnancies until a decision to deliver is made, which may be in an emergency. Thus, in this study I have demonstrated that recruitment of participants and third trimester sample collection in the pre-eclampsia and FGR cohorts, whilst unpredictable, is feasible.

Strengths and Limitations

These findings demonstrate reliability in the novel method used to isolate T cells from decidua to provide reliable and reproducible results. However, this study was not able to reproduce the findings from other studies that showed a difference in T cell composition in peripheral blood between pre-eclampsia and healthy pregnancy, since it has previously been demonstrated that there is a significantly higher percentage of memory T cells compared with naïve T cells in the blood of pre-eclamptic women

compared with normal pregnancy. Additionally there was a wide variation in PD-1 levels within cohorts. Both of these limitations may be due to the effect of a relatively small sample size compared with previously published work. Indeed a bigger sample size may show that the difference in this study between naïve T cell compositions in control blood compared with blood from FGR pregnancies may not remain significant. The samples used from this study were mostly from late-onset PE and FGR and it is possible that a higher number of samples from early-onset disease may give rise to a different T cell phenotype.

We recognise that in the group's previous work on healthy pregnancies, placentas were deliberately collected from women who were delivering by elective CS. This is an important decision in patient recruitment, since none of the patients would have had rupture of the amniotic membrane, and therefore no ascending migration of vaginal bacteria or other flora to cause a local inflammatory reaction. However, in our cohorts, it was not possible to get an adequate sample size from restricting recruitment to women undergoing elective CS, since many women with PE or FGR will undergo induction of labour when a decision for early delivery is made due to these diseases. To determine the effect of ruptured membranes on T cell composition we compared T cell numbers in those who had normal delivery vs. those who had a CS without ruptured membranes and found no significant difference. Burton et al. identified that mode of delivery can alter gene expression in placental tissue, with activation of the stress response in labour causing subsequent up- and down-regulation of certain pathways, that may in turn affect immune responses, cell death and anti-oxidant mechanisms[83]. Although our initial analysis found no effect of mode of delivery on T

cell composition, the inevitable effect of ruptured membranes and the stress of uterine contractions in labour on the analysis of placental tissue should be considered.

## **FUTURE WORK** (Grant applications)

We have successfully demonstrated the feasibility of collecting and sampling placental tissue from cohorts affected by malplacentation. The preliminary data outlined above was used to support PhD grant applications to Wellbeing of Women, Action Medical Research, Medical Research Council and the British Heart Foundation, as outlined below.

### Hypothesis

T cell mediated maternal anti-fetal rejection (MAFR) can contribute to the development PE and FGR. Our group demonstrated previously that a maternal cellular immune response against the fetus is present in healthy pregnancies but is regulated by expression of inhibitory checkpoint proteins and T regulatory cells within the maternal decidua. We also showed that decidual CD4+ and CD8+ T cells in healthy pregnancy display a strong transcriptional response to IFN signalling.

### Aim

Investigate the features of T cell MAFR in PE and FGR and uncover potential immune-mediated pathways to guide development of predictive markers and targeted immunotherapy.

### Objectives

1. Interrogate the phenotypic and functional features of peripheral and decidual T cells in MAFR to identify potential immunopathological mechanisms of PE and FGR.
2. Determine the peptide specificity of maternal T cell recognition of fetal tissue.

3. Quantify type I IFN in healthy and pathological pregnancies and determine whether type I IFN signalling is dysregulated in pathological pregnancy.
4. Develop immune markers for the potential prediction or novel management of MAFR-mediated disorders.

Study Design

Participants will be recruited from Birmingham Women's Hospital. Eligibility criteria include:

- Over 18 years old
- Understands and speaks English
- PE as defined by the NICE: BP ≥140/90 and urine PCR ≥30mg/mmol >20 weeks gestation)[15]
- No sepsis or chorioamnionitis in labour
- FGR, defined as either an EFW or AC plotting below the 3rd centile or plotting below the 10th centile with evidence of placental dysfunction (abnormal uterine artery Doppler between 20-24 weeks' or abnormal umbilical artery Doppler) in a non-smoker and not secondary to an underlying congenital anomaly[20].

Written consent will be sought from all participants and written information about the study will be provided. Maternal blood will be collected during venous cannulation in labour or prior to CS. Once delivered, cord blood will be aspirated from the placental umbilical cord and all samples will subsequently be transferred to the University of Birmingham Cancer Sciences laboratory for the experiments outlined below.

Sample size

Total sample size of n=150, with n=50 (control), n=50 (PE), n=50 (FGR) is in line with a power rate of 0.80 and type I error (incorrect rejection of the null hypothesis) rate of 0.05. Statistical power is difficult to define with confidence, as the effect size of immune correlates on clinical phenotype is difficult to assess at this stage[12].

Methodology

*TASK 1: Characterise the phenotypic, functional and metabolic features of decidual maternal T cells in malplacentation pregnancies (Objectives 1 & 2: 24 months)*

a. Samples of maternal blood and decidua will be obtained from pregnancies affected by PE or FGR and compared to normal pregnancy matched for gestational age. Each cohort (normal, PE and FGR) will comprise extreme early-onset disease (delivery at 24-32 weeks; n=10), early-onset (32-36 weeks; n=10) and late-onset (36-42 weeks; n=10).

b. Mononuclear cells will be isolated from the samples by density centrifugation and detailed flow cytometric analysis using CyTOF technology (35-plex) will determine T cell memory subset distribution, degree of differentiation, ratio of T regulatory to T effector cells and expression of checkpoint proteins such as PD-1.

c. Cytokine production and cytotoxic phenotype of decidual and peripheral T cells will be measured by mitogenic stimulation followed by intracellular cytokine analysis using flow cytometry for inflammatory cytokines and those associated with T cells such as IFN-γ, TNF-α, IL-10 and IL-4, and intracellular staining of perforin and granzyme.

d. I will set up mixed lymphocyte reaction assays[2] (n=10) to determine if decidual T cells exhibit increased immune recognition of fetal tissue in women with

malplacentation syndromes, using decidual and peripheral blood effector T cells on co-culture with cord blood collected at the time of delivery. To investigate the potential importance of T regulatory cells in modulating fetal-specific responses in these disorders, these assays will be performed prior to, and following, depletion of the $CD25^{bright}$ T regulatory subset.

e. Fluorescent metabolic indicators will be used to study the metabolic capacity of effector CD4+ and CD8+ T cells using flow cytometry (n=10). Glucose uptake capacity of cells, mitochondrial mass, activity, oxygen consumption and lactate production will be measured. This will determine potential functional differences in these cells between pathological and control pregnancies.

f. I will determine the number of fetal-specific T cells in malplacentation syndromes using HLA-peptide tetramers specific for peptides from the Y chromosome to directly visualise fetal-specific CD4+ and CD8+ T cells (n=10). These analyses will determine the number of such 'alloreactive' T cells and their membrane phenotype with a focus on memory and differentiation status. The pattern of cytokine production by fetal reactive T cells within each group will also be assessed. A recent innovation is our exploitation of HLA class II MHC-peptide tetramers to identify antigen-specific CD4+ T cells.

*TASK 2: Transcriptional analysis of CD4+ and CD8+ decidual T cells (Objective 3: 6 months)*

a. Building on our recent transcriptional analysis of decidual effector cells in healthy pregnancy, I will perform RNA-sequencing (RNA-seq) on CD4+ and CD8+ T cells from matched maternal blood and decidual samples from patients with PE and FGR

compared with data from normal pregnancy matched for gestational age (n=36 i.e. n=6 per sample type per cohort). Flow cytometric cell sorting will be used to isolate CD4+ and CD8+ EM cells and extract RNA, prior to total RNA-Seq Pico labelling and a High Output NextSeq 500 Flow Cell pv2 array for transcriptome analysis.

*TASK 3: Investigate the role of retroelement transcription and IFN expression in decidual T cell regulation (Objective 3: 12 months)*

a. Taking advantage of our RNA-seq dataset, I propose to assess retroelement transcription in isolated CD4+ and CD8+ T cells from the different cohorts of placental tissue. Protein production will be assessed by Western blot analysis for syncytin proteins.

b. Single-molecule array (Simoa) digital ELISA technology will be used to record attomolar ($10^{-18}$) concentrations of IFN-α in decidua and blood of normal and diseased pregnancies. This is in collaboration with Professor Yanick Crow, a world leader in interferonopathies, whose team pioneered this technology. I aim to isolate plasma and supernatant samples from matched cord, maternal blood and decidual samples of normal, PET and FGR pregnancies (n=10 each) prior to transport and assay in Edinburgh.

*TASK 4: Develop a predictive assay for pathological MAFR (Objective 4: 36 months)*

Using these findings, I propose to develop a blood test that might correlate with and predict pathological MAFR and pave the way for development of targeted immunotherapy. Recent evidence has shown CXCL10 may be a strong correlate of MAFR, a finding that concurs with our previous observations in graft versus host disease[13].

Statistical Methods

Flow cytometry data will be analysed using Flowjo software and statistical analysis will be performed using a dedicated statistics package (known as “R”). Non-Gaussian distribution will be assumed for all samples. Wilcoxon matched-pairs signed rank test will be used to identify significant differences between matched samples within a cohort; samples from different cohorts will be compared using the Mann-Whitney test. Statistically significant results will have $p<0.05$.

For transcriptional analysis, volcano plots will be generated based upon individual probes log-fold change and p values associated between a) peripheral blood and decidua and b) decidua of healthy and pathological pregnancies (using post-hoc Bayesian analysis ($B\geq5$)). Genes with differential expression between these groups will be analysed by Database for Annotation, Visualisation & Integrated Discovery (DAVID), and up- and down-regulated pathways generated and corrected by Analysis of Variance (ANOVA) with control non-coding RNAs.

To assess retroelement transcription, the viGEN pipeline can be applied to quantify expression of viral genes for subsequent downstream differential expression analysis and to identify viral variants. Other existing tools could be applied to identify viral integration and mRNA fusions (ViFi) or to detect the presence of viral species in human tissue RNA-seq datasets (VirTect).

The laboratory team has two embedded bioinformaticians to support data analysis from the latest technology and excellent training and professional support is available to analyse all data that arise from the Fellowship.

Anticipated Barriers

1. *Preterm control samples:* obtaining control samples matched for preterm gestational age will be difficult, as preterm delivery is generally associated with inflammatory pathology. However, higher order pregnancies (triplets and quadruplets) are routinely delivered electively preterm and may be considered for controls, though it is recognised that multiple pregnancies are not strictly “normal” pregnancies.
2. *Missed samples:* Obstetric emergencies and precipitate labours have led to missed samples during preliminary data collection. To address this, I created “packs” in hospital notes containing instructions and equipment to aid sample collection. The research team at Birmingham Women’s Hospital has successfully facilitated collection of over 500 samples since 2015, so I am confident that a sufficient sample size will be reached.

Proposed Patient and Public Involvement (PPI)

PPI will confirm the most suitable time considered by patients to collect blood samples, develop patient information leaflets and determine the most valuable ways to disseminate the research findings. I will attend university-led workshops to ensure I incorporate PPI effectively.

# CONCLUSION

Human studies of placental disorders are crucial to improve our understanding of common and potentially devastating diseases such as PE and FGR. We have demonstrated the feasibility in collecting blood and decidual tissue from patients affected by these diseases at different gestations to investigate the role of T cells in these diseases, about which there is a lot more to learn. This preliminary work provides the basis for the methods of data collection and T cell isolation that we anticipate to use in future experiments. In completing these subsequent experiments, we aim to gain a thorough understanding of the phenotypical, functional and genetic differences of T cells in the decidua and peripheral blood of pregnancies affected by PE and FGR at different gestations. In doing so we endeavour to uncover potential therapeutic targets that could alter the course of these diseases in further studies.

# PROJECT 2

## Published article

**C**ause **O**f Intrauterine and Neonatal **D**eath **I**n **T**win Pregnancies (CoDiT): development of a novel classification system.

Gulati N, Mackie FL, Cox P, Marton T, Heazell AEP, Morris RK, Kilby MD.

BJOG 2020;127:1507–1515.

**Contribution to authorship**

NG, FLM, RKM, PC, MDK co-designed the classification system.

NG, FLM, PC, TM tested the classification system by assigning cause of death to cases in the cohort to assess inter-disciplinary agreement.

AEH independently assigned cause of death to a subset of cases to assess inter-rater agreement.

NG re-classified a subset of cases to determine intra-rater agreement.

All authors contributed to the write-up and approval of the final manuscript.

## ABSTRACT

Objective: Twin pregnancies have a significantly higher perinatal mortality than singleton pregnancies. Current classification systems for perinatal death lack twin-specific categories, potentially leading to loss of important information regarding cause of death. We introduce and test a classification system designed to assign a cause of death in twin pregnancies (CoDiT).

Design: Retrospective cross-sectional study.

Setting: Tertiary maternity unit in England with a perinatal pathology service.

Population: Twin pregnancies in the West Midlands affected by fetal or neonatal demise of one or both twins between 1 January 2005 and 31 December 2016 in which post-mortem examination was undertaken.

Methods: A multidisciplinary panel designed CoDiT by adapting the most appropriate elements of singleton classification systems. The system was tested by assigning cause of death in 265 fetal and neonatal deaths from 144 twin pregnancies. Cause of death was validated by another obstetrician blinded to the original classification.

Main outcome measures: Inter-rater, intra-rater, inter-disciplinary agreement and cause of death.

Results: Cohen's Kappa demonstrated 'strong' (>0.8) inter-rater, intra-rater and inter-disciplinary agreement (95% CI 0.70–0.91). The commonest cause of death irrespective of chorionicity was the placenta; twin-to-twin transfusion syndrome (TTTS) was the commonest placental cause in monochorionic twins and acute chorioamnionitis in dichorionic twins.

Conclusions: This novel classification system records causes of death in twin pregnancies from post-mortem reports with high inter-user agreement. We highlight differences in aetiology of death between monochorionic and dichorionic twins.

Tweetable abstract: New classification system for #twin cause of death ‘CoDiT’ shows high rater agreement.

Disclosure of interests: FLM is a BJOG editor. The remaining authors have no disclosures.

Details of ethical approval: The project was registered as a service evaluation project by the unit’s clinical governance team in 2017.

Funding: none

# INTRODUCTION

Twin pregnancies account for 0.5-2% of all pregnancies worldwide[1]. Compared with singleton pregnancies, they are associated with an increased risk of adverse maternal and perinatal outcomes, particularly prematurity and stillbirth[1]. In the UK the rate of stillbirth in twin pregnancies is 6.99 per 1,000 total births, and for neonatal deaths (NND), the rate is 5.45 per 1,000 live births[2]. This is compared with a stillbirth rate of 3.51 per 1,000 total births in singleton pregnancies and a neonatal death rate of 1.64 deaths per 1,000 live births[3]. Twin pregnancies account for 20% of all preterm births in the UK, many of which are less than 28 weeks' gestation[4,5]. Risks to the mother of carrying a twin pregnancy include anaemia, pre-eclampsia and postpartum haemorrhage.

## Embryology of twin pregnancy

Development of a twin pregnancy occurs in two main ways. Simultaneous fertilisation of two separate oocytes by two different sperm, forming two zygotes, each with their own unique combination of genetic material, leads to non-identical (dizygotic) twins, each with their own placenta and in their own amniotic sac (dichorionic, diamniotic (DCDA)). This accounts for approximately two-thirds of all twins. Alternatively, fertilisation of a single oocyte, which then splits in to two equal zygotes, forms identical (monozygotic) twins that carry the same genetic material. In this scenario, the timing of this split determines the chorionicity of the twin pregnancy. If cleavage occurs by day 3, before implantation, two separate blastocysts will form, and as a result there will be two separate sites of implantation and the result is a DCDA twin pregnancy in which the twins are genetically identical, which accounts for up to 30% of monozygotic twins.

However, for the majority of monozygotic twins, cleavage occurs after day 3, when the blastocyst has already formed resulting in a twin pregnancy with a single placenta (monochorionic pregnancy). If the cleavage occurs between day 4 and day 8, each twin will have their own amniotic sac resulting in a monochorionic, diamniotic (MCDA) twin pregnancy. However, a small number of monozygotic twins, approximately 2%, will cleave between day 8 and 13, when it is too late for two separate amniotic sacs to form for each fetus, leading to a single placenta and a single sac for both twins. This is called a monochorionic, monoamniotic (MCMA) twin pregnancy. In one in 90 000 to 100 000 pregnancies, blastocyst cleavage occurs after day 13, leading to incomplete division of an embryo, resulting in conjoined twins that by definition will be MCMA[6].

Twin pregnancy complications

As previously mentioned, twin pregnancies are at increased risk of complications common to singleton pregnancies, such as pre-eclampsia, fetal growth restriction, postpartum haemorrhage, as well as postnatal infant feeding problems and postnatal depression[6]. In addition, there is a risk of complications unique to twin pregnancies. In particular monochorionic placentas, which almost always have inter-twin vascular anastomoses that allow bi-directional blood flow between the two fetuses, can be affected by a selection of pathologies caused by an imbalance in the net inter-twin transfusion, as described below[6,7]:

1) Twin-to-twin transfusion syndrome (TTTS):

TTTS, in which predominantly unidirectional arteriovenous anastomoses lead to haemodynamic imbalance, affects up to 15% of monochorionic pregnancies[6]. As a result, the fetus from which the net flow of blood is moving away becomes the “donor”,

and the other twin consequently becomes the “recipient” of the imbalanced circulation. Hallmark antenatal ultrasound features assist with the diagnosis and for staging the severity of TTTS. The Quintero staging system classifies these sonographic features to indicate severity of the disease and help to provide prognostic value[8]. Significant discordance in fetal weight of >20% and amniotic fluid volumes are seen in Stage I of the disease, the latter of which represents an effect of the haemodynamic imbalance on fetal renal function causing a reduced urine output in the donor twin which leads to oligohydramnios (deepest vertical pool <2cm) and polyuria in the recipient causing polyhydramnios (>8cm before 20 weeks or >10cm after 20 weeks of gestation). In Stage II, the bladder is absent in the donor, indicating anuria in that twin. This is followed by measurable evidence of haemodynamic compromise through abnormal arterial and venous Dopplers in Stage III. In Stage IV there is evidence of fluid overload and cardiac failure such as ascites, pericardial or pleural effusions, scalp oedema, or hydrops, which can develop in either twin[8]. Stage V of TTTS is classified as death of one or both babies.

Fetoscopic laser ablation (FLA) of the arterio-venous anastomoses to separate the fetal circulations has improved fetal and neonatal outcomes for this otherwise highly morbid condition, which until the introduction of FLA 20 years ago, was symptomatically treated by amnioreduction or septostomy[9]. A Cochrane review of interventions to treat TTTS found that more babies were alive without neurological abnormality at the age of six years after FLA compared with amnioreduction and concluded that FLA should be considered in the treatment of all stages of TTTS to improve neurodevelopmental outcomes[10]. Selective laser ablation through a sequential approach, whereby the anastomoses are first mapped and coagulated

sequentially, first arteriovenous, then veno-arterial, then arterio-arterial, has become the gold-standard technique and is associated with improved perinatal survival of both twins and less fetal demise than other approaches[11].

The Royal College of Obstetricians and Gynaecologists (RCOG) recommends 2-weekly ultrasound examinations for monochorionic twins between 16 and 26 weeks' gestation to monitor for signs of TTTS to allow timely referral to a fetal medicine centre for review and treatment. After 26 weeks' development of TTTS is rare but can occur in addition to selective growth restriction (see below) and so 2-weekly ultrasound scans are also recommended after 26 weeks [6].

2) Twin Anaemia-Polycythaemia Sequence (TAPS)

Another manifestation of chronic fetofetal transfusion due to inter-twin placental vascular anastomoses, TAPS is characterised by a significant discordance in haemoglobin levels between twins without the discordant amniotic fluid volumes seen in TTTS. TAPS affects 13% of monochorionic twins post TTTS but can occur spontaneously in 2%[6]. Antenatally the discordant haemoglobin levels are diagnosed by an increased MCA-PSV in the donor twin, indicating fetal anaemia (>1.5 MoM) and a reduced MCA-PSV in the recipient twin (<1.0 MoM), indicating polycythaemia[6,12].

The development of TAPS occurs due to the presence of micro-vessels (<1mm diameter) on the placental surface that form tiny arterio-venous anastomoses which allow the slow transfusion of blood from the donor to the recipient, creating discordant haemoglobin levels of ≥80g/L without the oligo-polycythaemia sequence seen in TTTS. These vessels can be seen on postnatal placental injection studies, which are

crucial to differentiating TAPS from acute transfusional events, which involve larger superficial low-resistance anastomoses[13].

The Solomon technique for FLA aims to minimise development of TAPS by coagulating the superficial micro-vessels between the ablated anastomotic sites on the chorionic plate following sequential selective laser occlusion, to create two distinct vascular territories[12].

3) Selective growth restriction (sGR)

Unequal placental sharing, inter-twin placental anastomoses and abnormal cord insertions can give rise to selective growth restriction, in which there is a significant discordance in the estimated fetal weight of >20% on ultrasound scan. This complication can be seen in 15% of monochorionic twins in the absence of TTTS and in over half of those affected by TTTS. In isolated sGR without TTTS there will typically be oligohydramnios in the smaller twin and a normal liquor volume in the bigger twin. Fortnightly umbilical artery Dopplers measurements taken from 20 weeks' gestation provide a prognosis for monochorionic pregnancies affected by sGR and is described as 3 types. In Type I sGR there is discordant growth but positive umbilical artery end diastolic flow (EDF) and planned delivery between 34-36 weeks' gestation is associated with a >90% perinatal survival As the EDF becomes absent or reversed in one or both twins in Type II sGR, prognosis worsens with a 29% risk of intrauterine demise of the growth-restricted twin and/or preterm delivery. Type III sGR, in which there is a cyclical umbilical artery diastolic waveform with positive followed by absent then reversed end-diastolic flow over several minutes, is associated with a 10–20% risk of unexpected in-utero death of the smaller twin and a subsequent 10–20% risk of

neurological injury in the larger twin. As a result planned delivery by 32 weeks' gestation for Type II and II sGR should be considered.

4) Twin reversed arterial perfusion sequence (TRAP)

A rare complication affecting 1% of monochorionic pregnancies, TRAP is characterised by the presence arterio-arterial anastomoses causing reversed perfusion of blood from a normally formed donor pump twin to an acardiac recipient twin. It is thought that the acardiac twin develops as a direct result of the abnormal anastomoses leading to early tissue hypoxia and subsequent atrophy of the heart and other organs, most commonly cranial and thoracic structures, known as the acardiac acephalus phenotype[14]. This hyperdynamic circulation can lead to high output cardiac failure, hydrops, polyhydramnios and preterm delivery for the pump twin[15,16]. In one-third of TRAP pregnancies, death of the pump twin occurs by 18 weeks' gestation[17]. Intervention through selective termination of the acardiac twin aims to unburden the pump twin, particularly if there are signs of growth discordance with a large acardiac twin or evidence of cardiac strain in the pump twin[6,16]. Methods for selective reduction in these cases include cord coagulation or ligation, photocoagulation of the anastomoses or intrafetal methods such as radiofrequency ablation. Though the optimal method and timing for intervention is yet to be established, a 2013 retrospective cohort study showed that conservative management of TRAP led to intrauterine death of all cases by 17 weeks', compared with intrafetal laser therapy, which was associated with an 82% survival rate for the pump twin and found that adverse pregnancy outcomes such as preterm prelabour rupture of membranes (PPROM) and chorioamnionitis, were lower when laser therapy was done before 16 weeks' gestation[16].

Perinatal Mortality Classification Systems

Classification systems to standardise causes of perinatal death are crucial to reducing perinatal mortality rates[18]. They provide a means by which causes can be accurately recorded to enable analysis that in turn can impact clinical care, facilitate comparisons within and between countries and inform public health policies and research[19,20]. A 2016 systematic review identified that 81 new or modified classification systems had been used between 2009 and 2014 to record causes of perinatal death[21.] In spite of this, none of them provide an adequate platform to record causes of perinatal death in twin pregnancies, since they do not acknowledge the unique risks of multiple pregnancies[20]. At best, TTTS is the only twin-specific cause of death acknowledged, as in ReCoDe (Relevant Condition at Death), or alternatively multiple pregnancy is identified as an associated cause of perinatal death as in Codac (Cause of death and Associated Conditions)[19,20,22]. A globally accepted classification system is crucially important, since a greater proportion of perinatal deaths occur in developing countries and yet most research into perinatal deaths occur in developed countries. The World Health Organisation (WHO) 10$^{th}$ Revision of the International Classification of Disease (ICD-10) is currently the only global perinatal death classification system and yet not only fails to adequately account for twin-specific causes of death, it falls short of allowing users to sufficiently classify causes of in-utero demise that are specific to pathologies during fetal life[19,23].

Recognising the need for a standardised system that would sufficiently capture the underlying cause of death as well as enable users to capture important additional information, a Delphi consensus study was undertaken by a group of international experts in perinatal death classification, to agree on the most important characteristics

required for a globally-acceptable classification system[23].They identified 10 structural and 7 functional characteristics that were crucial (Table 1).

| **Structural Characteristics** | **Functional Characteristics** |
|---|---|
| Accommodates both stillbirths and neonatal deaths. | Shows high inter- and intra-rater reliability. |
| Distinguishes antepartum from intrapartum conditions. | Has clear guidelines for use and definitions for all terms used. |
| Requires neonatal deaths to be clearly distinguished from stillbirths. | Is easy to use. |
| Requires the single most important factor leading to the death to be recorded. | Produces data that are easily understood and valued by users. |
| Allows associated factors to be recorded and clearly distinguished from causes of death. | Allows easy access to the data by the end-users. |
| Has a small number of main categories. | Is available in different formats including inexpensive ehealth and mhealth options, and in multiple languages. |
| Is multi-layered, to accommodate varying levels of available information, in particular the low levels of data available in may LMIC settings. | Uses rules to ensure valid assignment of cause of death categories. |

| | |
|---|---|
| Includes a sufficiently comprehensive list of categories to minimise the proportion of deaths classified as “other”. | |
| Ensures cause of death categories are relevant in all settings. | |
| Includes the level of data available to assign the cause of death (e.g. verbal autopsy only, placental histology, autopsy etc.). | |

**Table 1:** The 17 most important characteristics required for a globally-acceptable perinatal death classification system according to a 2016 Delphi consensus study[23].

Assessment of the 81 pre-existing classification systems against these characteristics showed that 82% were aligned with less than 24% (4/17) of these characteristics, and even the most aligned system, Codac, was only aligned with 9/17 of these characteristics[24]. However, it is recognised that development of a globally-accepted system should draw on the strengths of the most aligned systems[20,25].

It has also been recognised that in order to be a useful tool for improving perinatal outcomes, a classification system should be acceptable and valuable to different disciplines, not only obstetricians, but also perinatal pathologists, neonatologists, researchers and epidemiologists, to facilitate a holistic improvement in services[19].

Subsequently there is a need for a globally-accepted perinatal classification system that captures the distinctive pathologies of multiple pregnancies, in sufficient detail, so that causes of death in this high risk group can be effectively recorded.

The aim of this project was to devise a classification system specifically to assign the underlying cause of perinatal death in twin pregnancies and then test the global usability of the system based on the level of inter-rater, intra-rater and inter-disciplinary agreement.

# METHODS

## Developing the classification system

### 1) The categories

A multidisciplinary panel of four obstetricians and two perinatal pathologists was drawn to devise an original classification system for assigning cause of perinatal death in twin pregnancies. It was important that the system should fulfil as many of the structural and functional characteristics identified in the Delphi consensus study as essential for a globally-acceptable classification system[23]. The current classification systems that are most aligned with these characteristics are Codac, which aligns with 9 out of 17 characteristics and Tulip, which aligns with 7 characteristics[19,23,26]. Consequently these systems were used as a framework for our original classification system, known from herein as CoDiT (Cause of Death in Twins) (Appendix 1).

The main categories of the Tulip classification system adequately cover most of the broader categories of underlying aetiologies for perinatal death in twins as well as singletons, and as such feature as some of the main categories for cause of death in CoDiT. These categories are:

1. Congenital abnormality
2. Placenta
3. Prematurity
4. Infection
5. Other
6. Unknown

Unlike Tulip, Codac recognises umbilical cord events as a main category for cause of death, which is an important finding in MCMA twin pregnancies and so this cause also features as a main category in CoDiT.

Each of the 7 main categories in CoDiT has subcategories, which the panel agreed reflects the range of underlying causes of death that affect twin pregnancies, many of which are common to singleton pregnancies, but importantly, and unique to CoDiT, there are crucial subcategories which do not feature in current classification systems to adequately assign cause of perinatal death in a twin pregnancy. For example, in congenital abnormality, the first main category, conjoined twins and TRAP feature as subcategories to allow a more detailed assignment of cause of death. Furthermore, CoDiT allows the user to distinguish between the pump twin and the acardiac twin within the TRAP subcategory. In the second main category, placenta, TTTS has been added as a subcategory, with distinction between acute and chronic disease, antepartum or intrapartum TTTS, and whether death due to TTTS was in spite of treatment or without treatment. By adding a clinical dimension to an otherwise pathology-based aetiology, CoDiT provides a deeper understanding to the underlying cause of death by allowing important additional information to be captured whilst still ensuring that only a single cause of death can be recorded, thus fulfilling 2 of the important structural characteristics for a globally-acceptable classification system (Table 1). Users can also record important descriptive information about the placenta within the placental section of CoDiT itself, which is distinguishable from the cause of death but enable a more holistic analysis of the post-mortem findings. In the unknown category, the panel agreed that it was important to be able to distinguish between the cause of death being unknown “despite thorough investigation” or unknown due to

“important information missing”. As a result, these feature as subcategories and it was anticipated that this would provide a valuable insight in to why cause of death cannot be ascertained in certain cases.

2) Additional information

Recognising that CoDiT was being developed as a tool to facilitate research in to perinatal mortality in twins, the panel devised a section to record important maternal demographic information such as age, ethnicity, parity, BMI, past medial and obstetric history. Basic relevant background information about the current pregnancy can also be recorded on the system such as chorionicity and amnionicity (according to ultrasound scan) and whether the conception was spontaneous or assisted.

Since the basic principle of a classification system is to identify the underlying aetiology for perinatal death, it was crucial that CoDiT should distinguish between the cause of death and mode of death. The classification system is therefore prefaced with a section that prompts users to record mode of death through multiple choice options i.e. spontaneous death, termination of the whole pregnancy or selective reduction of one fetus, or even whether the death(s) occurred within a week of intrauterine intervention (such as FLA). Other important information recordable includes the gestation at death (or age at death if neonatal death) and gestation at delivery, to enable those analysing data to account for significant delays in delivery after intrauterine death. Birth weight and post-mortem weight can be documented, as can whether post-mortem chromosome testing was done, and if so, a free-text option for the level of testing is available.

3) User guidelines

To facilitate reproducibility of the classifications and standardise the use of a system, a set of user guidelines to accompany a classification system is identified as an important functional characteristic of a globally-acceptable classification system[23]. As a result, a set of guidelines was agreed containing explanations of the categories and subcategories most likely to be open to misinterpretation (Appendix 1).

Testing CoDiT

Once the content of CoDiT and the final version of the classification system and the accompanying set of guidelines had been agreed by the panel, the system was tested using post-mortem reports obtained from a West Midlands perinatal pathology unit within a tertiary maternity hospital. Fetal and neonatal post-mortems from twin pregnancies conducted between 1st January 2005 and 31st December 2016 from a pre-existing database of all post-mortems conducted at the unit were identified. All post-mortems had been conducted with parental consent and were performed by a specialist perinatal pathologist. Each twin pregnancy in the dataset was given a case number followed by the letter “A” for twin 1 and “B” for twin 2.

These post-mortem reports acted as the main source of information with which users would test CoDiT. Users to test the system comprised of 2 obstetricians and 2 perinatal pathologists. The standardised format of the post-mortem reports begins with a summary of maternal demographic information, gestation and the clinical history. This is followed by a summary of the main post-mortem findings, external examination comments and then a detailed description of the macroscopic and appearances of each external and internal organ. After this there is a detailed description of the placenta, including chorionicity, vascular territories, and, if injection studies were done,

there is a description of the anastomoses seen. Injection studies were performed only if the pathologist suspected TTTS, either from the clinical history given or if there was a significance discordance in body or organ weight between the twins. Histological findings of the all the organs and the placenta then follow as well as other relevant tests such as imaging, microbiology and chromosome testing.

Each post-mortem report in the cohort was examined independently by the obstetricians and the perinatal pathologists to assign a cause of death on to CoDiT and results were then use to determine inter-disciplinary agreement (Figure1). After independent classification, consensus on the cause of death in each case was drawn among users in order to enable to data to be analysed.

In order to determine the level of inter-rater agreement, an external obstetrician, blinded to the original classification, independently classified one-third of cases that were randomly selected using Microsoft Excel's random number generator function. To calculate intra-rater agreement, one of the original obstetricians to assign cause of death to each case, re-classified the same randomly selected one-third after a 2-year interval.

Cohen's Kappa was used through a hand-programmed matrix in Microsoft Excel to determine the level of inter-rater, intra-rater and inter-disciplinary agreement. A Kappa value of <0.4 suggested minimal agreement, 0.4-0.59 was weak agreement, 0.6-0.79 was moderate agreement, 0.8-0.9 strong agreement, and >0.9 suggested almost perfect agreement[27].

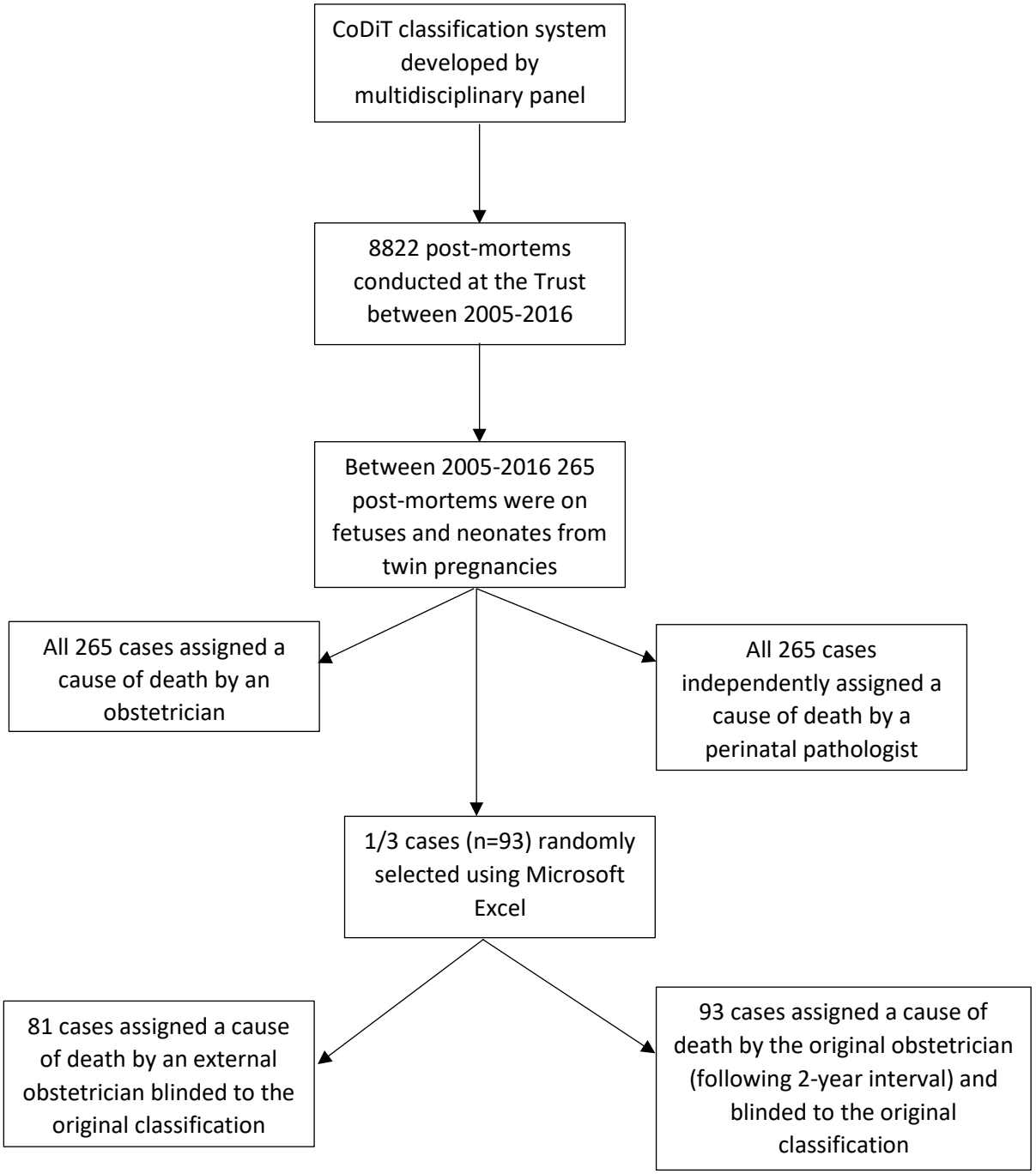



**Figure 1:** Flowchart demonstrating method of case identification following development of CoDiT classification and assignment of cause of death.

# RESULTS

## General Findings

Between 1st January 2005 and 31st December 2016, 265 fetal and neonatal post-mortems from 144 twin pregnancies were performed. These cases were referred from 14 hospitals throughout the West Midlands. Table 2 summarises the maternal demographic and descriptive data. Forty-six percent of deaths occurred in monochorionic twins (122/265 post-mortems). In 18% of cases (47/265) chorionicity could not be assigned at post-mortem as the placenta had either not been submitted for examination or was too fragmented or had been incorrectly fixed and thus unsuitable for assessment. Most demises were in-utero (miscarriages and stillbirths); 7% were NND (18/265), of which 61% (11/18) were dichorionic pregnancies. Most deaths, irrespective of chorionicity, were double-twin deaths (246/265; 93%); a greater proportion of single twin deaths occurred in dichorionic pregnancies compared to monochorionic pregnancies (8% vs. 5%). Mean gestation at death was 19-20 weeks (range 8-37 weeks). Most deaths were spontaneous (219/265; 83%); selective reduction and death within a week of medical intervention solely affected monochorionic pregnancies (9% of monochorionic deaths (11/122).

Irrespective of chorionicity, the most commonly assigned main category for cause of death was “placental” (117/265) (Figure 2), followed by “unknown” (65/265), of which 80% (52/65) were unknown “despite thorough post-mortem investigation”. The remaining 20% (13/65) were subcategorised as unknown due to “important information missing”, most commonly referring to the placenta not being submitted.

| | Monochorionic | Dichorionic | Total |
|---|---|---|---|
| **No. pregnancies** | 66 | 78 | 144 |
| **Mean maternal age** | 29.1 (95% CI 27.4-30.7) | 30.2 (95% CI 28.2 - 32.1) | 29.6 (95% CI 28.5-30.7) |
| **Mean maternal BMI** | 27.7 (95% CI 25.6-29.8) | 26.3 (95% CI 24.4-28.3) | 26.7 (95% CI 25.4-28.1) |
| **% Nulliparity** | 31/66 (47%) | 32/78 (41%) | 73/144 (51%) |
| **Total perinatal deaths** | 122 | 96 | 265 (unknown chorionicity in 47) |
| **No. fetal deaths** | 117/122 (96%) | 85/96 (89%) | 247/265 (93%) |
| **No. NNDs** | 5/122 (4%) | 11/96 (11%) | 18/265 (7%) |
| **No. Single deaths** | 6/122 (5%) | 8/96 (8%) | 19/265 (7%) |
| **No. Double deaths** | 116/122 (95%) | 88/96 (92%) | 246/265 (93%) |
| **Mean gestation at death (weeks)** | 19.6 (95% CI 18.3-20.8) | 19.7 (95% CI 18.5-21.0) | 19.6 (95% CI 18.8 - 20.5) |
| **No. spontaneous deaths** | 95/122 (78%) | 85/96 (89%) | 219/265 (83%) |
| **No. terminations of whole pregnancy** | 9/66 (14%) | 6/78 (8%) | 17/144 (6%) |
| **No. selective reductions** | 4/122 (3%) | 0 | 4/265 (2%) |
| **No. deaths within 7 days of medical intervention** | 7/122 (6%) | 0 | 9/265 (3%) |

**Table 2:** Demographic information of all post-mortem reports from twin pregnancies between Jan 2005-Dec 2016 at a West Midlands Tertiary Hospital. The chorionicity of 47 cases was unknown and so the total does not reflect the sum of monochorionic and dichorionic deaths.

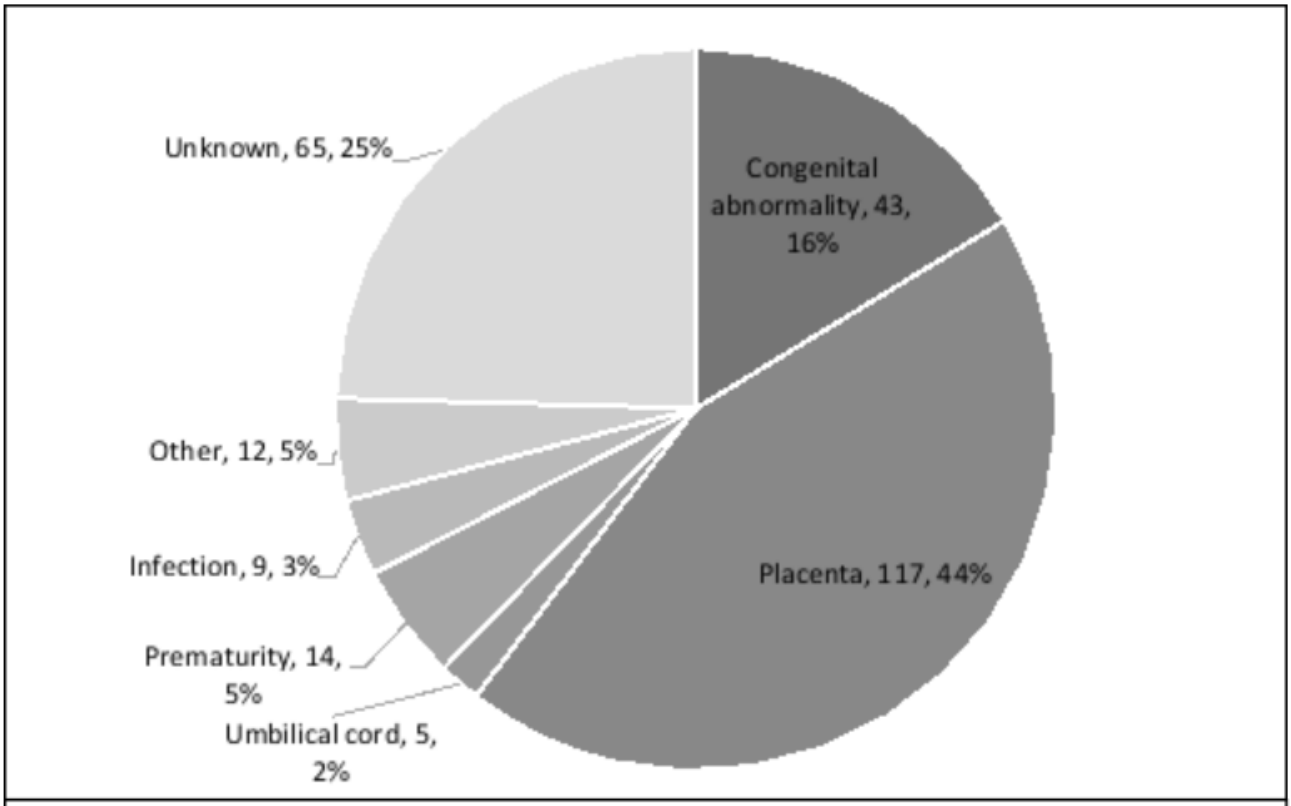



**Figure 2: Distribution of causes of perinatal death in twin pregnancies between 2005 and 2016 in the West Midlands irrespective of chorionicity.**

Inter-rater, intra-rater and inter-disciplinary agreement

The Kappa coefficient for the main cause of death was “strong” for all measured combinations of agreement; inter-rater 0.80 (95% CI 0.70-0.91), intra-rater 0.80 (95% CI 0.71-0.90) and an inter-disciplinary agreement 0.81 (95% CI 0.75-0.87). This indicates that the main cause of death was consistently and reproducibly categorised by different users irrespective of specialty.

Agreement was “minimal” and “weak” when all 51 subcategories were considered, with a Kappa co-efficient of 0.39 (95% CI 0.27-0.51) for inter-rater agreement, 0.33 (95% CI 0.22-0.43) for intra-rater, and 0.4 (95% CI 0.34-0.47) for inter-disciplinary

agreement. This may reflect the large number of subcategories. Nevertheless, percentage agreement in the main and sub- categories for all combinations of users was high; 86% (70/81) inter-rater main category agreement and 83% (67/81) subcategory agreement, 86% (80/93) intra-rater main category agreement and 76% (71/93) subcategory agreement and 86% (228/265) inter-disciplinary main category agreement and 82% (216/265) subcategory agreement.

The commonest subcategory disagreement was acute chorioamnionitis versus ascending infection, representing 29% (4/14) of inter-rater, 18% (4/22) of intra-rater and 16% (8/49) of interdisciplinary disagreements. Although user guidance states that ascending infection should only be assigned when there is proven colonisation from organisms present in the birth canal, users argued that acute chorioamnionitis often derives from ascending infection.

Cause of death by chorionicity

Cause of death by chorionicity and amnionicity are summarised in Figure 3. A Chi-squared test demonstrated that cause of death was significantly different between monochorionic and dichorionic twins ($p<0.01$). Specifically, significantly more monochorionic twins died of a congenital abnormality ($p<0.05$) or umbilical cord events ($p<0.05$) than dichorionic twins and significantly more dichorionic twins died of infection compared with monochorionic twins ($p<0.001$).

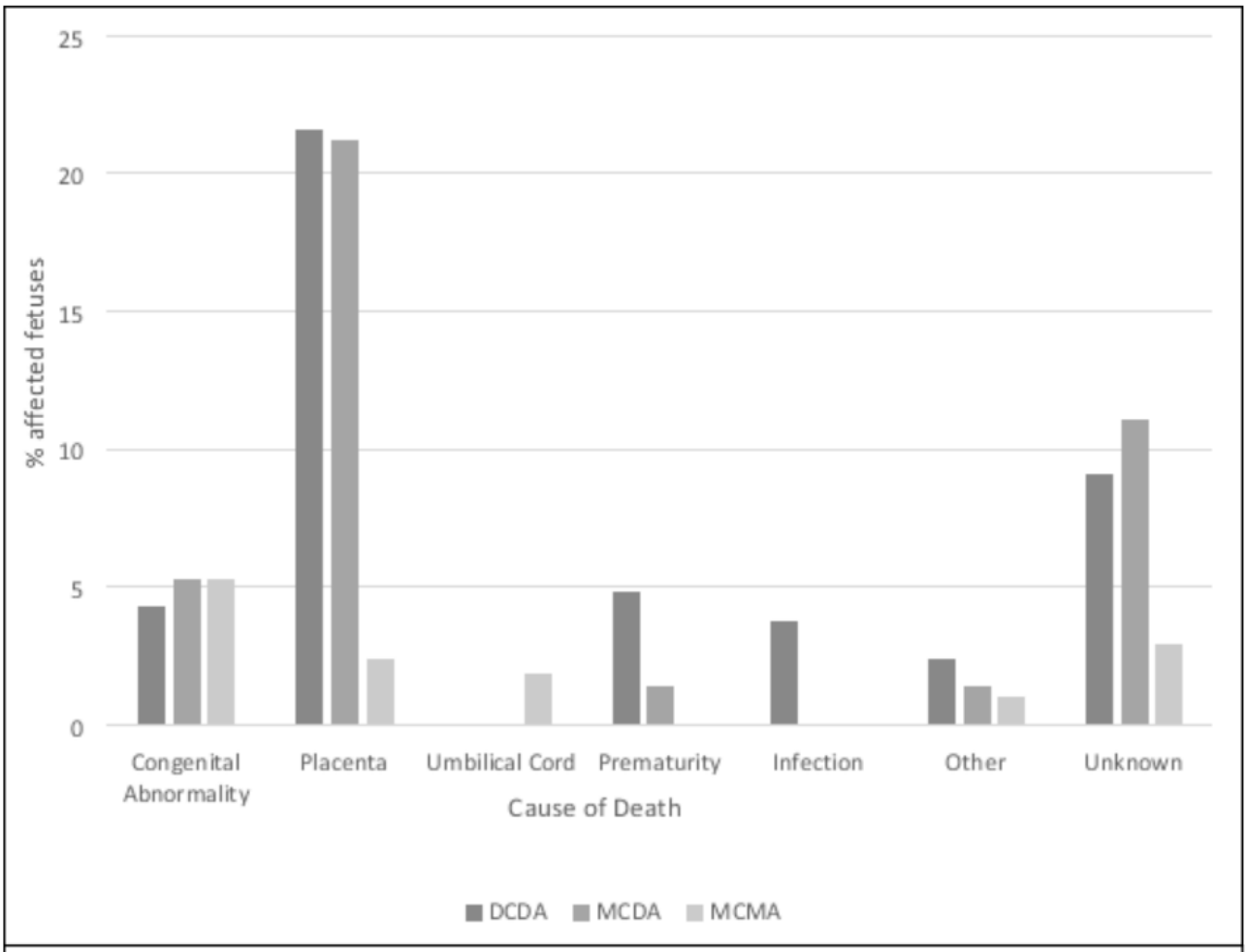



**Figure 3: Distribution of causes of death by chorionicity and amnionicity, as a percentage of the total number of fetuses about which chorionicity was known (n=208).**
**DCDA = dichorionic diamniotic; MCDA = monochorionic diamniotic;**
**MCMA = monochorionic monoamniotic**

Dichorionic twins

Placental causes represented 47% (45/96) of deaths in dichorionic pregnancies, with acute chorioamnionitis being the commonest subcategory (40/45; 89%). An "unknown" cause of death was the second commonest classification in dichorionic twins (19/96; 20%), all "despite thorough investigation", except one, classified as unknown due to important information missing because parental consent was for a limited post-mortem.

Prematurity, the third commonest cause of death in dichorionic twins (10/96; 10%), was secondary to PPROM in 6/10 cases, spontaneous preterm labour in 3/10, and complications of prematurity in one 22-day old neonate, born at 31 weeks' gestation. The 8 deaths due to infection (8/96; 8%) were double twin deaths from 4 pregnancies, 3 of which were ascending infections and 1 transplacental infection. Congenital abnormalities affected 8 fetuses, of which 2 were co-twins affected by lethal urogenital anomalies. All but one of the dichorionic pregnancies affected by congenital abnormalities resulted in death of the co-twin within two weeks of death of the abnormal twin. The 5 deaths categorised as "other" were from 3 dichorionic pregnancies; 2 sets of twins that died secondary to maternal diseases, and a NND at 17 minutes of age at 25 weeks gestation due to fetal trauma at birth.

Monochorionic twins

Placental causes of death were most common in monochorionic twins (53/122; 43%); here TTTS was the commonest subcategory (36/53; 68%). In total, 51 deaths were categorised as TTTS but in 15/51 of these cases (29%), the placenta was not submitted or too fragmented to confirm chorionicity, and TTTS was diagnosed from supporting information using the clinical history and ultrasound findings provided by the referring hospital and examining the fetuses. Most TTTS deaths were chronic and untreated (40/51; 78%). Only 20 of the 36 cases of TTTS with a placenta sent to pathology underwent injection studies, representing 39% (20/51) of the total number of fetuses categorised as TTTS. Of the 4 fetal deaths secondary to chronic treated TTTS, one set of twins died at $27^{+2}$ weeks' gestation following amnioreduction, 1 fetus died spontaneously more than 7 days after FLA, with survival of its co-twin, and the other died within 4 days of FLA with subsequent death of its co-twin due to acute

TTTS. In 47% (24/51) of TTTS cases, clinical information from the referring hospital provided no information about possible evidence of TTTS antenatally and as such TTTS was diagnosed solely based on post-mortem findings. For 13 of these 24 cases diagnosed with TTTS solely at post-mortem, the diagnosis was made without a suitable placenta to examine and was based on features noted on fetal examination such as body or organ weight discordance. Of the 20 cases in which injection studies were performed, TTTS was diagnosed solely on the basis of injection studies in 20% (n=4).

An “unknown” cause of death was the second commonest category in monochorionic twins (29/122; 24%), all of which were categorised as such “despite thorough investigation”. A third of these “unknown” cases underwent injection studies (10/29; 34%), all demonstrating a balanced circulation.

The third commonest cause was congenital abnormalities, representing 23% of monochorionic twin deaths (28/122), of which TRAP (acardiac and pump) was the commonest abnormality (13/28; 46%). There were 28 MCMA pregnancies, representing 23% of the monochorionic pregnancies in this cohort with a gestational age at death ranging from 8 to 28 weeks’ gestation. The commonest cause of death in MCMA twins was congenital abnormalities (11/28), of which almost two-thirds were twin-specific (7/11) (i.e. TRAP or conjoined twins). All deaths due to umbilical cord events (4/122; 3%) occurred in MCMA pregnancies.

Cause of death by double and single-twin demise

In monochorionic pregnancies, 95% were double twin deaths (116/122), with 44% (51/116) classified as placental, 24% (28/116) due to congenital abnormalities, 22%

(25/116) as unknown, and the remaining due umbilical cord events (n=4), prematurity (n=3) and “other” (n=5). In TTTS, 98% of deaths were double twin deaths (50/51). In dichorionic pregnancies, 92% of deaths were double twin deaths (88/96), with 50% (44/88) classified as placental and 15% (13/88) as unknown. All deaths due to prematurity, infection, “other” causes and congenital abnormalities in dichorionic twins were double-twin deaths.

Of the 19 single-twin deaths, 58% had an unknown cause of death (11/19), all of which were spontaneous fetal losses with a mean gestational age at death of 23 weeks (range 12-$20^{+3}$ weeks’ gestation). Four of these single-twin deaths with an unknown cause were from monochorionic pregnancies, six from dichorionic pregnancies and one did not have a placenta submitted to confirm chorionicity.

Gestation at death and delivery

Table 3 outlines the causes of death by gestational age and chorionicity. The most common gestation for death irrespective of chorionicity was before 24 weeks’ gestation (129/218; 59%), representing 60% of deaths in monochorionic (73/122) and 58% of deaths in dichorionic twins (56/96). In both chorionicities, the most common cause of death before 24 weeks was placental, affecting 41% of monochorionic twins (30/73) that died before 24 weeks and 43% of dichorionic twins (24/56).

In 59% of cases, data for gestation at death and delivery was provided, allowing calculation of the interval (Figure 4). Overall, 11% of fetuses delivered more than 4 weeks after death (n=17). In most of these, cause of death was unknown (65%; n=11) and 76% were <24 weeks’ gestation (13/17). Of the 4 deaths >24 weeks’ gestation that delivered more than 4 weeks after death, three were single intrauterine deaths in

dichorionic pregnancies, and were delivered with the surviving co-twin between $34^{+0}$ and $40^{+2}$ weeks, and one was a monochorionic twin that died at 24 weeks' gestation with termination of the co-twin at 32 weeks, when both twins were delivered together.

| | <24 weeks gestation | | >24 weeks gestation | | Neonatal death | | Unknown gestation at death | |
|---|---|---|---|---|---|---|---|---|
| **Cause of Death** | **MC n (%)** | **DC n (%)** | **MC n (%)** | **DC n (%)** | **MC n (%)** | **DC n (%)** | **MC n (%)** | **DC n (%)** |
| **Congenital Abnormality** | 23 (32%) | 5 (9%) | 1 (5%) | 1 (11%) | 1 (20%) | 1 (9%) | 3 (13%) | 2 (10%) |
| **Placental** | 30 (41%) | 24 (43%) | 9 (43%) | 4 (44%) | 2 (40%) | 4 (36%) | 12 (52%) | 13 (65%) |
| **Umbilical cord** | 3 (4%) | 0 (0%) | 1 (5%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| **Prematurity** | 0 (0%) | 4 (7%) | 0 (0%) | 0 (0%) | 1 (20%) | 5 (45%) | 2 (9%) | 1 (5%) |
| **Infection** | 0 (0%) | 8 (14%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| **Other** | 4 (5%) | 2 (4%) | 1 (5%) | 2 (22%) | 0 (0%) | 1 (9%) | 0 (0%) | 0 (0%) |
| **Unknown** | 13 (18%) | 13 (23%) | 9 (43%) | 2 (22%) | 1 (20%) | 0 (0%) | 6 (26%) | 4 (20%) |
| **Total** | **73** | **56** | **21** | **9** | **5** | **11** | **23** | **20** |

**Table 3:** Distribution of gestational age at death by cause and chorionicity with numbers and percentage within each group.

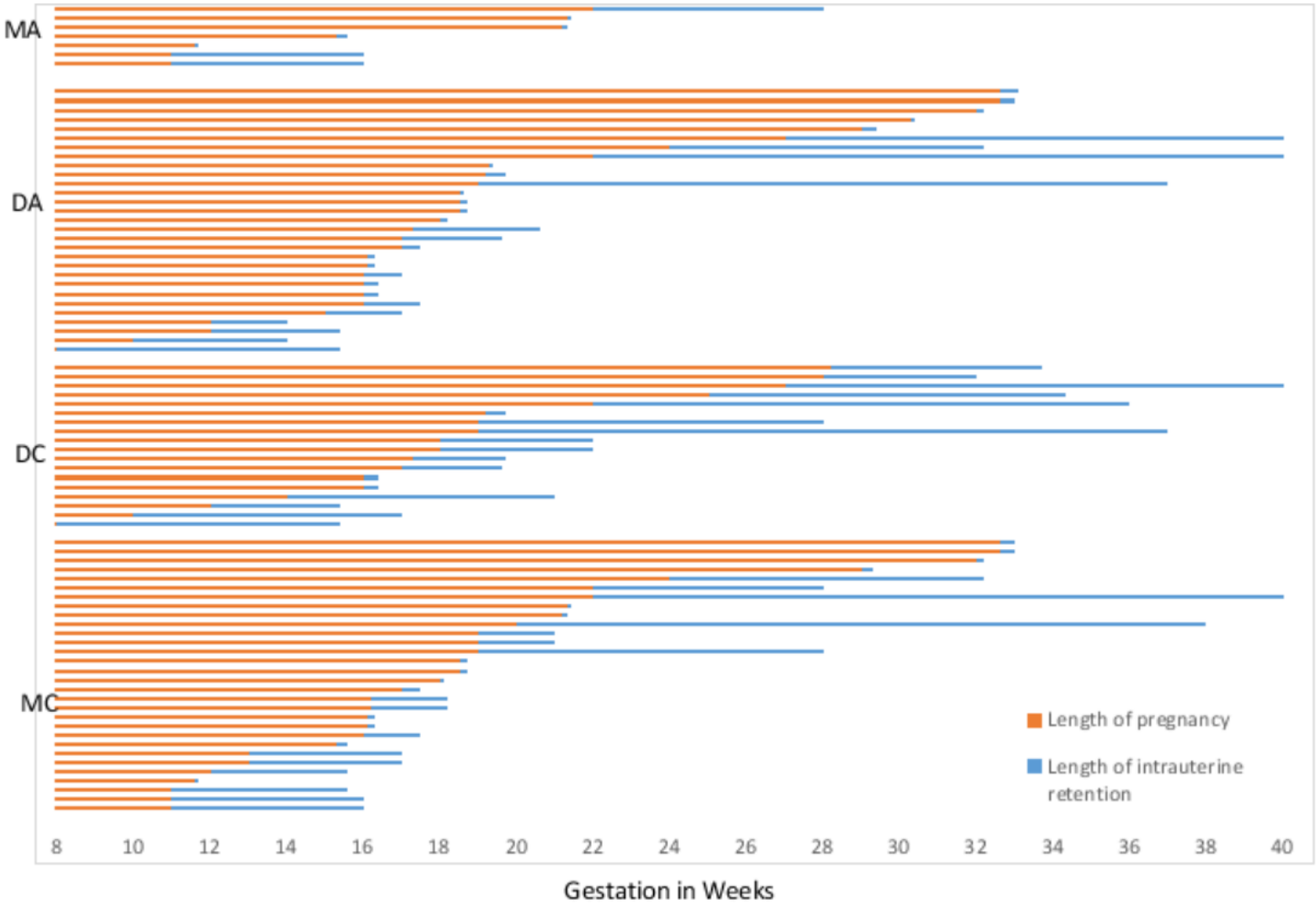



Figure 4: Gestation at death and gestation at delivery demonstrating length of retention in-utero, by chorionicity and amnionicity.

# DISCUSSION

## Main Findings

CoDiT is a classification system with high inter- and intra-rater reliability designed specifically for twin pregnancies using post-mortem reports as the primary source of information. Initial testing of CoDiT demonstrates high inter-and intra-rater agreement for main cause of death and provides important insight into causes of death in twins. The commonest cause of death overall was placental conditions, with acute chorioamnionitis the commonest subcategory in dichorionic twins, and TTTS in monochorionic twins. Most deaths were double demises and irrespective of chorionicity, most deaths occurred before 24 weeks' gestation. Delivery more than 4 weeks after death was associated with increased likelihood of the cause of death being unknown.

## Strengths and Limitations

Using post-mortems as the source of information to classify cause of death may have introduced case selection bias, since parental consent is required to conduct a post-mortem examination. Therefore, the cohort may include disproportionately fewer terminated pregnancies due to a known abnormality, fewer single deaths with prolonged in utero retention or perhaps a higher number of spontaneous deaths without an obvious antenatal cause.

In the 2017 UK Perinatal Mortality Surveillance Report, only 50% of parents of stillborn babies and 28% of parents affected by NND consented to post-mortem[28]. Restricting the use of CoDiT to cases in which post-mortem was conducted limits its general usability, especially as a tool for global use, since facilities for perinatal post-mortem

examination are extremely limited in developing countries. As such, most pre-existing classification systems rely on clinical information to determine cause of death. Furthermore, even within developed countries, the quality of perinatal post-mortem is variable. It is known that high quality reporting is directly related to the probability of a post-mortem yielding clinically relevant information[29]. As a result, the Royal College of Pathologists has published guidelines for fetal autopsy, third trimester antepartum and intrapartum stillbirths and neonatal deaths. These guidelines provide an auditable standard for autopsy procedure and reporting, although specific guidelines for multiple pregnancies and performing injection studies in monochorionic placentas do not currently exist. In spite of such guidelines, post-mortem reports still depend on the local expertise of the pathologist and the quality of clinical information received by the pathology department. It is the responsibility of the requesting clinician to provide a detailed clinical summary to inform the autopsy and facilitate a meaningful clinico-pathological correlation[30]. A standardised approach to submitting clinical information, and for conducting and reporting post-mortem examinations and placental injections studies in multiple pregnancies would improve the accuracy of assigning cause of death. Despite these limitations, post-mortem findings provide new information to change the diagnosis in 9-11%, and additional information in 22-76% of cases, and as such are a powerful tool when assigning cause of death in a classification system[31-36]. Placental examination is also associated with a significant reduction in “unexplained” deaths (OR 0.17; 95% CI 0.04-0.70) and is more cost-effective than post-mortem of the baby or cytogenetics[35,36].

The 2020 MBRRACE perinatal confidential enquiry in to stillbirths and neonatal deaths in twin pregnancies concluded that “pathological examination of the placenta is an

integral part of the investigation of stillbirth and the placenta should be submitted for examination in all cases. Placental examination directly contributes to the understanding of the cause of death and should be encouraged in all cases, even if post-mortem examination is declined by the family"[30]. Therefore a classification system that solely relies of clinical information is unlikely to capture important details that will arise from examination of the placenta. We have demonstrated the value of using post-mortem reports to assign cause of death since the most common cause of death overall was placental and in many cases the cause of death was only confirmed following placental examination. In addition, the MBRRACE report recommended that a specialist perinatal pathologist should examine the placenta in cases of stillbirth of either a singleton or a twin pregnancy[30]. Thus an important strength of CoDiT lies in the fact that it was created by a panel that included expert perinatal pathologists, and subsequently tested the system using post-mortem reports written by perinatal pathologists.

For twins, placental examination also confirms chorionicity and amnionicity and in monochorionic twins, injection studies determine whether inter-twin transfusional processes contributed to demise[28]. Lopriore et al. suggest that injection studies should be performed on all monochorionic placentas, irrespective of the birth outcome, to evaluate the effect of any FLA and to understand the pathophysiology of disorders affecting monochorionic pregnancies, potentially uncovering patterns associated with fetal demise[37-40].

Comparison with the Delphi consensus study

CoDiT already aligns with 6/17 of the essential characteristics outlined in the Delphi consensus study – accommodates fetal death, stillbirths and NND; distinguishes between stillbirth and NND; has a small number of main categories; shows strong inter- and intra- rater agreement; allows associated factors such as placental descriptions to be recorded and distinguished from the cause of death; requires the single most important factor to be recorded[23]. CoDiT will require external validation to demonstrate that it meets other characteristics – ease of use; clear guidelines; produces data that are easily understood; has a sufficiently comprehensive list of categories to minimise the proportion of deaths classified as "unknown"[23].

An important characteristic we were unable to assess was the ability of CoDiT to distinguish between antepartum and intrapartum deaths, as our cohort contained no intrapartum deaths. As a higher proportion of twin pregnancies are delivered electively by Caesarean section (CS), a lack of intrapartum deaths may reflect the reduced number of women who labour with twin pregnancies.

Comparison with other classification systems

The proportion of cases classified as unknown (25%) is comparable to Codac but higher than Tulip (11%), which captured late fetal losses, stillbirths, NNDs and infant deaths, and ReCoDe (15%), which only applies to stillbirths[19,22,26]. CoDiT was designed to be used across all gestations including the first trimester. Determining cause of death from early gestation fetal post-mortems can be extremely difficult. However, we have shown the value of incorporating these early gestations. In our cohort, 20% (54/265) of fetuses were <16 weeks' gestation and the commonest cause

of death <24 weeks in our cohort was placental (54/129; 42%); moreover TTTS, which was the commonest cause of monochorionic twin death, typically occurs between 16 and 24 weeks' gestation.

CoDiT is the first classification system designed specifically for perinatal deaths in twin pregnancies. One study concluded that no pre-existing classification system was suitable to classify causes of death in twin pregnancies as they did not reflect the diversity of diagnoses in twins; at best, they identified twin pregnancies as subcategories under "other conditions"[41]. Another study identified that a major risk factor for double fetal deaths in twins was monochorionicity, which is reflected in the higher proportion of double monochorionic twin deaths compared to dichorionic deaths in our cohort[42]. Using Codac to assign cause of death in twins and singletons found a higher prevalence of twin pregnancies in cases with a placental and unknown cause of death, aligning with the overall top two causes in our cohort[43]. A large retrospective cohort analysis comparing the risks and causes of stillbirths in singletons and twins using ReCoDe, found that stillbirths were mainly due to TTTS in monochorionic twins, as in our cohort, but unlike our cohort, congenital anomalies were the biggest cause of death in dichorionic twins and singletons[44]. In line with our cohort, the 2020 MBRRACE report in to perinatal loss in twins, which sampled 118 twin pregnancies over 22 weeks' gestation, identified TTTS as the most common cause of perinatal loss in monochorionic twins[30]. Extreme preterm labour was the most frequent cause of perinatal loss among the 70 dichorionic pregnancies in the MBRRACE cohort, compared with acute chorioamnionitis in our cohort of 78 dichorionic pregnancies, though the latter includes losses in all gestations, which may account for this discrepancy[30].

Although there was recurring disagreement between users when assigning cause of death as either acute chorioamnionitis or ascending infection, the user guidelines clearly state that ascending infection should be assigned when there is proven colonisation in the birth canal. In other classification systems, cases that clearly did not follow the user guidance were identified as “misinterpretation” of the guidelines and excluded from the study. Had this been considered in CoDiT, is it probable that inter-user agreement would have been even stronger.

Future work

CoDiT requires validation using external cohorts (i.e. other tertiary UK maternity units with dedicated perinatal pathology services) and external panels to determine its suitability as a global classification system for twins. The guidelines may require modification to clarify how users should distinguish ascending infection from acute chorioamnionitis. Adding a subcategory to distinguish between deaths due to prematurity from PPROM with or without evidence of infection may be useful. Furthermore, comparative studies to evaluate the performance of CoDIT against other classification systems in twin pregnancies will determine whether the system employed affects the classification of cause of death and the frequency of unexplained deaths.

## CONCLUSION

We introduce the first classification system for perinatal mortality specifically designed for twin pregnancies. Testing CoDiT demonstrated the commonest causes of death in this cohort of twins, and the gestation with the highest incidence of fetal demise. CoDiT yields reproducible outcomes and already meets many characteristics required for a global classification system. Whilst external validation is required, CoDiT has the potential to be a powerful tool in furthering our understanding of deaths in twin pregnancies and a catalyst to improve their management.

# REFERENCES

**PROJECT 1:** Investigating the T-cell repertoire in malplacentation disorders


1. Carter AM. Animal models of human placentation–a review. Placenta. 2007;28:S41-S47.
2. Mikkelsen E, Lauridsen H, Nielsen PM, Qi H, Nørlinger T, Andersen MD et al. The chinchilla as a novel animal model of pregnancy. R Soc Open Sci. 2017;4(4): 161098. doi: 10.1098/rsos.161098.
3. Burton GJ, Fowden AL. The placenta: a multifaceted, transient organ. Philos Trans R Soc Lond B Biol Sci. 2015;370(1663): 20140066. doi: 10.1098/rstb.2014.0066.
4. Menon R, Lappas M, Zakar T. Editorial: The Role of the Fetal Membranes in Pregnancy and Birth. Front Physiol. 2021;12: 653084. doi: 10.3389/fphys.2021.653084.
5. Bourne G. The foetal membranes. A review of the anatomy of normal amnion and chorion and some aspects of their function. Postgrad Med J. 1962;38(438): 193-201. doi: 10.1136/pgmj.38.438.193.
6. Roberts RM, Ezashi T, Das P. Trophoblast gene expression: transcription factors in the specification of early trophoblast. Reprod Biol Endocrinol. 2004;2: 47. doi: 10.1186/1477-7827-2-47.
7. Turco MY, Moffett A. Development of the human placenta. Development. 2019;146(22): dev163428. doi: 10.1242/dev.163428.

8. Wang Y, Zhao S. Vascular Biology of the Placenta. In *Integrated Systems Physiology: from Molecules to Function to Disease.* San Rafael (CA): Morgan & Claypool Life Sciences; 2010. DOI: 10.4199/C00016ED1V01Y201008ISP009
9. Zeldovich V, Bakardjiev A. Host Defense and Tolerance: Unique Challenges in the Placenta. PLoS Pathogens 2012;8(8): e1002804. https://doi.org/10.1371/journal.ppat.1002804.
10. Hazan AD, Smith SD, Jones RL, Whittle W, Lye SJ, Dunk CE. Vascular-leukocyte interactions: mechanisms of human decidual spiral artery remodeling in vitro. Am J Pathol. 2010;177(2): 1017-30. doi: 10.2353/ajpath.2010.091105.
11. Man J, Hutchinson JC, Heazell AE, Ashworth M, Jeffrey I, Sebire NJ. Stillbirth and intrauterine fetal death: role of routine histopathological placental findings to determine cause of death. Ultrasound Obstet Gynecol. 2016;48(5): 579-584. doi: 10.1002/uog.16019.
12. Burton GJ, Redman CW, Roberts JM, Moffett A. Pre-eclampsia: pathophysiology and clinical implications. BMJ. 2019;366: l2381.
13. Roberts JM, Redman CWG. Pre-eclampsia: more than just pregnancy-induced hypertension. The Lancet 1993; 341(8858): 1447-1451.
14. Gutierrez Henares J, Gutierrez Henares R, Perry H, Khalil A, Thilaganathan B. Maternal cardiovascular potential and kinetic energy indices in pre-eclamptic and small-for-gestational-age pregnancies. To be published in *Ultrasound Obstet Gynecol.* [Preprint] 2021. doi: 10.1002/uog.24768.
15. National Institute for Health and Care Excellence (NICE), *Hypertension in pregnancy: diagnosis and management [NG133].* 2019. Available from: https://www.nice.org.uk/guidance/ng133 [Accessed 10th Oct 2021].

16. Raymond D, Peterson E. A critical review of early-onset and late-onset preeclampsia. Obstet Gynecol Surv. 2011;66(8): 497-506. doi: 10.1097/OGX.0b013e3182331028.
17. Robson SC, Martin WL, Morris RK on behalf of the Royal College of Obstetricians and Gynaecologists. *The Investigation and Management of the Small–for–Gestational–Age Fetus. Green-top Guideline No.31.* 2014. Available from: https://www.rcog.org.uk/en/guidelines-research-services/guidelines/gtg31/ [Accessed 11th Jan 2022]
18. Gordijn SJ, Beune IM, Thilaganathan B, Papageorghiou A, Baschat AA, Baker PN et al. Consensus definition of fetal growth restriction: a Delphi procedure. Ultrasound Obstet Gynecol. 2016;48(3): 333-9. doi: 10.1002/uog.15884.
19. Audette MC, Kingdom JC. Screening for fetal growth restriction and placental insufficiency. Seminars in Fetal and Neonatal Medicine. 2018;23(2): 119-125. doi: 10.1016/j.siny.2017.11.004.
20. NHS England. Saving Babies' Lives Care Bundle Version 2. 2019. Available from: https://www.england.nhs.uk/wp-content/uploads/2019/07/saving-babies-lives-care-bundle-version-two-v5.pdf [Accessed 14th July 2022]
21. Kho EM, McCowan LM, North RA, Roberts CT, Chan E, Black MA et al; SCOPE Consortium. Duration of sexual relationship and its effect on preeclampsia and small for gestational age perinatal outcome. J Reprod Immunol. 2009;82(1): 66-73. doi: 10.1016/j.jri.2009.04.011.
22. Kieffer TEC, Laskewitz A, Scherjon SA, Faas MM, Prins JR. Memory T Cells in Pregnancy. Front Immunol. 2019;10: 625. doi: 10.3389/fimmu.2019.00625.

23. Saito S, Shiozaki A, Nakashima A, Sakai M, Sasaki Y. The role of the immune system in preeclampsia. Mol Aspects Med. 2007;28(2): 192-209. doi: 10.1016/j.mam.2007.02.006.

24. Lissauer D, Kilby MD, Moss P. Maternal effector T cells within decidua: The adaptive immune response to pregnancy? Placenta. 2017;60: 140-144. doi: 10.1016/j.placenta.2017.09.003.

25. Chaplin DD. Overview of the immune response. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S3-23. doi: 10.1016/j.jaci.2009.12.980.

26. Nicholson LB. The immune system. Essays Biochem. 2016;60(3): 275-301. doi: 10.1042/EBC20160017.

27. Lu HQ, Hu R. The role of immunity in the pathogenesis and development of pre-eclampsia. Scand J Immunol. 2019;90(5): e12756. doi: 10.1111/sji.12756.

28. Wissinger E. *CD8+ T cells.* Cells: Bite-size immunology. British Society of Immunology. Available from: https://www.immunology.org/public-information/bitesized-immunology/cells/cd8-t-cells [Accessed 11th Jan 2022]

29. Sargent IL, Borzychowski AM, Redman CW. Immunoregulation in normal pregnancy and pre-eclampsia: an overview. Reprod Biomed Online. 2006;13(5): 680-6. doi: 10.1016/s1472-6483(10)60659-1.

30. Lissauer D, Piper K, Goodyear O, Kilby MD, Moss PA. Fetal-specific CD8+ cytotoxic T cell responses develop during normal human pregnancy and exhibit broad functional capacity. J Immunol. 2012;189(2): 1072-80. doi: 10.4049/jimmunol.1200544.

31. Kahn DA, Baltimore D. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. Proc Natl Acad Sci USA. 2010;107: 9299–304. doi: 10.1073/pnas.1003909107

32. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature. 2012;490: 102–6. doi: 10.1038/nature11462

33. Ashkar AA, di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. Journal of Experimental Medicine. 2000;192: 259–270.

34. Bilinski MJ, Thorne JG, Oh MJ, Leonard S, Murrant C, Tayade C et al. Uterine NK cells in murine pregnancy. Reprod Biomed Online. 2008;16(2): 218-26. doi: 10.1016/s1472-6483(10)60577-9.

35. Greenwood JD, Minhas K, di Santo JP, Makita M, Kiso Y, Croy BA. Ultrastructural studies of implantation sites from mice deficient in uterine natural killer cells. Placenta. 2000;21(7): 693-702. doi: 10.1053/plac.2000.0556.

36. Wallace AE, Fraser R, Cartwright JE. Extravillous trophoblast and decidual natural killer cells: a remodelling partnership. Hum Reprod Update. 2012;18(4): 458-71. doi: 10.1093/humupd/dms015.

37. Powell RM, Lissauer D, Tamblyn J, Beggs A, Cox P, Moss P et al. Decidual T cells exhibit a highly differentiated phenotype and demonstrate potential fetal-specificity and a strong transcriptional response to interferon. Immunol. 2017; 199(10): 3406–3417. doi: 10.4049/jimmunol.1700114.

38. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev. 2010;236: 219-42. doi: 10.1111/j.1600-065X.2010.00923.x.
39. Ozkaynak E, Wang L, Goodearl A, McDonald K, Qin S, O'Keefe T et al. Programmed death-1 targeting can promote allograft survival. J. Immunol. 2002;169: 6546–6553. DOI: 10.4049/jimmunol.169.11.6546
40. Veras E, Kurman RJ, Wang TL, Shih IM. PD-L1 Expression in Human Placentas and Gestational Trophoblastic Diseases. Int J Gynecol Pathol. 2017;36(2): 146-153. doi: 10.1097/PGP.0000000000000305.
41. Redman CW, Sargent IL. Immunology of pre-eclampsia. Am J Reprod Immunol. 2010;63(6):534-43. doi: 10.1111/j.1600-0897.2010.00831.x.
42. Mor G, Cardenas I. The immune system in pregnancy: a unique complexity. Am J Reprod Immunol. 2010;63(6): 425-33. doi: 10.1111/j.1600-0897.2010.00836.x.
43. Zenclussen AC, Fest S, Joachim R, Klapp BF, Arck PC. Introducing a mouse model for pre-eclampsia: adoptive transfer of activated Th1 cells leads to pre-eclampsia-like symptoms exclusively in pregnant mice. Eur J Immunol. 2004;34(2): 377-87. doi: 10.1002/eji.200324469.
44. Harmon AC, Ibrahim T, Cornelius DC, Amaral LM, Cunningham MW Jr, Wallace K et al. Placental $CD4^+$ T cells isolated from preeclamptic women cause preeclampsia-like symptoms in pregnant nude-athymic rats. Pregnancy Hypertens. 2019;15: 7-11. doi: 10.1016/j.preghy.2018.10.007.
45. Dhillion P, Wallace K, Herse F, Scott J, Wallukat G, Heath J et al B. IL-17-mediated oxidative stress is an important stimulator of AT1-AA and hypertension

during pregnancy. Am J Physiol Regul Integr Comp Physiol. 2012;303(4): R353-8. doi: 10.1152/ajpregu.00051.2012.

46. Novotny S, Wallace K, Herse F, Moseley J, Darby M, Heath J et al. $CD4^+$ T Cells Play a Critical Role in Mediating Hypertension in Response to Placental Ischemia. J Hypertens (Los Angel). 2013 Jun;2: 14873. doi: 10.4172/2167-1095.1000116

47. Wallace K, Richards S, Dhillon P, Weimer A, Edholm ES, Bengten E et al. CD4+ T-helper cells stimulated in response to placental ischemia mediate hypertension during pregnancy. Hypertension. 2011;57(5): 949-55. doi: 10.1161/HYPERTENSIONAHA.110.168344.

48. Wallace K, Novotny S, Heath J, Moseley J, Martin JN Jr, Owens MY et al. Hypertension in response to CD4(+) T cells from reduced uterine perfusion pregnant rats is associated with activation of the endothelin-1 system. Am J Physiol Regul Integr Comp Physiol. 2012;303(2): R144-9. doi: 10.1152/ajpregu.00049.2012.

49. Nevers T, Kalkunte S, Sharma S. Uterine Regulatory T cells, IL-10 and hypertension. Am J Reprod Immunol. 2011;66 Suppl 1(Suppl 1): 88-92. doi: 10.1111/j.1600-0897.2011.01040.x.

50. Harmon A, Cornelius D, Amaral L, Paige A, Herse F, Ibrahim T et al. IL-10 supplementation increases Tregs and decreases hypertension in the RUPP rat model of preeclampsia. Hypertens Pregnancy. 2015;34(3): 291-306. doi: 10.3109/10641955.2015.1032054.

51. Ibrahim T, Przybyl L, Harmon AC, Amaral LM, Faulkner JL, Cornelius DC et al. Proliferation of endogenous regulatory T cells improve the pathophysiology associated with placental ischaemia of pregnancy. Am J Reprod Immunol. 2017;78(5): 10.1111/aji.12724. doi: 10.1111/aji.12724.
52. Schraven B, Kalinke U. CD28 superagonists: what makes the difference in humans? Immunity. 2008;28(5): 591-5. doi: 10.1016/j.immuni.2008.04.003.
53. Shima T, Inada K, Nakashima A, Ushijima A, Ito M, Yoshino O et al. Paternal antigen-specific proliferating regulatory T cells are increased in uterine-draining lymph nodes just before implantation and in pregnant uterus just after implantation by seminal plasma-priming in allogeneic mouse pregnancy. J Reprod Immunol. 2015;108: 72-82. doi: 10.1016/j.jri.2015.02.005.
54. Tsuda S, Nakashima A, Shima T, Saito S. New Paradigm in the Role of Regulatory T Cells During Pregnancy. Front Immunol. 2019;10: 573. doi: 10.3389/fimmu.2019.00573.
55. Guleria I, Khosroshahi A, Ansari MJ, Habicht A, Azuma M, Yagita H et al. A critical role for the programmed death ligand 1 in fetomaternal tolerance. J Exp Med. 2005;202(2): 231-7. doi: 10.1084/jem.20050019.
56. Habicht A, Dada S, Jurewicz M, Fife BT, Yagita H, Azuma M et al. A link between PDL1 and T regulatory cells in fetomaternal tolerance. J Immunol. 2007;179(8): 5211-9. doi: 10.4049/jimmunol.179.8.5211.
57. Tian M, Zhang Y, Liu Z, Sun G, Mor G, Liao A. The PD-1/PD-L1 inhibitory pathway is altered in pre-eclampsia and regulates T cell responses in pre-eclamptic rats. Sci Rep. 2016;6: 27683. doi: 10.1038/srep27683.

58. Milosevic-Stevanovic J, Krstic M, Stefanovic M, Zivadinovic R, Vukomanovic P, Trajkovic-Dinic SP et al. T lymphocytes in the third trimester decidua in preeclampsia. Hypertens Pregnancy. 2019;38(1): 52-57. doi: 10.1080/10641955.2019.1575393.
59. Darmochwal-Kolarz D, Kludka-Sternik M, Tabarkiewicz J, Kolarz B, Rolinski J, Leszczynska-Gorzelak B et al. The predominance of Th17 lymphocytes and decreased number and function of Treg cells in preeclampsia. J Reprod Immunol. 2012;93(2): 75-81. doi: 10.1016/j.jri.2012.01.006.
60. Nguyen TA, Kahn DA, Loewendorf AI. Maternal-Fetal rejection reactions are unconstrained in preeclamptic women. PLoS One. 2017;12(11): e0188250. doi: 10.1371/journal.pone.0188250.
61. Rieger L, Segerer S, Bernar T, Kapp M, Majic M, Morr AK et al. Specific subsets of immune cells in human decidua differ between normal pregnancy and preeclampsia--a prospective observational study. Reprod Biol Endocrinol. 2009;7: 132. doi: 10.1186/1477-7827-7-132. PMID: 19930648;
62. Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA et al. Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. J Immunol. 2008;180(8): 5737-45. doi: 10.4049/jimmunol.180.8.5737.
63. Sasaki Y, Darmochwal-Kolarz D, Suzuki D, Sakai M, Ito M, Shima T et al. Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. Clin Exp Immunol. 2007;149(1): 139-45. doi: 10.1111/j.1365-2249.2007.03397.x.

64. Tsuda S, Zhang X, Hamana H, Shima T, Ushijima A, Tsuda K, et al. Clonally expanded decidual effector regulatory T cells increase in late gestation of normal pregnancy, but not in preeclampsia, in humans. Front Immunol. 2018;9: 1934. doi: 10.3389/fimmu.2018.01934.
65. Darmochwal-Kolarz D, Saito S, Tabarkiewicz J, Kolarz B, Rolinski J, Leszczynska-Gorzelak B, et al. Apoptosis signaling is altered in CD4(+)CD25(+)FoxP3(+) T regulatory lymphocytes in pre-eclampsia. Int J Mol Sci. 2012;13: 6548–60. doi: 10.3390/ijms13066548.
66. Hsu P, Santner-Nanan B, Dahlstrom JE, Fadia M, Chandra A, Peek M et al. Altered decidual DC-SIGN+ antigen-presenting cells and impaired regulatory T-cell induction in preeclampsia. Am J Pathol. 2012;181(6): 2149-60. doi: 10.1016/j.ajpath.2012.08.032.
67. Meggyes M, Miko E, Lajko A, Csiszar B, Sandor B, Matrai P et al. Involvement of the PD-1/PD-L1 Co-Inhibitory Pathway in the Pathogenesis of the Inflammatory Stage of Early-Onset Preeclampsia. Int J Mol Sci. 2019;20(3): 583. doi: 10.3390/ijms20030583.
68. Chaiworapongsa T, Gervasi MT, Refuerzo J, Espinoza J, Yoshimatsu J, Berman S et al. Maternal lymphocyte subpopulations (CD45RA+ and CD45RO+) in preeclampsia. Am J Obstet Gynecol. 2002;187(4): 889-93. doi: 10.1067/mob.2002.127309.
69. Faas MM, Schuiling GA, Baller JF, Visscher CA, Bakker WW. A new animal model for human preeclampsia: ultra-low-dose endotoxin infusion in pregnant rats. Am J Obstet Gynecol. 1994;171:158-64. doi: 10.1016/0002-9378(94)90463-4.

70. Hill JA, Devoe LD, Bryans CI Jr. Frequency of asymptomatic bacteriuria in preeclampsia. Obstet Gynecol 1986;67: 529-32.
71. Herrera JA, Chaudhuri G, López-Jaramillo P. Is infection a major risk factor for preeclampsia? Med Hypotheses. 2001;57(3): 393-7. doi: 10.1054/mehy.2001.1378.
72. Moffett A, Chazara O, Colucci F. Maternal allo-recognition of the fetus. Fertil Steril. 2017;107(6): 1269-1272. doi: 10.1016/j.fertnstert.2017.05.001.
73. Dahl M, Klitkou L, Christiansen OB, Djurisic S, Piosik ZM, Skovbo P et al. Human leukocyte antigen (HLA)-G during pregnancy part II: associations between maternal and fetal HLA-G genotypes and soluble HLA-G. Hum Immunol. 2015;76(4): 260-71. doi: 10.1016/j.humimm.2015.01.015.
74. Tersigni C, Redman CW, Dragovic R, Tannetta D, Scambia G, Di Simone N et al. HLA-DR is aberrantly expressed at feto-maternal interface in pre-eclampsia. J Reprod Immunol. 2018;129: 48-52. doi: 10.1016/j.jri.2018.06.024.
75. Tamblyn JA, Lissauer DM, Powell R, Cox P, Kilby MD. The immunological basis of villitis of unknown etiology - review. Placenta. 2013;34(10): 846-55. doi: 10.1016/j.placenta.2013.07.002.
76. Derricott H, Jones RL, Heazell AE. Investigating the association of villitis of unknown etiology with stillbirth and fetal growth restriction - a systematic review. Placenta. 2013;34(10): 856-62. doi: 10.1016/j.placenta.2013.07.003.
77. Derricott H, Jones RL, Greenwood SL, Batra G, Evans MJ, Heazell AE. Characterizing Villitis of Unknown Etiology and Inflammation in Stillbirth. Am J Pathol. 2016;186(4): 952-61. doi: 10.1016/j.ajpath.2015.12.010.

78. Bezemer RE, Schoots MH, Timmer A, Scherjon SA, Erwich JJHM, van Goor H et al. Altered Levels of Decidual Immune Cell Subsets in Fetal Growth Restriction, Stillbirth, and Placental Pathology. Front. Immunol. 2020;11: 1898. doi: 10.3389/fimmu.2020.01898
79. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol. 1999;163(10): 5211-8.
80. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133(5): 775-87. doi: 10.1016/j.cell.2008.05.009.
81. Turco MY, Gardner L, Kay RG, Hamilton RS, Prater M, Hollinshead MS et al. Trophoblast organoids as a model for maternal-fetal interactions during human placentation. Nature. 2018;564(7735): 263-267. doi: 10.1038/s41586-018-0753-3.
82. Bilardo CM, Hecher K, Visser GHA, Papageorghiou AT, Marlow N, Thilaganathan B et al; TRUFFLE Group. Severe fetal growth restriction at 26-32 weeks: key messages from the TRUFFLE study. Ultrasound Obstet Gynecol. 2017;50(3): 285-290. doi: 10.1002/uog.18815.
83. Burton GJ, Sebire NJ, Myatt L, Tannetta D, Wang YL, Sadovsky Y et al. Optimising sample collection for placental research. Placenta. 2014;35(1):9-22. doi: 10.1016/j.placenta.2013.11.005. Erratum in: Placenta. 2014;35(4):289.

**PROJECT 2:** Development of a novel classification system to assign **C**ause of **D**eath in **T**wins: **C**o**D**i**T**


1. Esteves-Pereira AP, da Cunha AJLA, Nakamura-Pereira M, Moreira ME, Domingues RMSM, Viellas EF et al. Twin pregnancy and perinatal outcomes: Data from 'Birth in Brazil Study'. PLoS One. 2021;16(1): e0245152. doi: 10.1371/journal.pone.0245152.
2. Chepkin S, Prince S, Johnston T, Boby T, Neves M, Smith P et al. *Learning from Standardised Reviews When Babies Die.* National Perinatal Epidemiology Unit: Oxford. 2019. Available from: https://www.npeu.ox.ac.uk/pmrt/reports [Accessed 10th Oct 2021].
3. Draper ES, Gallimore ID, Smith LK, Fenton AC, Kurinczuk JJ, Smith PW et al. *MBRRACE-UK Perinatal Mortality Surveillance Report UK Perinatal Deaths for Births from January to December 2018.* National Perinatal Epidemiology Unit: Oxford. 2020. Available from: https://www.npeu.ox.ac.uk/mbrrace-uk/reports [Accessed 13/01/2022].
4. Blondel B, Macfarlane A, Gissler M, Breart G, Zeitlin J; PERISTAT Study Group. Preterm birth and multiple pregnancy in European countries participating in the PERISTAT project. BJOG. 2006;113(5): 528–35. doi: 10.1111/j.1471-0528.2006.00923.x.
5. Khalil A. Unprecedented fall in stillbirth and neonatal death in twins: lessons from the UK. Ultrasound Obstet Gynecol. 2019;53(2):153–7. doi: 10.1002/uog.20107.
6. Kilby MD, Bricker L on behalf of the Royal College of Obstetricians and Gynaecologists*. Management of Monochorionic Twin Pregnancy. Green-top Guideline No. 51.* BJOG. 2017;124(1): e1-e45. doi: 10.1111/1471-0528.14188.

7. Slaghekke F, Kist WJ, Oepkes D, Middeldorp JM, Klumper FJ, Vandenbussche FP et al. TAPS and TOPS: two distinct forms of feto-fetal transfusion in monochorionic twins. Z Geburtshilfe Neonatol. 2009;213(6): 248-54. doi: 10.1055/s-0029-1241884.
8. Quintero RA, Morales WJ, Allen MH, Bornick PW, Johnson PK, Kruger M. Staging of Twin-Twin Transfusion Syndrome. Journal of Perinatology. 1999;19(8 Pt 1): 550-5. doi: 10.1038/sj.jp.7200292.
9. Dhillon RK, Hillman SC, Pounds R, Morris RK, Kilby MD. Comparison of Solomon technique with selective laser ablation for twin-twin transfusion syndrome: a systematic review. Ultrasound Obstet Gynecol. 2015; 46(5):526-33. doi: 10.1002/uog.14813.
10. Roberts D, Neilson JP, Kilby MD, Gates S. Interventions for the treatment of twin-twin transfusion syndrome. Cochrane Database Syst Rev. 2014;(1): CD002073. doi: 10.1002/14651858.CD002073.pub3.
11. Quintero RA, Ishii K, Chmait RH, Bornick PW, Allen MH, Kontopoulos EV. Sequential selective laser photocoagulation of communicating vessels in twin-twin transfusion syndrome. J Matern Fetal Neonatal Med. 2007;20(10): 763-8. doi: 10.1080/14767050701591827.
12. Glennon CL, Shemer SA, Palma-Dias R, Umstad MP. The History of Treatment of Twin-to-Twin Transfusion Syndrome. Twin Res Hum Genet. 2016;19(3): 168-74. doi: 10.1017/thg.2016.27.
13. Slaghekke F, Kist WJ, Oepkes D, Pasman SA, Middeldorp JM, Klumper FJ et al. Twin anemia-polycythemia sequence: diagnostic criteria, classification, perinatal

management and outcome. Fetal Diagn Ther. 2010;27(4): 181-90. doi: 10.1159/000304512.

14. Weisz B, Peltz R, Chayen B, Oren M, Zalel Y, Achiron R et al. Tailored management of twin reversed arterial perfusion (TRAP) sequence. Ultrasound Obstet Gynecol. 2004;23(5): 451-5. doi: 10.1002/uog.1040.
15. Tsao K, Feldstein VA, Albanese CT, Sandberg PL, Lee H, Harrison MR et al. Selective reduction of acardiac twin by radiofrequency ablation. Am J Obstet Gynecol. 2002;187(3): 635-40. doi: 10.1067/mob.2002.125242.
16. Pagani G, D'Antonio F, Khalil A, Papageorghiou A, Bhide A, Thilaganathan B. Intrafetal laser treatment for twin reversed arterial perfusion sequence: cohort study and meta-analysis. Ultrasound Obstet Gynecol. 2013;42(1): 6-14. doi: 10.1002/uog.12495.
17. Lewi L, Valencia C, Gonzalez E, Deprest J, Nicolaides KH. The outcome of twin reversed arterial perfusion sequence diagnosed in the first trimester. Am J Obstet Gynecol 2010;203(3): 213.e1–4. doi: 10.1016/j.ajog.2010.04.018.
18. Wigglesworth JS. Classification of perinatal deaths. Soz Praventivmed. 1994;39(1): 11-4. doi: 10.1007/BF01369938.
19. Frøen JF, Pinar H, Flenady V, Bahrin S, Charles A, Chauke L et al. Causes of death and associated conditions (Codac)—a utilitarian approach to the classification of perinatal deaths. BMC Pregnancy Childbirth 2009;9: 22. doi: 10.1186/1471-2393-9-22.
20. Gulati N, Mackie FL, Cox P, Marton T, Heazell AEP, Morris RK et al. Cause of intrauterine and neonatal death in twin pregnancies (CoDiT): development of a

novel classification system. BJOG 2020;127(12): 1507–1515. doi: 10.1111/1471-0528.16291.

21. Leisher SH, Teoh Z, Reinebrant H, Allanson E, Blencowe H, Erwich JJ et al. Seeking order amidst chaos: a systematic review of classification systems for causes of stillbirth and neonatal death, 2009-2014. BMC Pregnancy Childbirth. 2016;16(1): 295. doi: 10.1186/s12884-016-1071-0.
22. Gardosi J, Kady SM, McGeown P, Francis A, Tonks A. Classification of stillbirth by relevant condition at death (ReCoDe): population based cohort study. BMJ. 2005;331(7525):1113-7. doi: 10.1136/bmj.38629.587639.7C.
23. Wojciezek AM, Reinebrant H, Leisher SH, Allanson E, Coory M, Erwich JJ et al. Characteristics of a global classification system for perinatal deaths: a Delphi consensus study. BMC Pregnancy Childbirth. 2016;16: 223. doi: 10.1186/s12884-016-0993-x.
24. Leisher SH, Teoh Z, Reinebrant H, Allanson E, Blencowe H, ErwichJJ et al. Classification systems for causes of stillbirth and neonatal death, 2009–2014: an assessment of alignment with characteristics for an effective global system. BMC Pregnancy Childbirth. 2016;16: 269. doi: 10.1186/s12884-016-1040-7.
25. Gordijn SJ, Korteweg FJ, Erwich JJHM, Holm JP, van Diem MT, Bergman KA et al. A multi-layered approach for the analysis of perinatal mortality using different classification systems. Eur J Obstet Gynecol Reprod Biol 2009;144(2):99–104. doi: 10.1016/j.ejogrb.2009.01.012.
26. Korteweg FJ, Gordijn SJ, Timmer A, Erwich JJ, Bergman KA, Bouman K et al. The Tulip classification of perinatal mortality: introduction and multidisciplinary

inter-rater agreement. BJOG 2006;113(4): 393–401. doi: 10.1111/j.1471-0528.2006.00881.x.

27. McHugh ML. Interrater reliability: the kappa statistic. Biochem Med(Zagreb). 2012;22(3): 276– 82.
28. Draper ES, Gallimore ID, Smith LK, Kurinczuk JJ, Smith PW, Boby T et al. *MBRRACE-UK Perinatal Mortality Surveillance Report UK Perinatal Deaths for Births from January to December 2017.* National Perinatal Epidemiology Unit: Oxford. 2019. Available from: https://www.npeu.ox.ac.uk/mbrrace-uk/reports [Accessed 13/01/2022].
29. Royal College of Obstetricians and Gynaecologists and Royal College of Pathologists. *Fetal and Perinatal Pathology. Report of a Joint Working Party.* RCOG. 2001.
30. Draper ES, Gallimore ID, Kurinczuk JJ, Kenyon S (Eds.) on behalf of MBRRACE-UK. *MBRRACE-UK 2019 Perinatal Confidential Enquiry: Stillbirths and neonatal deaths in twin pregnancies.* National Perinatal Epidemiology Unit: Oxford. 2021. Available from: https://www.npeu.ox.ac.uk/mbrrace-uk/reports [Accessed 10/10/2021].
31. Faye-Petersen OM, Guinn DA, Wenstrom KD. Value of perinatal autopsy. Obstet Gynecol. 1999;94(6): 915-20. doi: 10.1016/s0029-7844(99)00468-8.
32. Kock KF, Vestergaard V, Hardt-Madsen M, Garne E. Declining autopsy rates in stillbirths and infant deaths: results from Funen County, Denmark, 1986-96. J Matern Fetal Neonatal Med. 2003;13(6): 403-7. doi: 10.1080/jmf.13.6.403.407.

33. Cartlidge PH, Dawson AT, Stewart JH, Vujanic GM. Value and quality of perinatal and infant postmortem examinations: cohort analysis of 400 consecutive deaths. BMJ. 1995;310(6973): 155-8. doi: 10.1136/bmj.310.6973.155.
34. Cernach MC, Patrício FR, Galera MF, Moron AF, Brunoni D. Evaluation of a protocol for postmortem examination of stillbirths and neonatal deaths with congenital anomalies. Pediatr Dev Pathol. 2004;7(4): 335-41. doi: 10.1007/s10024-001-0211-2.
35. Saller DN Jr, Lesser KB, Harrel U, Rogers BB, Oyer CE. The clinical utility of the perinatal autopsy. JAMA. 1995;273(8):663-5. doi: 10.1001/jama.273.8.663.
36. Gordijn SJ, Erwich JJ, Khong TY. Value of the perinatal autopsy: critique. Pediatr Dev Pathol. 2002;5(5): 480-8. doi: 10.1007/s10024-002-0008-y.
37. Lopriore E, Slaghekke F, Middeldorp JM, Klumper FJ, van Lith JM, Walther FJ, et al. Accurate and simple evaluation of vascular anastomoses in monochorionic placenta using colored dye. J Vis Exp. 2011;(55):e3208. doi: 10.3791/3208.
38. Lopriore E, Middeldorp JM, Sueters M, Vandenbussche FP, Walther FJ. Twin-to-twin transfusion syndrome: from placental anastomoses to long-term neurodevelopmental outcome. Curr Pediatr Rev. 2005;1:191– 203.
39. Lopriore E, Sueters M, Middeldorp JM, Vandenbussche FP, Walther FJ. Haemoglobin differences at birth in monochorionic twins without chronic twin-to-twin transfusion syndrome. Prenat Diagn. 2005;25: 844– 50. doi: 10.1002/pd.1175.
40. Papathanasiou D, Witlox R, Oepkes D, Walther FJ, Bloemenkamp KW, Lopriore E. Monochorionic twins with ruptured vasa previa: double trouble! Fetal Diagn Ther. 2010;28(1): 48– 50. doi: 10.1159/000315493.

41. Skeie A, Frøen JF, Vege A, Stray-Pedersen B. Cause and risk of stillbirth in twin pregnancies: a retrospective audit. Acta Obstet Gynecol Scand. 2003;82: 1010–6. doi: 10.1034/j.1600-0412.2003.00288.x.
42. Rydhstroem H. Pregnancy with stillbirth of both twins. BJOG. 1996;103(1): 25–32. doi: 10.1111/j.1471-0528.1996.tb09511.x.
43. Helgadóttir LB, Turowski G, Skjeldestad FE, Jacobsen AF, Sandset PM, Roald B, et al. Classification of stillbirths and risk factors by cause of death—a case-control study. *Acta Obstet Gynecol Scand.* 2013;92(3): 325-33. doi: 10.1111/aogs.12044.
44. Russo FM, Pozzi E, Pelizzoni F, Todyrenchuk L, Bernasconi DP, Cozzolino S et al. Stillbirths in singletons, dichorionic and monochorionic twins: a comparison of risks and causes. Eur J Obstet Gynecol Reprod Biol. 2013;170(1): 131–6. doi: 10.1016/j.ejogrb.2013.06.014.

# APPENDIX: CoDiT Classification system

**Case number**:
(i.e. 1A = case 1, twin 1, 1B = case 1, twin 2)

| **Maternal Demographics** | **Pregnancy details** |
|---|---|
| **Age at booking:** | **Chorionicity according to Scan:** MC / DC / not reported |
| **Gravida:** | **Amnionicity according to scan:** MA / DA / not reported |
| **Parity:** | **Conception:** spontaneous / assisted / not reported |
| **BMI:** | **Twin demise:** single / double / not reported |
| **Ethnicity:** | **Mode of demise:** spontaneous / termination of whole pregnancy / selective reduction of that fetus / within 7 days of intervention / not reported |
| **Medical history (e.g. diabetes mellitus):** | **Gestation at death if intra-uterine death:** |
| | **Age at death if neonatal death:** |
| **Delivered at:** | **Gestation at delivery:** |
| | **Birth weight:** **PM weight:** |
| | **Chromosome testing done:** Y/N |
| | **If yes, level of testing conducted + result:** |

Please circle as many options as found in the post-mortem but only select one main cause of death

| **Cause of death** | **Subclassification** | **Other details** |
|---|---|---|
| **1. Congenital abnormality** | 1. Chromosomal defect<br>a. Numerical<br>b. Structural<br>c. Microdeletion/uniparental disomy | |
| | 2. Syndrome<br>a. Monogenic<br>b. Other | |
| | 3. Central nervous system | |
| | 4. Heart and circulatory system | |
| | 5. Respiratory system | |
| | 6. Digestive system | |
| | 7. Urogenital system | |
| | 8. Musculoskeletal system | |
| | 9. Endocrine/metabolic system | |
| | 10. Neoplasm | |
| | 11. Other<br>a. Single organ<br>b. Multiple organ | |
| | 12. Twin specific<br>a. TRAP – acardiac twin<br>b. TRAP – pump twin<br>c. Conjoined twins | |
| **2. Placenta** | See placental table | |
| **3. Umbilical cord** | See placental table | |
| **4. Prematurity** | 1. PPROM | |

| | | |
|---|---|---|
| | 2. Spontaneous preterm labour | |
| | 3. Cervical dysfunction | Diagnosed on scan: Y/N |
| | 4. Iatrogenic | Details: |
| | 5. Other | |
| **5. Infection** | 1. Transplacental | Details of infection:<br>(If chorioamnionitis please document in placental table under "membranes"). |
| | 2. Ascending | |
| | 3. Neonatal | |
| | 4. NOS | |
| **6. Other** | 1. Fetal hydrops of unknown origin | |
| | 2. Maternal disease | |
| | 3. Trauma | a) Fetal<br>b) Maternal |
| | 4. Exceptional circumstances (i.e. uterine rupture) | |
| | 5. Following medical intervention | Details: |
| | 6. Iatrogenic | |
| **7. Unknown** | 1. Despite thorough investigation | |
| | 2. Important information missing | |

**Placental table**

| | | |
|---|---|---|
| **Overview** | **Chorionicity on Pathology**<br>a. Monochorionic<br>b. Dichorionic<br>c. Not reported<br><br>**Amnionicity on PM**<br>a. Monoamniotic<br>b. Diamniotic<br>c. Not reported<br><br>Placental weight (g):<br>Fetal:placental weight ratio:<br><br>Placentas fused or separate:<br><br>Comment on vascular territory for each fetus (MC only): Y/N<br>If yes, details:<br><br>If MC, placental injection studies performed: Y/N<br>If yes, details of anastomoses: | Same as scan findings: Y/N<br>If no, details: |
| **Description of Placenta**<br>(Important relative descriptive factors that are NOT a COD | **Please select if any of these descriptors are relevant:**<br>• **Placenta praevia**<br>• **Placenta accreta/increta/percreta**<br>• **Nucleated RBC**<br>• **Immaturity**<br>• **Advanced maturation** | |

| | | |
|---|---|---|
| | • **Mild villitis of unknown aetiology (not severe)**<br>• **Umbilical cord – details_____** | |
| **Cause of death** | **Subclassification** | **Other details** |
| **2. Placenta** | 1. Low placental weight (<10th centile for gestational age)<br>2. High placenta weight (>90th centile for gestational age)<br>3. Thin placenta (average thickness <2cm)<br>4. Placental haemorrhage<br>a. Retroplacental haematoma<br>b. Subchorionic thromboses (extensive) | |
| 2a. Vascular abnormalities | 1. TTTS – **IS THIS A COD: Y / N**<br>a. Chronic – untreated<br>b. Chronic – treated (state treatment modality in next column)<br>c. Acute (2nd twin)<br>i. Neurological damage: Y/N<br>2. Fetal chorionic vessel thrombosis – **IS THIS A COD: Y / N** | Treatment modality for TTTS:<br><br>Clinical evidence of:<br>TAPS: Y/N<br>TOPS: Y/N |
| 2b. Placental chorionic villi and intervillous space abnormalities | 1. Extensive placental infarction<br>2. Vasculo-syncytial membrane deficiency<br>3. Extensive perivillous fibrin deposition<br>4. Maternal floor infarct | |
| 2c. Villitis | 1. Severe villitis of unknown aetiology<br>2. Histiocytic intervillositis | |
| 2d. Membranes | 1. Acute chorioamnionitis<br>2. Chronic chorioamnionitis | **Is this a COD: Y / N** |
| 2e. Angiomas | 1. Angioma of the cord | |
| 2f. Placenta | 1. Decidual vasculopathy<br>2. Other | |
| **3. Umbilical cord** (only select if a COD – if present but **not** a direct COD please list in "Description of placenta" section) | 1. Short cord (<32m)<br>2. Long cord (>100cm)<br>3. Marginal cord insertion<br>4. Velamentous insertion<br>5. Overcoiling of cord (>1coil/5cm)<br>6. Undercoiling of cord (<1coil/5cm)<br>7. Tight true knot<br>8. Single umbilical artery<br>9. Thrombosis of umbilical cord vessels<br>10. Cord entanglement<br>11. Other | |

**Guidance for completing the CoDiT data collection form**

**(1) Congenital anomaly:** the cause of death is explained by a genetic or a structural defect incompatible with life or potentially treatable but the primary cause of death. Assignment to this group is justified if

the congenital anomaly is the actual cause of death and no other major category of causes of death has initiated the causal pathway leading to death. Termination of pregnancy because of a congenital anomaly is also classified in this group. Any chromosome testing and details of the level of testing should also be recorded i.e. PCR, standard G band karyotype, microarray, genetic level.

**(2) and (3) Placenta and Umbilical cord:** the cause of death is explained by a placental or umbilical cord abnormality supported by the clinical findings. Cause of death should be recorded in the dedicated placental table which also requests descriptive details of the placenta including whether injection studies were performed in MC placentas and details of the findings.

**(4) Prematurity:** the cause of death is explained by the initiation of preterm delivery only and in the case of neonatal death also, with the associated problems of prematurity/immaturity.

- (4.1) Preterm prelabour rupture of membranes (PPROM) initiates preterm delivery.
- (4.2) Preterm labour where uterine contractions initiate preterm delivery.
- (4.3) Cervical dysfunction initiates preterm delivery.
- (4.5) Other where prematurity is the cause of death but it is not clear how preterm delivery was initiated.

**(5) Infection:** the cause of death is explained by an infection resulting in sepsis and stillbirth or neonatal death. There is a clear microbiological evidence of infection with matching clinical and pathological findings.

- (5.1) Transplacental where there is a haematogenous infection through the spiral arteries, the placenta and the umbilical cord to the fetus such as Parvovirus infection. If there is evidence of chorioamnionitis on the membranes, this should be documented in the placental table.
- (5.2) Ascending where there is an ascending infection from colonisation of the birth canal such as Streptococci group B infection.
- (5.3) Neonatal where there is infection acquired after birth such as Escherichia coli sepsis–meningitis.
- (5.4) Not otherwise specified (NOS) where there is infection, but it cannot be discerned whether the infection was transplacental, ascending or acquired after birth.

**(6) Other:** the cause of death is explained by another specific cause not mentioned in the previous groups of cause of death.

- (6.1) Fetal hydrops of unknown origin.
- (6.2) Maternal disease is severe enough to jeopardise the fetus or the neonate, initiating death. Examples might be severe maternal sepsis or alloimmunisation. For most maternal medical conditions, this classification will only apply when the disease leads directly to perinatal death, as in diabetic ketoacidosis. Otherwise, the condition is a risk factor.
- (6.3) Trauma
    - (6.3.a) Fetal such as selective reduction or birth trauma.
    - (6.3.b) Maternal such as severe road traffic accidents.
- (6.5) Following medical intervention is to be selected if an intentional intervention such as fetoscopic laser ablation or selective reduction for a co-twin has led to unintentional death.
- (6.6) Iatrogenic cause of death is assigned when intentional twin reduction or termination of pregnancy has occurred without an underlying lethal cause of death.