Villitis of Unknown Etiology (VUE): Unravelling Placental Dysfunction and Causes of Stillbirth and Fetal Growth Restriction

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

Doctor of Philosophy

in the Faculty of Medical and Human Sciences

2015

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Word Count: 43962

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LIST OF ABBREVIATIONS

Ab	antibody
Ag	antigen
AGA	appropriate-for-gestational age
CD	cluster of differentiation
CIUE	chronic intervillositis of unknown etiology
CK7	cytokeratin 7
CMRL	Connaught Medical Research Laboratories
CTB	cytotrophoblast
CTL	cytotoxic T lymphocyte
DAB	3,3'-diaminobenzidine
DAMP	damage-associated molecular pattern
DAPI	4',6-diamino-2-phenylindole
ELISA	enzyme-linked immunosorbent assay
EVT	extravillous trophoblast
FBS	fetal bovine serum
FGR	fetal growth restriction
H&E	haematoxylin and eosin
hCG	human chorionic gonadotropin
HBC	Hofbauer cell
HCQ	Hydroxychloroquine
HLA	human leukocyte antigen
HMGB1	high mobility group box 1

hPL	human placental lactogen
IBC	individualised birthweight centile
IBR	individualised birthweight ratio
IFN-γ	interferon-γ
IHC	immunohistochemistry
IL	interleukin
IUGR	intrauterine growth restriction
M mAb	mouse monoclonal antibody
MAC	membrane attack complex
meAIB	N-methylated aminoisobutyric acid
MFHRG	Maternal and Fetal Health Research Group
MHC	major histocompatibility complex
MVM	microvillous membrane
NBF	neutral buffered formalin
NE	neutrophil elastase
OCT	optimal cutting temperature
ONS	Office for National Statistics
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PGH	placental growth hormone
PlGF	placental growth factor
PMSF	phenylmethanesulfonylfluoride

PRR	pattern recognition receptor
PTLI	pre-term labour with infection
R pAb	rabbit polyclonal antibody
RPMI	Roswell Park Memorial Institute
RFM	reduced fetal movement
SGA	small-for-gestational age
STB	syncytiotrophoblast
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TGF-β	transforming growth factor-β
Th	T helper
TLR	Toll-like receptor
TNFα	tumour necrosis factor α
uNK	uterine natural killer
VIE	villitis of infectious etiology
VUE	villitis of unknown etiology
WHO	World Health Organisation

SCIENTIFIC ABSTRACT of thesis entitled **"Villitis of Unknown Etiology (VUE): Unravelling Placental Dysfunction and Causes of Stillbirth and Fetal Growth Restriction"** submitted by **Hayley Derricott** for the Degree of **Doctor of Philosophy** from the **University of Manchester, September 2015**

Many researchers in the academic and clinical communities theorise that inflammation may underpin the placental dysfunction to which the majority of fetal growth restriction (FGR) and stillbirth cases are attributed. Villitis of unknown etiology (VUE) is an inflammatory condition of the placenta characterised by lesions of macrophages and T cells in the villous stroma.

This study addressed the hypothesis that VUE is a maternal-mediated immune reaction that contributes to FGR and stillbirth by detrimentally affecting placental function. The hypothesis was tested by: 1) completing a systematic review of the literature to confirm implied links of VUE to poor pregnancy outcome, 2) performing a detailed characterisation of the cellular phenotype of VUE in stillbirth, 3) developing an *in vitro* model of VUE and 4) examining the functional effects of VUE using this model.

A systematic review of the literature revealed that VUE occurred in 28.6% of placentas from FGR pregnancies compared to 15.6% of placentas from appropriately grown infants ($p<0.0001$), confirming the implied association. There were insufficient published studies to be able to corroborate a link with stillbirth.

Elevated numbers of macrophages, CD4 and CD8 T cells were quantified in VUE lesions. There were significant increases in pan-placental CD4 and CD8 T cell presence in placentas from stillborn infants with VUE ($p<0.0001$). A greater staining area of pro-inflammatory cytokines interleukin (IL)-2 ($p<0.05$) and IL-12 ($p<0.0001$) was recorded in VUE lesions and a reduction in the anti-inflammatory cytokine IL-4 in the stillbirth with VUE cohort. Dual immunofluorescence of cell markers and cytokines implies that the immune response in VUE is directed towards Th1-type cell-mediated immunity.

An *in vitro* model of VUE was developed that enabled co-culture of explants with fluorescently labelled T cells isolated from matched maternal whole blood samples. Placental tissue and T cells could be maintained in culture for the required duration of the experiment and placental function was not affected by preparation and culture conditions. *In vitro* co-culture with maternal T cells resulted in a significant reduction in placental function as measured by hCG secretion ($p=0.015$). There were significant increases in culture supernatant concentrations of IL-1β ($p=0.008$), IL-10 ($p=0.02$), interferon-γ ($p=0.02$) and IL-1Ra ($p=0.05$) and tissue lysate concentrations of IL-6 ($p=0.008$) and IL-1β ($p=0.02$). Culture of explants with a combination of IL-2, IL-12 and anti-IL-4 significantly reduced hCG secretion compared to control ($p=0.03$).

These studies indicate that VUE involves a Th1-type immune response that may affect placental function, the impact of which might be impaired fetal growth that could contribute to stillbirth. The novel *in vitro* model facilitates future investigations into the pathophysiology of VUE.

LAY ABSTRACT of thesis entitled **"Villitis of Unknown Etiology (VUE): Unravelling Placental Dysfunction and Causes of Stillbirth and Fetal Growth Restriction"** submitted by **Hayley Derricott** for the Degree of **Doctor of Philosophy** from the **University of Manchester, September 2015**

Stillbirth (the death of a baby before birth) affects 10 families every day in the UK. In 60% of stillbirth cases the placenta, the organ that allows nutrients and oxygen to move from mother to baby, goes wrong. This somehow prevents essential nutrients and oxygen from being transferred to the baby, which can reduce its growth (fetal growth restriction; FGR). If FGR is severe the baby is at a much greater risk of being stillborn.

There is a great deal of interest in the role of the mothers' immune system in pregnancy complications. An increasing body of evidence suggests that inflammation may be important in many cases of FGR and stillbirth. Our study focussed on a condition called villitis of unknown etiology (VUE), which is believed to represent a mother's immune system recognising the placenta as foreign and rejecting it. VUE is thought to impair placental function which might then go on to cause FGR and/or stillbirth.

Previously published studies related to VUE were obtained and analysed. Their results established that VUE is found in significantly more placentas from FGR babies compared to placentas from healthy pregnancies. However, too few studies have been published to enable a definitive link of VUE with stillbirth and this warrants further investigation.

This study examined cell types in areas of VUE in the placentas of stillborn infants. The microscopic lesions in the placenta in VUE are made up of specific white blood cells called macrophages, CD4 T cells and CD8 T cells. We also observed, for the first time, evidence that T cells were present in areas of placenta that looked otherwise healthy. The location and amount of the chemical messengers (cytokines) that these cells produce was also altered in stillbirth with VUE.

An experiment was devised that allowed growth of fragments of placenta with T cells that had been isolated from mothers' blood samples. Initial experiments showed that tissue and cells could be prepared without affecting the way that either cell type functioned. Culturing tissue and cells together for a period of 4 days altered the function of the placenta; it produced less of an important hormone called hCG and more cytokines that are known to be associated with inflammation. Culturing tissue with cytokines also caused a decrease in hCG secretion.

These studies have shown that the cells and cytokines involved in VUE have the potential to damage the functional ability of the placenta. The new experiments can be used as a basis for further studies into the effect of inflammation on the way this critical organ works.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

COPYRIGHT STATEMENT

ACKNOWLEDGEMENTS

I express my sincere gratitude to my supervisors Dr Alexander Heazell, Dr Rebecca Lee Jones and Dr Susan Greenwood. Your patience, knowledge and guidance has driven me to keep going when things weren't working quite as I would have hoped. You have encouraged me to be self-reliant and helped me to believe that I was capable of completion. Thanks to you I have been able to present my work at local, national and international meetings, travel to new places and meet some truly inspirational people. The last three years could have been so different if I hadn't had my supervisory 'dream team'.

Thanks also go to the women who have donated placentas and bloods for my studies – I literally could not have done it without you. I'm grateful to the recruiters, midwives and Anthony Nolan team who work tirelessly to consent women so that we have tissue to work with. I am especially grateful for the times when I requested (and got) maternal bloods. Dr Mark Wareing co-ordinates the Biobank and organises the many requests each day, without him chaos would ensue. I am totally indebted to Sue for the time she spent with me, training me in explant culture, tying off explants and supervising me on uptake days. I don't know if you'll ever realise how much I appreciate your support. Thanks to Rebecca and Sylvie for their help with everything immunohistochemical and immunological. Everyone at MFHRG welcomed me into the lab and tolerated a myriad of questions about antibodies, solutions, ordering, microscopes, glassware, protocols etc. Moreover, my penchant for labelling everything seems to have been accepted and for that I thank you all. Thanks to my advisor, Dr Jo Pennock, who has been on hand to answer my numerous questions of an immunological nature.

Last, but by absolutely no means least, huge thanks to my family for their unwavering support. To know that you have been behind me 100% makes the long hours and long days worthwhile. This is as much for you as it is me. Dave; you have helped to make this a reality and, I'm sorry, but you will have to call me Dr Derricott. Mum; no I have not, and probably never will have finished all my work.

THE AUTHOR

HD graduated from the University of Manchester in 2002 with a BSc. (Hons) in Anatomical Sciences. She worked as an Anatomy Prosector and Teaching Fellow at Keele University for 9 years before starting her PhD in the Maternal and Fetal Health Research Group in October 2012.

ALTERNATIVE FORMAT

An alternative format thesis is presented by the postgraduate doctoral student as a series of sections suitable for submission to a peer-reviewed journal. Article-style results in the thesis include those which are solely/jointly authored by the student and should represent an original contribution to the field of research.

Alternative format was chosen for this thesis as Chapter 3, a systematic review of the literature, has been published in the journal *Placenta.* Subsequent laboratory work and their corresponding results chapters naturally formed chapters suitable for publication. Chapters 4 and 5 are currently in review with the *American Journal of Pathology* and the *Journal of Biological Methods,* respectively. Chapter 6 is being prepared for submission to the *Journal of Immunology.*

CHAPTER 1: Introduction

Why would a pregnancy devoid of any obvious complications result in the death of the unborn child? The question has been asked by parents, obstetricians, pathologists and researchers over many years. In 1938, Mabel Potter discussed "fetal mortality in the offspring of apparently healthy women" (1) and in 1967, Benirschke and Driscoll talked of "burgeoning interest" in fetal health (2). Successful term pregnancy is influenced by a great many variables yet these same variables also have the potential to exert a negative influence. It is only when we understand and appreciate the normal development and function of the structures involved that we can begin to unravel the complications that occur. The placenta is an organ pivotal to the success of pregnancy; knowing its origin, structure and function is key to interpreting its role in fetal growth and survival.

Adverse outcomes of pregnancy can describe maternal or fetal disorders such as, pre-eclampsia/eclampsia or fetal growth restriction (FGR) resulting in preterm birth, maternal death or stillbirth. Risk factors that can co-exist and may be associated with a poor pregnancy outcome include maternal obesity, advanced maternal age, pre-existing or acquired maternal illness and recurrent pregnancy failure, amongst others (3). In addition, abnormalities of placentation and/or placental dysfunction underpin many pregnancy complications (4).

This project investigated villitis of unknown etiology (VUE), an inflammatory condition of the placenta thought to be a contributor to pregnancy complications such as FGR and stillbirth. An association of VUE with pregnancy complications was first established, then the morphology of VUE lesions was comprehensively described. Finally, an *in vitro* model of VUE was developed to examine the effects of inflammation on placental function. This introductory chapter provides an overview of poor pregnancy outcomes and describes the global nature of their occurrence. It will describe the development of the placenta and its mature morphology along with key terms critical for understanding the discussions on this topic. There follows summarised information key to understanding the basics of the immune system and how it specifically changes during pregnancy. Inflammatory conditions of the placenta are discussed along with the potential influence they may have on pregnancy outcome.

1.1 Stillbirth

As an expectant parent, experiencing a stillbirth is a profoundly traumatic event that has enduring physical, economic, psychological and social consequences (5,6). Worldwide, the World Health Organisation (WHO) reported an estimated 2,600,000 stillbirths in 2009, a rate of 18.9 per 1,000 live births, 98% of which occur in low- and middle-income countries (7). In 2013, according to the Office for National Statistics (ONS), 3,284 stillbirths occurred in England and Wales; this equates to 4.7 per 1,000 or 1 in 212 live births. The stillbirth record

in the UK is poor, ranking 33 out of 35 high-income countries. UK stillbirth rates have seen little improvement over the period 1998-2013, with a peak at 5.8 per 1,000 live births in 2003 (8). There is a disparity in the rates seen in different countries, confounded by the fact that reports are estimates, obstetric care is variable and the definition of stillbirth is not consistent worldwide (Table 1). High income countries report a rate of approximately 3 per 1,000 live births (≥28 weeks) whereas in Sub-Saharan Africa this rate can reach 28 per 1,000 live births. A reduction in global incidence of 1.1% per year has been documented in the past decade, yet this rate is slower than that seen in maternal and neonatal mortality rates. Indeed, WHO Millennium Development Goals do not record reducing stillbirth rates as a priority. A 2011 Lancet series on stillbirth highlighted the ongoing ignorance of fetal mortality and stated that this is an important health issue on a global scale (9).

Whilst some causes of stillbirth are known and risk factors that contribute are recognised, historically approximately half of all cases remain unexplained (10–12). Analysis of studies carried out to assess causes of, and associations with, stillbirth is impeded by different definitions that are in place in different countries. WHO defined stillbirth as "late fetal deaths weighing at least 1000 grams or occurring at or beyond 28 weeks gestation" (7). However, many countries do not use this definition and it is not routinely used in studies investigating the phenomenon. In addition, many systems exist that attempt to classify causes of stillbirth in an effort to reduce the number of deaths reported as 'unexplained'. Table 1 details some of the differing definitions of stillbirth cited in the literature.

Table 1: Examples of different definitions of stillbirth

Author/Country	**Definition of Stillbirth**
WHO (7)	≥28 weeks gestation or ≥1000g weight
Rayburn et al., 1985 (13) Bukowski, 2010 (14) United States (15)	≥20 weeks gestation
Gardosi et al., 1998 (10) United Kingdom (16) Del Rosario et al., 2004 (17)	≥24 weeks gestation
Romero-Gutiérrez et al., 2005 (18)	≥21 weeks gestation
Vergani et al., 2008 (19)	≥22 weeks gestation or >500g birthweight
Walsh et al., 2008 (20)	In utero fetal death ≥20 weeks gestation
Hulthén Varli et al., 2012 (21)	≥ 22nd week of pregnancy

Over 30 fetal/neonatal death classification systems exist, each with their own approach and focus (4,22). More recent systems aim to reduce the number of stillbirths where cause of death is reported as 'unknown' (22–24). Wigglesworth's classification system (1980) reports

66% of stillbirths as 'unexplained' whereas this is reduced to approximately 15% with Tulip and ReCoDe and to 10% with Codac classification systems (4,12,19,23). It is not within the scope of this chapter to analyse the merits of each system, though it is worth appreciating the diversity of classification methods and their effects on the understanding of causative and associated factors in cases of stillbirth.

A comprehensive review of contemporary classification systems, published in 2009, demonstrated that placental pathologies are the most frequent abnormality associated with stillbirth, accounting for up to 60% of stillbirths (4). It is these placental pathologies, and particularly inflammatory conditions, that are specifically relevant to this project.

1.2 Fetal Growth Restriction

Fetal growth restriction (FGR) or intrauterine growth restriction (IUGR) is defined as "a birth weight below the 10th percentile for gestational age" and refers to a fetus' failure to reach its genetically predetermined potential weight (25–28). Studies investigating this phenomenon tend to use the terms "fetal growth restriction" and "intrauterine growth restriction" interchangeably, though FGR is the more commonly accepted term today. In some studies small-for-gestational age (SGA) is used, although this descriptor can be interpreted in different ways. The term constitutionally small implies that, though small, the infant has reached its genetically predetermined growth potential. Pathologically small, however, is described in similar terms to IUGR and FGR (11). Reliably identifying FGR infants is problematic and birthweight alone cannot be relied upon. One approach, individualised birthweight ratio or centile (IBR/IBC) measures the difference between actual and predicted birthweight (29). Predicted birthweight is calculated from a variety of contributory factors including maternal height and weight, ethnic origin and infant sex. However, recently published results from the INTERGROWTH study have suggested that fetal growth is consistent irrespective of ethnicity (30). Nevertheless, the lower the infants IBR, the greater the likelihood it is related to FGR (11,31,32). Approximately 30% of infants below the 10th centile display FGR increasing to around 70% in infants below the 3rd centile (32). FGR occurs in approximately 5-10% of pregnancies, yet remains undiagnosed in up to 50% of cases (33,34). Various underlying factors may influence the inability to reach true potential size, including environmental, genetic, vascular and placental abnormalities (25). More than 50% of stillborn infants whose cause of death is classed as unknown show growth restriction (23,34). FGR is associated with perinatal morbidity and mortality (35,36) and a 7-fold increase in the risk of stillbirth (14,21).

1.3 Placenta

The placenta is the organ that acts as both a barrier and an interface between mother and fetus, so its role is undeniably critical to the pregnancy outcome. The placenta transports

gases and nutrients, removes waste and secretes hormones whilst maintaining a distinct separation between maternal and fetal circulations. Events occurring very early in placental development can have downstream effects that manifest much later in pregnancy (37). Indeed, events that occur throughout the whole pregnancy can increase the risk of cardiovascular and metabolic diseases in postnatal life (38). Though the understanding of placental morphology and the complexity of the process that brings this structure into being are well described, there remain aspects that are still poorly understood.

1.3.1 Development of the Placenta

In preparation for implantation of the embryo, maternal endometrium undergoes a remodelling process called decidualisation. This also occurs normally, though less extensively, as part of the menstrual cycle (39). Decidualisation involves transformation of the uterine stroma, arteries and glands and an influx of uterine natural killer (uNK) cells. Though maternal decidual function is not completely understood it is believed to restrict excessive trophoblast invasion (39,40). In the embryo, prior to implantation, trophectoderm cells completely surround the inner cell mass as it changes from morula to blastocyst. The precursor cells of parts of the placenta, the cytotrophoblasts, begin to differentiate around 4-5 days post-fertilisation (41). Once implantation has occurred the trophoblast cells begin further differentiation, producing a multi-nucleated invasive syncytial layer. This results in syncytiotrophoblast (STB) and cytotrophoblast (CTB) layers surrounding the embryo. CTB cells continue proliferating throughout pregnancy, differentiating and fusing into the STB to replenish it. However, the CTB does not expand at the same rate as the rest of the placenta. Thus, the initially continuous cell layer appears to become discontinuous over time (42) though it has been hypothesised that CTB cells continue to communicate throughout pregnancy by means of interdigitating cellular processes (43).

At the beginning of the second week post-conception, extracellular vacuoles begin to appear in the STB that will combine to form lacunae. Initially lacunae are filled with tissue fluid and uterine secretions, separated by columns of STB (trabeculae). Eventually the trophoblast will erode maternal capillaries, superficial venous plexuses and finally spiral arteries leading to maternal blood filling the lacunae (27,42,44). Thus, the intervillous space is formed (Figure 1). Maternal spiral arteries are invaded and remodelled by extravillous trophoblasts (EVT) that replace the smooth muscle of the vessels (27,42,45). There is also evidence that the spiral arteries are invaded by decidual macrophages and natural killer (NK) cells (46–48). EVTs, therefore, are fetal-derived cells that are the first to come into direct contact with the maternal immune cells and potentially expose the mother to foreign antigen. They may also interact to bring about successful spiral artery remodelling (49).

A villous tree must be established in order that efficient transfer of nutrients can take place as the fetus grows and demands are increased. As the STB layer invades the decidua, column-like syncytial sprouts elongate and begin to form the morphology of the villous tree. By 14 days post conception these sprouts are invaded by CTB cells, forming a primary villus that protrudes into the newly formed intervillous space (Figure 1). However, to become a functional villus blood vessels need to inhabit it. The blood vessels are formed from extraembryonic mesoderm that migrates from the inner cell mass. The mesoderm contributes to the chorionic plate and the umbilical vein and arteries as well as the villus vessels. The migration of mesoderm into the core of the trophoblastic structure establishes a secondary villus; tertiary villi, with capillary networks, will be established by 20 days post conception.

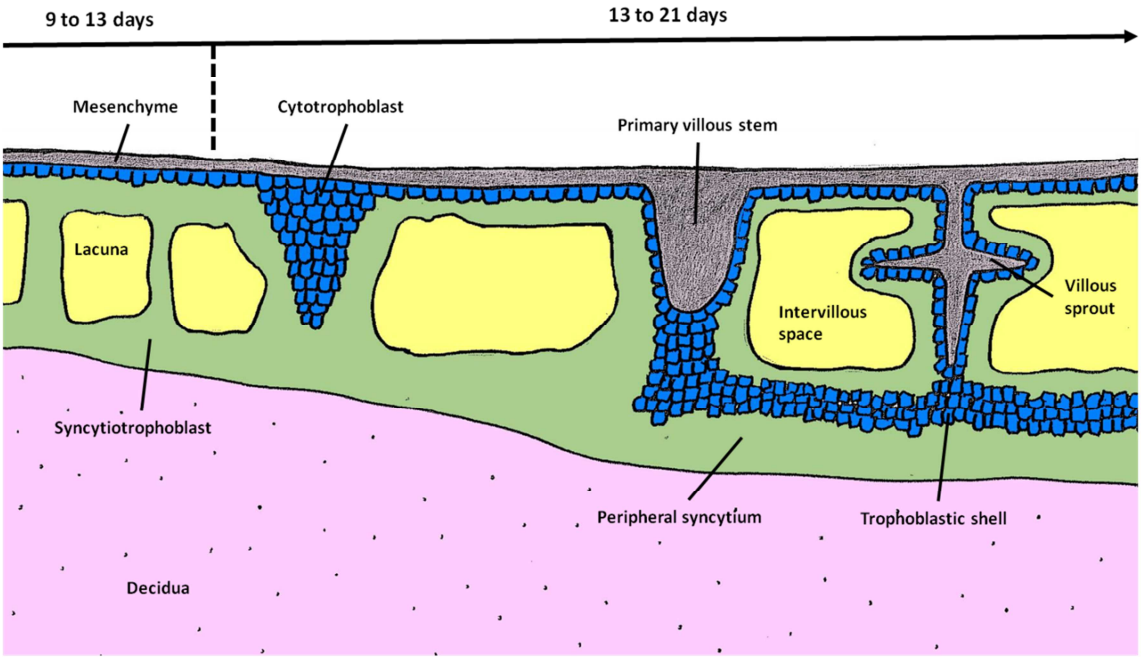

Figure 1: Simplified diagram of the development of the placenta. Redrawn from Fox, Pathology of the Placenta (42)

Fetal macrophages (Hofbauer cells (HBC)) found in the villous stroma are thought to initially differentiate from villous mesenchyme before fetal bone marrow assumes responsibility once the fetal circulation is established (42,50). HBCs are thought to play a role in angiogenesis and establishing the villous tree (50). As blood and endothelial cells begin to develop and the placental vessel network is established, the tertiary villi are formed (Figure 2). The placental and chorionic vessels develop separately from the fetal circulation itself then join at a later stage.

There are several different types of villi that form throughout placental development, each displaying a distinct morphology (41). Of particular relevance to VUE are the stem and terminal villi. Understanding the normal structure and cellular phenotype of the villi assists when examining the consequences of alterations to villous morphology.

Stem villi are large (up to 3000μm in diameter) and serve mainly as structural support to the villous tree. They characteristically display relatively large blood vessels in the centre of the villi and are surrounded by fibrous tissue. Due to the morphology of these villi and their central vessels it is thought that they play little role in maternal-fetal nutrient and gas exchange (41,51).

The terminal villi are the ends of the branches and the smallest diameter structures (around 80μm). They are the functional subunits of the placenta. They are inhabited by many capillaries and, in cross-section, about half of their area is occupied by these vessels (41,51). Additionally, the STB is thinner, the CTB cells have become discontinuous and the capillary and STB share a basement membrane (vasculosyncytial membrane). The STB is the interface between the maternal blood in the intervillous space and the fetal capillaries and is also the solute transport epithelium of the human placenta (39). In a morphology roughly analogous to the alveoli of the lung, the vasculosyncytial membrane permits efficient gas exchange through a very narrow area (Figure 2).

The placenta transfers oxygen and nutrients from maternal blood to the fetus and transfers the waste products of fetal metabolism to maternal blood for elimination. Several mechanisms exist to effect the movement of substances across the plasma membranes of STB, from diffusion in the case of gas exchange to active transport e.g. amino acids (27) (see Section 1.3.3).

The mothers' contribution to the placenta, the decidua basalis, forms the maternal aspect of the interface. As detailed previously, fetal EVTs can be localised to the walls of the spiral arteries. Non-uterine maternal cells present in the decidua in large numbers are leukocytes, particularly macrophages and uNK cells (40). Immune modulation at the maternal-fetal interface is critical for a successful pregnancy and will be discussed in more detail in Section 1.7.4 (52–54).

1.3.2 Mature Placental Morphology

In order to understand abnormalities in the morphology of the third trimester placenta it is first necessary to appreciate its normal appearance. As described previously, during development and maturation the placenta undergoes a series of morphological changes, which permit the organ to become an efficiently functioning unit to adequately provide for third trimester metabolic demands of the fetus. Figure 2 shows a schematic representation of the microscopic appearance of a terminal villus and Figure 3 the macroscopic appearance of the mature placenta.

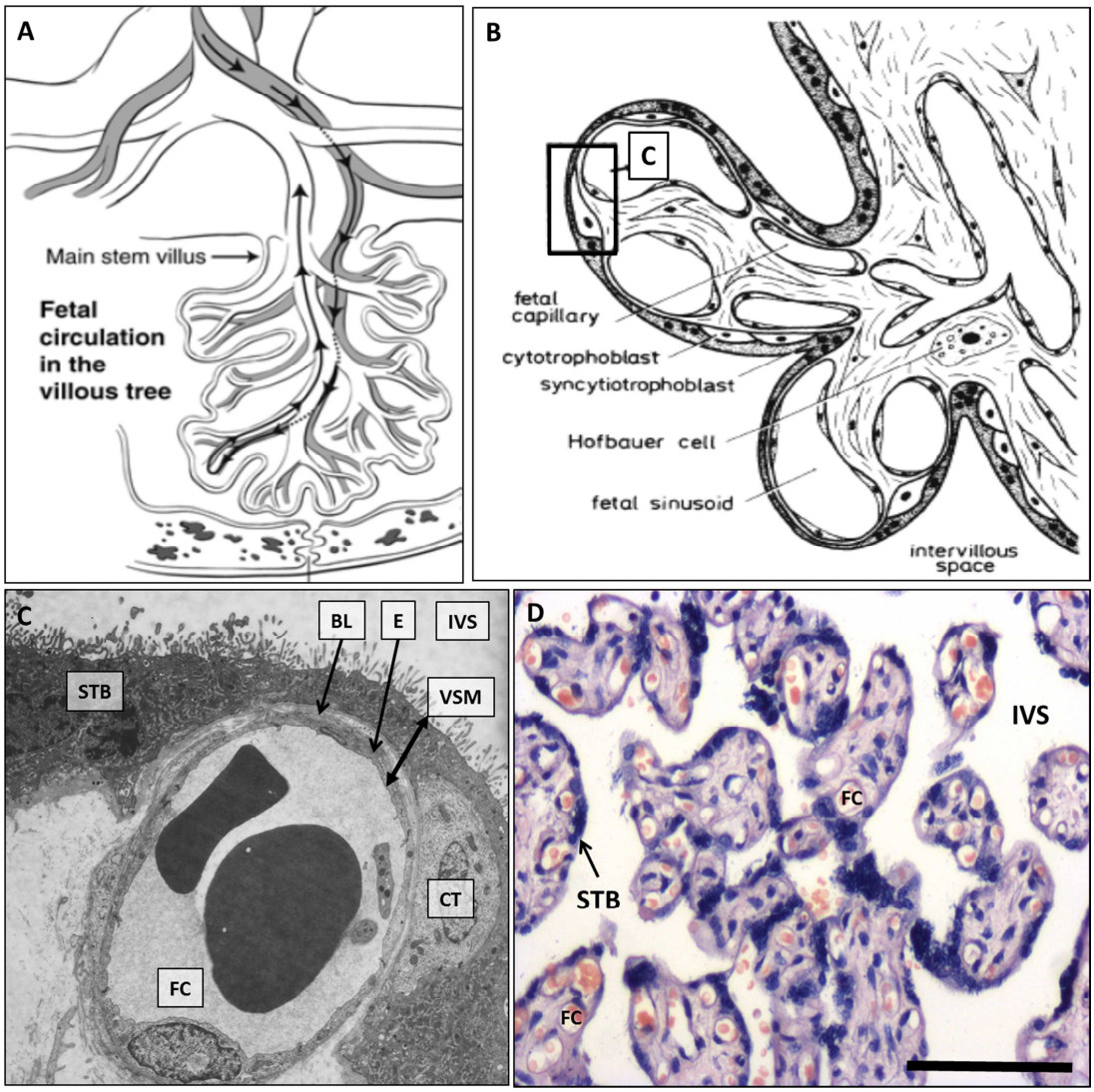

Figure 2: Diagram to show the structure of the villous tree and vasculosyncytial membrane.
A) The location of the fetal circulation in the villous tree, B) The microscopic appearance of a terminal villus showing the vasculosyncytial membrane, C) Electron micrograph of the vasculosyncytial membrane (VSM), D) H&E section of normal villous tissue, scale bar represents 100µm, original magnification x100. IVS: maternal blood-containing intervillous space, FC: fetal capillary containing fetal blood, STB: syncytiotrophoblast, CT: cytotrophoblast cell, BL: basal lamina, E: endothelium. Images A and B reproduced from Baergen, Manual of Benirschke and Kaufmann's Pathology of the Human Placenta (51), image C courtesy of Dr C. J. P. Jones, Maternal and Fetal Health Research Group, Manchester, image D courtesy of Miss S. Lean, Maternal and Fetal Health Research Group, Manchester.

Figure 2 and Figure 3 illustrate that the morphology of the placenta is critical to the outcome of the pregnancy. These diagrams show that the placental tissue develops in order to minimise the distance between the maternal and fetal circulations, thus maximising the transport of gases. Key to the functional capacity of the placenta is the continual turnover of the trophoblast layer. Mononucleated CTB cells proliferate throughout pregnancy and are critical for maintenance of the maternal facing STB layer. CTBs leaving the cell cycle have the appearance of intermediate cells with high concentrations of mitochondria, mRNA, endoplasmic reticulum and ribosomes. Eventually, these cells (and their contents) will fuse with the syncytial layer. The multinucleated STB is in contact with CTB and basal membrane and has an apical surface displaying microvilli. The STB is a single, continuous layer that

covers the villi in their entirety; the microvilli contribute to a sevenfold increase in surface area. The STB does not demonstrate any proliferative activity so the maintenance of this structure is entirely reliant on the continued incorporation of CTB throughout pregnancy. Effete syncytial nuclei form syncytial nuclear aggregates (SNAs) that are reported to be released into the mother's circulation (39,55–58). Numerous transporters are located on the maternal facing microvillous membrane (MVM) that are responsible for ensuring adequate nutrient transfer from mother to fetus (39,59–61) The fetus is highly metabolically active during the third trimester and has a large oxygen and nutrient demand so efficiency is key to a successful outcome. Disruption to the balance of cell turnover events that maintain STB can lead to loss of function and disease (62–64).

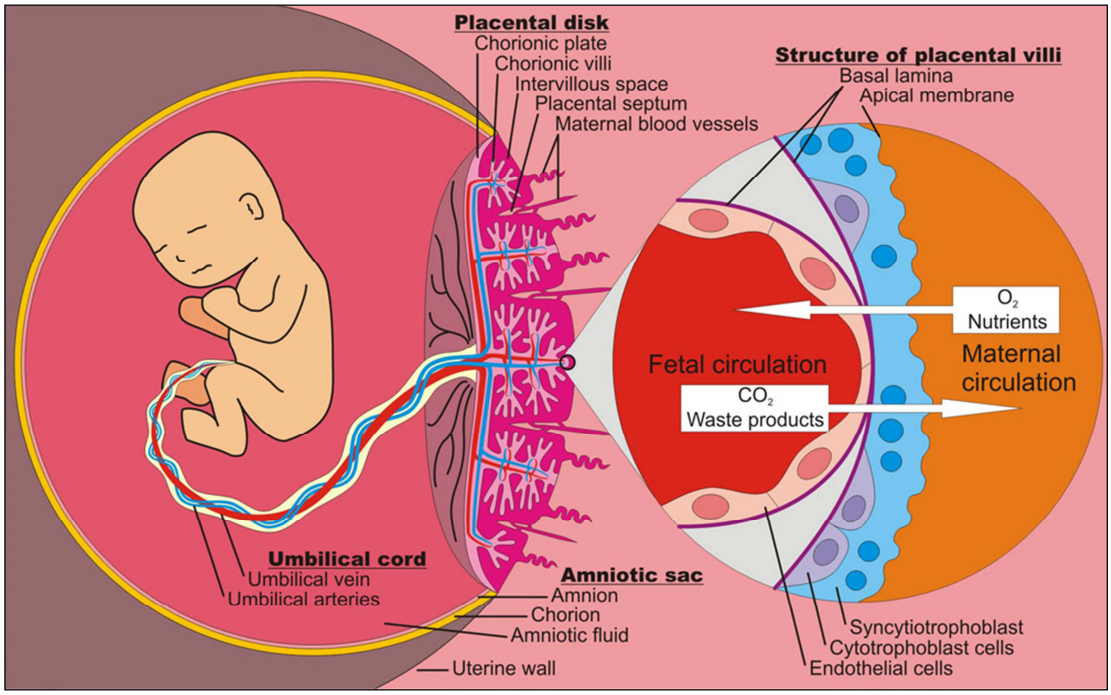

Figure 3: The macroscopic appearance of the mature placenta.
Reproduced from: "Placental Structure and Transport" Website of the Institute of Biochemistry and Molecular Medicine, University of Bern.
http://www.ibmm.unibe.ch/content/groups/albrecht_group/projects/transporters_and_human_placenta/placental_structure_and_transport/index_eng.html

1.3.3 Mature Placental Function

The key to a successful pregnancy is an efficiently functioning placenta. This specialised organ is required to transfer the majority of nutrients, gases and water needed by the fetus as well as returning waste products to the maternal circulation. Fetal growth, therefore, is intimately related to placental function. The placenta is also an endocrine organ that produces numerous hormones required to establish and maintain pregnancy. In addition, this organ contributes to the protection of the fetus from some infectious agents, xenobiotics and maternal diseases.

The STB permits the rapid diffusion of oxygen from maternal to fetal blood because of its permeability to this gas. Fetal haemoglobin also has a higher affinity for oxygen than maternal haemoglobin thus facilitating oxygen transfer to the fetus (39,61).

Where substrates are less permeable both active and passive transport mechanisms are required to fulfil the demands of the fetus. Transporters are necessary for transfer of substances such as amino acids and glucose. The fetus is capable of very little gluconeogenesis so this primary source of fetal energy must be obtained from the maternal circulation (39). Transfer across the placenta is achieved via various facilitated diffusion transporters (GLUTs). It is believed that GLUT1, expressed on both maternal facing MVM and fetal facing basal membrane, is primarily responsible for glucose transport in late pregnancy (39,60,65).

The fetus requires amino acids for protein synthesis, and whilst some can be synthesised, some (the 'essential' amino acids) cannot. The ratio of fetal to maternal plasma levels of most amino acids is greater than one suggesting an active transport mechanism is present in the placenta. At least 15 placental amino acid transporters have been identified (65) in the STB MVM. The characteristics and capabilities of these MVM-based transporters determine amino acid transfer and therefore fetal growth. Extensive literature exists in relation to transporters; one of the most well described is the system A transporter (39,60,61,65). System A is a sodium-dependent non-essential amino acid transporter, highly polarised to the MVM. It transports amino acids such as alanine, serine and glycine into STB against a concentration gradient. Reduced system A activity has been well documented in placentas from FGR pregnancies (60,61,65,66) and one report with maternal perception of reduced fetal movements (RFM) (67); which are both at increased risk of FGR and stillbirth (67,68).

Numerous hormones, produced by the placenta, may act in an autocrine, paracrine or endocrine manner. It is not feasible to describe them all here, however, the key contribution of some hormones should be recorded. The placenta produces progesterone that promotes uterine quiescence and thus suppresses the labouring process (39,69). It is also a major source of circulating oestrogens that act as growth factors for maternal reproductive organs (70). The trophoblast produces human chorionic gonadotropin (hCG) in both early (8-12 weeks) and late pregnancy. hCG stimulates CTB differentiation and cell fusion and ensures continued progesterone production (39,70,71). Human placental lactogen (hPL) is synthesised by STB and has numerous developmental and metabolic effects. Placental growth hormone (PGH) can alter insulin production and promote maternal insulin resistance to increase availability of glucose for the fetus (71). Placental growth factor (PlGF) is a potent angiogenic factor that may promote mobilisation of epithelial precursors to stimulate growth of new vessels (72). Evaluation of placental cell turnover, transport capability and hormone production are all used experimentally as proxy markers of placental function. A recent review

has suggested that biochemical assessment of placental function by hPL and PlGF levels in maternal serum could be used to identify adverse pregnancy outcomes mediated by placental insufficiency (73).

1.4 Placental Dysfunction

It has long been recognised that the fetus, umbilical cord and placenta are a single functional unit and "adequate placental function is paramount to fetal survival". Furthermore, "the placenta is the co-victim with the fetus in many antenatal disorders" (2). It has been suggested that failure of the terminal villi to develop may be responsible for inadequacy of blood flow or there may be an insufficient number of villous arteries (35). Fetal infection and inflammation may lead to tissue destruction. Similarly, maternal infection, inflammation or infarction could lead to placental dysfunction (23,24,27,42). Placental dysfunction is one of the major causes of pregnancy complications that include stillbirth and FGR, and has been described as the most common aetiology associated with term stillbirth (20,74). Placental insufficiency or dysfunction describes an inadequate supply of oxygen and nutrients to the fetus. The term incorporates dysfunctional blood flow and nutrient transport capacity as evidenced by altered umbilical artery Doppler (73,75) and system A activity (67), respectively. It is possible that underlying morphological differences may contribute to placental insufficiency though the functional significance of placental lesions is not yet clear (73).

1.5 Placental Pathology

In 1975, whilst reporting the incidence of placental lesions, Altshuler and Russell (76) recognised that potentially significant lesions could be overlooked if the role of the placenta is disregarded, a belief reiterated by Khong et al. many years later (77). The fact that many consider the placenta to be the "black box" of a pregnancy implies that much can be learned about pregnancy from the information it contains (78–80). Altshuler and Russell (76) further suggest that should such lesions occur in more comprehensively studied organs, they would be much more readily recognised and reported.

Placental pathologies can detrimentally reduce utero-placental and feto-placental blood flow and/or nutrient transport leading to clinically relevant conditions such as FGR and stillbirth (35,61,65). Inflammatory conditions are just one of a number of placental pathologies that have the capability of affecting fetal/neonatal health (81) and there are various associated aetiologies. Examples of inflammatory conditions affecting the feto-placental unit include: chorioamnionitis, funisitis, villitis and deciduitis. These inflammatory conditions may occur following bacterial, viral or parasitic infection and are associated with increased perinatal morbidity and mortality. This project focusses on inflammation in the villus parenchyma - villitis.

1.6 The Villitides

It is more than 35 years since Altshuler and Russell (76) intricately described the "human placental villitides" from both an aetiological and morphological perspective. These authors present a comprehensive list of infectious agents and their presentation in pregnancy pathologies. At the time of publishing it was presumed that infectious causes were responsible for all inflammatory placental disorders. Their work became a benchmark for the descriptions and characteristics of villitis that are still used today.

The authors described the histological appearance of villitis of increasing severity as follows (76):
Focal villitis: villous lesions in sporadic, isolated villi
Diffuse villitis: random groups of continuous villi involved, very rarely affecting the entire placenta
Proliferative villitis: increased numbers of inflammatory cells, mainly polymorphonuclear neutrophils, in the villous tissue
Necrotizing villitis: lesions showing evidence of necrosis
Reparative villitis: evidence of fibrosis indicating a stage of repair

Altshuler and Russell were the first to describe villitis of unknown etiology (VUE). Because the placentas they examined failed to present a known bacterial/viral cause, the nomenclature of VUE was selected. Today VUE is classified as low grade or high grade according to the classification system of Knox and Fox (82). Since Altshuler and Russell's study was published much work has been done to elucidate a causative agent underlying the cases where an infectious aetiology has been ruled out.

1.6.1 Villitis of Infectious Etiology

Though comprehensive in its description of inflammatory pathology of the placenta, Altshuler and Russell's 1975 work assumes that all villitis must be of an infectious origin (76). They also assume that, because the chorionic tissue is fetal in origin, then so too is the inflammatory response to the infectious agent. Thus, their definition of villitis became "an intrinsic inflammatory response occurring within one or more villi, the proliferating or infiltrating cells being fetal in origin." However, it has since become apparent that in only a minority of cases villitis has a known infectious etiology, with reported rates ranging from 0-20% (31,82–86). Commonly recognised infectious agents include those of the TORCH group of infections: *toxoplasma gondii*, rubella, cytomegalovirus and herpes. Histological examination of the placenta in these cases demonstrates an infiltration of plasma cells characteristic of an adaptive antibody-mediated immune response (Figure 4A). In addition, it has been shown that the infiltrating cells are of both maternal and fetal origin (87).

In 2012, the Hulthén Varli group (21) investigated the over-representation of growth restriction and inflammation due to infection in cases of stillbirth. Placental dysfunction and SGA was identified in 13% of 126 term stillbirths and counted as one of the most common causes of stillbirth. In common with other studies their results show 21.4% of stillborn infants were SGA compared to 2.9% of live born infants – a significantly higher proportion ($p<0.001$). In addition, histological examination demonstrated higher incidence of inflammatory conditions in both SGA groups. The definition of SGA in this study used the Swedish standard of birth weight <2 standard deviations below mean. Villitis, specifically, was seen in 18.3% placentas of stillborn infants compared to 5.1% in live born infants ($p<0.001$). Unfortunately, the study concentrated on chorioamnionitis so there was no differentiation between villitis of infectious and unknown origin. The analysis of results showed that villitis as a whole is seen three times more often in stillbirth compared to live births.

As villitis of an infectious etiology accounts for the minority of cases seen and is well documented elsewhere further detailed analysis of these studies is not within the scope of this chapter.

1.6.2 Villitis of Unknown Etiology

VUE has been of interest for several decades particularly because there does not appear to be a consistent cause or effect. VUE is reported to occur in 5-15% of all third trimester placentas (35,84,88–90), though its cited incidence varies greatly in different regions (from 7.6% in the UK to 68% in South America) (31,81,83,89). It is rarely seen in placentas prior to 32 weeks gestation, the majority of cases occurring post 37 weeks gestation. It has been implied that an infectious etiology should be suspected in cases of villitis observed in placentas prior to 32 weeks (88). Reported rates differ greatly because of a number of variables: the population studied, the classification of villitis and the placental sampling method used. Altshuler and Russell (76) attempted to correlate the histological appearance of VUE with that of known infectious origin. This was in part because the microscopic appearance (lymphocytic infiltrates) of placental sections with focal VUE in their study was similar to that seen in rubella. However, though comprehensive immunisation programmes have virtually eliminated the incidence of rubella in the UK, VUE still exists. Similarly, the incidence, recurrence rate, pattern of villus involvement and lack of seasonality of VUE distinguish it from villitis of infectious etiology (88).

VUE displays a histological appearance similar to that of villitis of infectious origin but, as yet, no causative agent or antigen has been identified so it is classified separately (88). In the placenta there is a characteristic infiltrate of inflammatory cells, usually in terminal villi and there is also a characteristic absence of villous plasma cells and an insignificant number of neutrophils, excluding bacterial infectious etiology (Figure 4B). Placental tissue appears to be

affected in a random focal fashion with non-affected tissue appearing as normal (88,91). Studies attempting to classify the cells involved have demonstrated infiltration by fetal macrophages (87,89,92) and maternal CD8 T cells (84,87,88) or CD4 T cells (89,93). There are a number of inflammatory conditions that have been reported to occur concurrently with villitis, namely: chronic deciduitis, chronic chorioamnionitis and chronic histiocytic intervillositis (81,84,89,94,95).

It has been suggested that VUE is linked to FGR and stillbirth in term pregnancies (21,76,84,88,96–98), yet it can also occur in pregnancies that deliver an apparently healthy appropriately grown infant (89,95). Redline (88) concluded that an association of VUE severity and distribution with pregnancy outcome exists, a statement that Faye-Peterson refutes (81). Pathak et al. (95) imply that correlation values between VUE and pathology are low. They found that following examination of the placenta they were unable to positively identify pregnancies that may have been at risk. Their study of an unselected population of both low- and high-risk pregnancies demonstrated VUE in 3.7% of both subsets, so not all cases of FGR demonstrated villitis and not all cases of villitis demonstrated a poor pregnancy outcome.

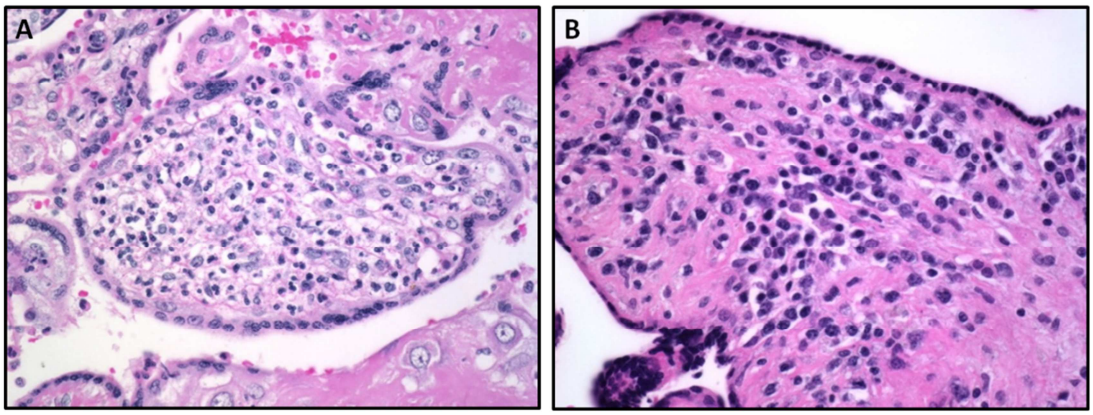

Figure 4: H&E stained villous tissue.
A) neutrophil infiltrate in VIE and B) lymphocyte infiltrate in VUE. Images reproduced from "Female Genital Tract", Web Pathology website. http://www.webpathology.com (99)

Reports of a higher risk (10-25%) of VUE occurring in subsequent pregnancies and increased severity when recurring led to the notion that it has a basis in immunity, possibly a graft rejection of the semi-allogeneic placenta (84,87,100). These observations were based on the work of Redline and Abramowsky (98) and Russell (31). Despite this hypothesis, the stimulus for initiation of the immune response remains unclear. Stated possible mechanisms for initiation of VUE include: breakdown of trophoblast layer exposing the villous stroma (92,98), graft-versus-host invasion (84,98,101) or complement deposition (102,103)

1.6.2.1 Diagnosis of VUE

One of the issues associated with the diagnosis of VUE is that it is a subjective evaluation by pathologists who may not be perinatal specialists, which could affect reliability and reproducibility (77,104). Knox and Fox stated that 4 specimen blocks were adequate to "detect all but a tiny minority of cases of villitis" (82) whereas Altemani et al. (86) specifically sought to ascertain how many specimen blocks were required to accurately diagnose VUE in placental tissue. They found that 8 blocks was the amount required to be able to identify 95% of villitis cases, with 85% accuracy.

Another issue is that the definition of VUE differs between studies, summarised in Table 2. Whilst some authors provide thorough detail of the discriminatory features used to diagnose villitis (95,101) others refer readers to previous work (90,92,97,98) or do not describe any features identified (105). Whilst it may be possible to work out what different researchers may class as VUE it is still notable that none of the methods employed use quantitative definitions. The analyses and results in all studies are qualitative, confirming the subjective nature of diagnosing this disorder. Without a definitive boundary, occurrence rates of VUE could be under- or over-estimated.

Finally, another issue related to interpretation of results is whether villitis of infectious etiology and VUE are regarded as separate entities. Unless specified it is not always clear whether the subsets of villitis are considered separately. In the case of earlier studies such as Altshuler and Russell (76) and Garcia (85) an infectious aetiology was assumed in all cases. Because of this, no attempt was made to differentiate between VIE or VUE as a cause.

1.6.2.2 VUE and Fetal Growth Restriction

VUE is suggested to be associated with fetal growth restriction and in some studies occurs more regularly in births where FGR is recorded (25,88,90,98,101). It is hypothesised that the inflammatory changes in the placenta affect the transfer of nutrients across the STB detrimentally impacting on fetal growth. The effects are particularly noted in the third trimester of pregnancy when fetal growth is at its highest rate and most reliant on the effectiveness of the placenta (28).

Most studies conducted on VUE and FGR examine tissue sections routinely stained with haematoxylin and eosin (H&E) (25,35,90,95,101), and this is also presumed to be the case in those studies that do not specify (21,96,105,108). Whilst H&E stained sections permit the recognition of an infiltration of leukocytes, there is not the cell specificity of staining associated with techniques such as immunohistochemistry.

Table 2: Comparison of the definitions of VUE described in different studies.
^Study designed to assess the optimum number of blocks required to identify villitis in placental parenchyma.

Author/Group	**Year**	**Description of VUE**
Altshuler et al. (106)	1975	Infiltration by inflammatory cells
Russell (107)	1980	Inflammatory foci of graded severity
Garcia (85)	1982	"Intrinsic inflammatory response occurring within the villi, due to proliferation and infiltration of fetal cells."
Knox and Fox (82)	1984	Inflammatory process occurring in varying foci affecting villous tissue
Mortimer (90)	1985	Criteria of Russell (31)
Redline and Abramowsky (98)	1985	Criteria of Altshuler and Russell (76)
Labarrere and Althabe (101)	1987	"Mononuclear (lymphohistiocytic) inflammatory cell infiltration of the villi, with or without necrosis or fibrosis."
Labarrere et al. (92)	1989	Criteria of Labarrere et al. (83)
Salafia et al. (105)	1992	No description
Altemani et al.^ (86)	2003	"Inflammatory cell infiltration of villi."
Becroft (91)	2005	"Lymphohistiocytic cellular infiltration and disruption or expansion of the stroma."
Aviram (96)	2010	"Villi infiltrated by lymphocytes."
Feeley and Mooney (97)	2010	"The presence of lymphocytes and/or histiocytes in villi graded from 1 to 4" (criteria of Knox and Fox (82))
Pathak (95)	2011	"Morphologically increased chronic inflammatory cells affecting groups of chorionic villi in the absence of known clinical or histological aetiological factor/organisms"
Vedmedovka (25)	2011	*Chronic villitis*: "infiltration of the villous stroma by maternal T-lymphocytes."
Almasry (35)	2012	Presence of cellular infiltration in the stem villi affecting 10 or more in 2 fields of view
Hulthén Varli (21)	2012	Villitis <1% or villitis ≥1% "Chronic villitis: presence of a mononuclear cell (lymphocyte, histiocyte and plasma cell) infiltrate in the villous stroma, often with destruction/necrosis of the villous parenchyma."

Comparison of studies relating to VUE and FGR is hampered by the different approaches that researchers have employed. Almasry et al. (35), for example, compared the placentas of SGA and AGA infants and described the histological appearance of villitis in stem villi. Their approach differed from that of Redline and Abramowsky (98) who only reported results from the terminal villi of placentas from cases of recurrent villitis. Different again were Althabe and Labarrere (109) who investigated growth restriction but included ponderal index (a measure of proportion) and recorded only live births. Aviram et al. (96) chose to exclude macroscopically normal placentas from their study. This is unfortunate as VUE is very rarely detectable macroscopically, so they could have overlooked some cases of VUE. It is interesting to note that in studies where normal placentas were examined some of the degenerative changes witnessed in FGR were also seen in AGA infants but without the poor outcome (25,35,95).

1.6.2.3 VUE and Stillbirth

Redline (84) suggests that VUE involving only a few terminal villi had little clinical significance, but the more extensive the lesions became, the more severe the clinical presentation, from FGR to stillbirth in extreme cases. This is in direct conflict with Becroft (91) who states that there is "no significant relationship between pathologic severity and SGA status".

There are few studies that concentrate on chronic inflammatory conditions in stillbirth, those that do link VUE and recurrent villitis (98). Recurrent reproductive failure due to VUE was detailed in one case report (107). The patient experienced five pregnancies, four of which resulted in the unexpected death of the fetus and one live born infant with severe FGR. Unfortunately, two of the four stillborn fetuses had neither autopsy nor placental examination performed therefore the authors statement of "reproductive failure due to severe placental villitis of unknown etiology" was only actually shown in two samples. Histologically, the three placentas studied (2 stillbirth, 1 live birth) presented similar results. The majority of cells found in the villi were lymphohistiocytic and the trophoblastic surrounding layer remained intact. Villitis was classed as severe because of the presence of large proliferating and/or necrotizing areas. Lymphocytes were also present in the decidua. In each of the cases the mother was asymptomatic and bacteriological and serological investigations of both mother and fetus proved negative. Of note is the description of the cause of death of the first two fetuses (where no post-mortem examination occurred), that of "idiopathic placental insufficiency". The authors suggest that a great many pathological cases related to villitis may have been overlooked in the past because of a differing nomenclature. This may have potentially severely underestimated the occurrence and relevance of this type of inflammation in stillbirth. Like Altshuler and Russell (76) this group assumed that the causal agent was an infection of some kind. They based this on the

histological similarity between VUE and villitis associated with rubella infection. This case report has been cited by many researchers as proof of the role of VUE in recurrent reproductive failure (84,88,91,98).

Another much cited study of 63 cases of VUE (76) showed 4 stillbirths in the group. Autopsy found no gross fetal abnormalities in three of the four cases (the fourth did not undergo an autopsy). Two patients presented with previous recurrent reproductive failure with one of these cases resulting in a stillbirth. The other patient produced a small-for-gestational-age infant (associated with pre-eclampsia) in the first pregnancy studied and a stillbirth in the second.

When addressing the issue of tissue sampling it is worth noting that many of the featured studies sample parenchyma from the central cord area and the periphery of the placenta and fail to sample the tissue in between. This leaves a large area of tissue that has not been sampled, which could be particularly problematic given the focal nature of the lesions being studied. If it is true that villitis potentially exists in all placentas one must consider the problem that Becroft highlights; that it is just not feasible to sample all of the parenchyma in order to find it (91).

One aspect of the subjective histological examination of tissue samples is the reproducibility of results. The Hulthén Varli group, by their own admission, failed to address the reproducibility of their study, though they were following standard protocols and collecting information for an established database (21). The fact that the slides in several studies were interpreted by a single pathologist (21,25,96,105,108) could easily affect the reproducibility in future studies, particularly if examined by a non-specialist pathologist. Additionally, there may be bias in the studies where the clinician was already aware of the outcome compared to those where the clinician was blinded to it.

It is difficult to accurately collate and compare the findings of these studies as they all have an emphasis on different aspects of pregnancy outcome, be it FGR, SGA, VUE or normal. VUE has rarely been the focus of research studies which makes confirming any association with stillbirth difficult. In fact it is more often reported as an aside to other results.

1.6.3 Intervillositis

Chronic intervillositis of unknown etiology (CIUE) is another inflammatory condition that is characterised by the presence of a maternal mononuclear cell infiltrate in the intervillous space. It is much rarer than VUE, has a very high recurrence rate, a much poorer prognosis and tends to occur at earlier gestations (110,111). The boundary between VUE and intervillositis remains unclear, with some authors suggesting it could be a severe variant of VUE (89,112). A recently published immunohistochemical study of CIUE by Labarrere et al.

(113) described similar cellular composition of CIUE and VUE. Like VUE, intervillositis has associations with increased severity on recurrence, FGR and stillbirth (89) and is often seen in conjunction with VUE.

1.7 Immunity

1.7.1 The Immune System

The body is constantly exposed to an assault of potential pathogens and foreign particles that, if left unchecked, have the potential to overwhelm and cause disease. The immune system is an integrated network of lymphoid cells, organs and soluble factors divided into innate and adaptive systems. Though the systems are described separately they are, in fact, intimately related and cross-talk between the two are essential for an appropriate and adequate response (114). It serves to protect the internal environment from the external one (Figure 5). Both innate and adaptive immunity have humoral (soluble) and cell-mediated components. In order to effectively protect the body without causing itself harm, this system should be able to recognise 'non-self', particularly bacteria, viruses and foreign cells, from 'self' (115).

1.7.2 Innate Immunity

Innate immunity is the more rudimentary of the two systems and though it incorporates physical barriers to foreign particles such as the skin, it usually refers to the protective elements of the immune system. It is essentially the first line of defence, is very fast-acting and always present however, its repertoire is limited. As stated previously, there are humoral and cellular components of the innate system.

1.7.2.1 Cells of the Innate Immune System

Cells of this system are polymorphonuclear granulocytes (neutrophils, eosinophils, basophils and mast cells), mononuclear cells (monocytes and macrophages) and natural killer (NK) cells (114). Neutrophils are found systemically i.e. they do not reside in one particular body compartment. Their recruitment is driven by macrophages and is a key feature of innate immunity. They are phagocytes that kill organisms with an oxygen-dependent respiratory burst or toxic proteins and enzymes. The other granulocytes (eosinophils, basophils, mast cells) represent a much smaller proportion of leukocyte total. They are involved in protection against parasitic infections but, in developed countries, are also implicated in pathological conditions such as asthma and anaphylaxis. Natural killer cells resemble lymphocytes morphologically, yet lack the specific antigen receptor of the lymphocyte. They are able to recognise antigen-antibody complexes and induce cell cytotoxicity (116,117). They also have the ability to recognise altered self cells and lyse their targets. Some of these cells play an important role in pregnancy and will be discussed in more detail in Section 1.7.4.

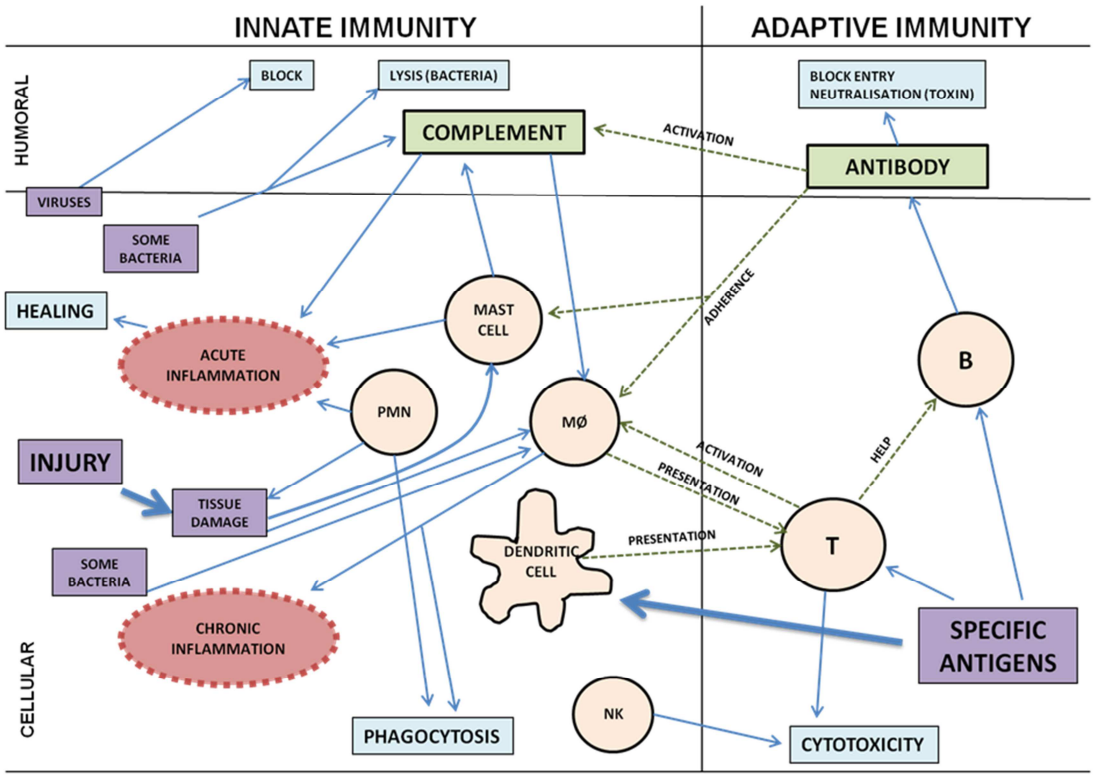

Figure 5: Representation of the components of the immune system and their interactions. Image redrawn from Playfair and Chain, Immunology at a Glance (115).

1.7.2.2 Complement

Complement is the humoral component of the innate immune system and comprises a series of at least 20 plasma proteins (114,118). Activation of complement occurs as a cascade and is amplified with each stage of the process, such that activation of a single complement molecule results in thousands of molecules at the end of the cascade. This system has three pathways of activation: classical, alternative and mannin-binding lectin. The classical pathway is activated via antigen-antibody interaction and the alternative by microbial polysaccharides. The mannin-binding lectin pathway is recently discovered, initiated by carbohydrates on microbial surfaces and feeds in to the classical pathway though bypasses the first stage in the process (89,92). The result in all pathways is chemotaxis of inflammatory cells and the production of a membrane attack complex (MAC) which leads to cell death by osmotic lysis (119). The cells of the host express complement regulatory proteins (decay accelerating factor and complement receptor type 1) which serve to prevent progression of the complement cascade and therefore self-immunoreactivity. Invading microbes do not possess either of these proteins which renders them susceptible to complement. In addition, components of the cascade contribute to other elements of immunity. Protein C3a causes activation and release of inflammatory mediators and can increase vascular permeability, an essential element of the inflammatory process, C3b acts as an opsonin 'coating' the antigen to enable its phagocytosis by macrophages and C5b is involved in recruitment of the membrane attack complex (MAC) (119). Finally, complement

can stimulate the adaptive immune system via an interaction with antibody-producing B cells.

1.7.2.3 The Innate Immune Response

An innate immune response is initiated when receptors (known as pattern recognition receptors (PRRs)) on specific cells recognise particular areas on invading microorganisms. PRRs recognise pathogen-associated molecular patterns (PAMPs) - specific highly conserved patterns found on microbes but not host cells. These exogenous sequences are largely recognised through the Toll-like receptors (TLR) (93,94). The result of PAMP binding to PRRs is one of three things: enhanced Ag presentation, cell activation or opsonisation. Despite this, there are limited outcomes to this type of stimulation: phagocytosis or lysis of bacteria, inhibition of viruses or release of cytokines. Ultimately the result is very similar to that seen in injury or tissue damage and culminates in inflammation; because the response tends to be poorly localised, tissue damage occurs in surrounding tissue. Numerous components of the innate system contribute to the overall end result: mast cells within tissues secrete inflammatory mediators, macrophages remove debris via phagocytosis, granulocytes release bactericidal enzymes, complement results in bacterial lysis and inflammation, cytokines (interferons) react to intracellular infection and dendritic cells process antigen for the cells of the adaptive system (115). Pathogens are not the only agents that can damage tissue and cells: trauma, hypoxia, wounds, chemical insults and lack of nutrients may also do this in a process deemed sterile inflammation. The term 'alarmins' has been cited to describe endogenous molecules that represent the result of cell or tissue damage. Combined, these immunostimulatory patterns are known as damage-associated molecular patterns (DAMPs). There are various features of alarmins related to their ability to produce inflammation: they are released following non-programmed cell death rather than apoptosis; they stimulate both the innate and adaptive immune systems and should contribute to the restoration of homeostasis following injury/trauma. Many putative alarmins have been proposed, with high mobility group box 1 (HMGB1), cell free DNA and uric acid representing three key molecules (120,122).

1.7.3 The Adaptive Immune System

The adaptive immune system provides the type of response that reacts to novel insults that may have evaded detection by or overcome the innate system. Adaptive immunity has an enormous repertoire of non-self antigens that it can potentially react to and additionally displays memory for previously encountered antigen. As with the innate system, there are cellular and humoral components of the adaptive system.

T (thymus-matured) and B (bone-marrow-matured) lymphocytes are the primary cells of the adaptive system. Morphologically, they are indistinguishable from each other and differ only

in their cell surface proteins and functional products. They express the antigen-specific receptors that underpin their targeted effects (116).

B cells are responsible for producing antibodies, the humoral component of adaptive immunity. Antibodies fulfil numerous roles including: opsonisation of bacteria (as a precursor to phagocytosis), neutralisation of toxins, prevention of organism adhesion and priming of infected/tumour cells before cytotoxic attack. Overall, the function of the B cell is to activate and enhance the response of the innate system. B cells exhibit membrane-bound antibody that acts as the B cell receptor; free antigen becomes bound to the antibody and is internalised, processed and then presented to T cells. There are several different classes of antibodies that are localised to different compartments of the body. Even so, an antigen eliciting a response at one site of the body will produce the same immune response in another compartment (116,123). Any one B-cell is programmed to make antibody of only one type – they do not produce a variety of antibodies in response to different stimuli.

In contrast to B cells, T cells are only able to recognise antigen when it is combined with a cell surface molecule called the major histocompatibility complex (MHC). MHC molecules are also known as human leukocyte antigen (HLA) and are classified as either type I, occurring on all nucleated cells or type II, occurring only on B cells, dendritic cells and macrophages which are antigen presenting cells (APC). T cells are further divided into subsets based on the cell surface markers that they display and the type of MHC they recognise. CD8+ T cells recognise antigen bound to MHC class I molecules and when stimulated differentiate into cytotoxic T lymphocytes (CTL). As their name suggests, CTLs are responsible for the destruction of infected cells and produce a targeted response (114,115,118). CD4+ T cells recognise antigen bound to MHC class II molecules and are classed T-helper (Th) cells as they assist in the generation of an immune response. Th cells can be further subdivided into Th1 and Th2 type cells. CD4+ Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-α and are associated with cell-mediated inflammatory responses. Conversely Th2 cells secrete IL-4, IL-5 and stimulate anti-inflammatory, antibody-mediated immunity (95). Overall, their effect is the stimulation of a broad range of additional cells. Because of the potential for widespread damage following the event of an inappropriate Th cell response, the number of cells capable of presenting MHCII and antigen is limited. The process of endocytosis of pathogens and subsequent presentation of antigen with MHC ensures that the peptides are recognised as foreign and that self-peptides are ignored. In addition, binding of T cell receptor to antigen/MHC alone is not sufficient to activate the lymphocytes, co-stimulatory interaction between APC and T cell is also required. T lymphocytes are also programmed to recognise the difference between MHC of 'self' and 'non-self' and it is this that forms the basis of immune rejection (see Section 1.7.5). Regulatory T cells (Treg) are another subset of CD4+ cells characterised by the transcription

factor Foxp3. They play a critical role in immune tolerance and immune response regulation (125).

1.7.4 The Immune System in Pregnancy

In 1953 Medawar first communicated the theory that there must be some kind of immunological privilege allowing a mother to accept and carry a fetus that is immunologically foreign (96). His suggestions were that: i) the maternal immune system is suppressed, ii) there is a distinct anatomical barrier between mother and fetus and iii) the fetus remains immunologically immature (97). However, the immune system must still function during pregnancy to safeguard the mother from environmental antigen. Pregnancy represents the confounding situation of a fetal allograft 'residing' within the maternal environment. Under normal circumstances, such foreign antigen would stimulate rejection by the immune system. Immune activation by the fetus can occur, as seen in the production of maternal antibodies to fetal rhesus antigen, though usually this is not the case (49,92). A fundamental difference between a true allograft and the fetal allograft is the scale and time span upon which antigen exposure occurs; a true allograft requires surgical trauma and delivers acute and large antigen exposure (128). It is now known that the maternal immune system is not suppressed, it 'recognises' pregnancy and the placenta is neither inert nor impenetrable so there must be mechanisms by which tolerance is achieved. It is likely that a variety of immunological mechanisms, initiated by both mother and fetus, work together to produce the resulting tolerance (53) though these systems are still incompletely understood. From a fetal perspective, several mechanisms for evading or type-shifting maternal immunity exist:

MHC expression: fetal STB and EVTs do not express MHC class II molecules or the highly polymorphic HLA-A or HLA-B MHC class Ia antigen presentation molecules that are the main targets for T cells in transplant rejection (43,92,97,99–101). The polymorphic class Ia HLA-C molecule is expressed by EVTs, though appears to evade immune-mediated fetal rejection (53) by downregulating maternal NK cell responses (128,129). They also express the much less variable Class Ib HLA-E, -F and -G molecules. HLA-G is expressed on both spiral artery invading and interstitial EVT cells (99). This class Ib molecule inhibits uNK cells; it does so by expressing MHC, therefore EVTs are not seen as 'MHC null' and are not deleted. Because HLA-G is not highly polymorphic it is also unlikely to be recognised as 'non-self' by the mother (49,92). It has been suggested that fetal HLA-G expression may ultimately lead to immune tolerance of the molecule in the adult resulting in a lack of maternal response to the marker (49,101). HLA-G has been shown to be upregulated in skin cancer, a disease characterised its ability to evade the immune system (131). Soluble HLA-G has also been suggested to promote Fas/FasL-mediated apoptosis of activated CD8+ T cells (130).

T Cell Inhibition: expression of the transmembrane receptor programmed death (PD)-1 has been shown to occur on trophoblast populations throughout pregnancy. The ligands that bind to PD-1 (PDL-1 and PDL-2) arise from activated APCs after they are activated or exposed to IFN-γ. Binding of these ligands to either receptor affects antigen-stimulated T cells by altering proliferative capacity, cytokine production or inducing apoptosis (119). Controlling the population of T cells by inducing apoptosis in paternal antigen reactive ones may be a mechanism of maintaining tolerance.

Complement evasion: trophoblast has been shown to express three complement regulatory proteins that inhibit complement at different stages of the activation cascade. These proteins are significant adaptations that protect the fetus from inflammation following maternal complement activation and deposition. In studies where complement regulatory proteins were inhibited trophoblast was found to be more susceptible to complement-mediated inflammation (119).

Cytokine and hormone production: the placenta produces progesterone, a hormone that maintains uterine quiescence throughout the majority of pregnancy. Pathologies such as infection or inflammation can reduce the effect of progesterone (119,132). In mice, the biological effects of progesterone include an alteration of cytokine balance towards T helper (Th)2-type (humoral immunity) and inhibition of NK cells (119). Th1 (cell-mediated) responses are suppressed by other placental products such as prostaglandin E_2, and anti-inflammatory cytokines IL-4 and IL-10 (128). Oestrogens exert an immunomodulatory effect by inhibiting the Th1 cytokines IL-12, TNF-α and IFN-γ and stimulating Th2-type anti-inflammatory cytokines IL-10, IL-4 and transforming growth factor-beta (TGF-β) (132). Local T cells produce cytokines, but the placenta appears to be a major alternative non-lymphoid site of Th2 cytokine production.

From the maternal perspective a variety of immunological modifications occur:

Innate immunity: Sacks et al. (128) suggest that rather than complete immunosuppression, it is only the adaptive immune system that is affected, swaying any immune response towards the innate. Thus, according to these researchers, monocytes and macrophages assume responsibility for immune responses during pregnancy. More specifically, it is thought that implantation brings about a change in leukocyte population within the decidua. This change is related to a prevalence of decidual innate immune cells (uNKs and macrophages), leaving the cells of acquired immunity (T- and B-lymphocytes) to reside deeper within the myometrium. It is thought that uNK cells have limited cytotoxicity compared to peripheral NK cells (47). Treg lymphocytes are the exception to this rule in that they reside in the decidua with the innate cells and are generated by interaction with fetal

HLA-G (99). They occur in higher numbers in pregnancy and are believed to induce tolerance (49,103).

Cytokine and hormone production: hormones with the ability to modulate immune responses are present during pregnancy as are chemokines that regulate cell numbers (53). It is additionally suggested that a shift of immune response from a Th1- to Th2-type occurs. A predominance of Th1-type responses is associated with poor pregnancy outcome (119,132). Endometrium expresses the anti-inflammatory cytokine TGF-β that inhibits Th1-type immune responses. Additionally, endometrial stromal cells and uNK cells express galectin-1, an immunosuppressive factor that, upon exposure to dendritic cells, induces differentiation towards a tolerogenic phenotype (130). Such a shift would direct an immune response away from an inflammatory one (97,99).

The implication that a system has evolved that enables the mothers' body to 'understand' that it is pregnant is an interesting one. The true extent to which immunity changes during pregnancy remains to be elucidated and more so the consequences of altered/inappropriate immune responses.

1.7.5 Immune Rejection

Immune rejection is the basis by which foreign antigen is removed from the body. Well known from the field of organ transplantation it has been suggested that immune rejection is the process that occurs in VUE (69,70,77,104). The process involves recognition, by the host (maternal) immune system, of 'non-self' MHC molecules on the surface of the transplanted organ/cells (the placenta). The immune hypothesis for VUE rests upon evidence that T cells and macrophages, both key in the rejection process, are present in the villous stroma during VUE (69,104). Several factors point towards an immune as opposed to infectious origin for VUE: there is not usually any evidence of an infection, non-affected tissue appears as normal, severity increases as VUE recurs, B cells and neutrophils are rarely seen and the presence of inflammation is not seasonal (88).

1.8 Summary

That inflammatory conditions of the placenta are still being investigated over 50 years since they were first described suggests that the underlying processes are still to be unravelled. Advances are being made in the field of pregnancy immunology but more research is required to enable understanding of the role in inflammation in poor pregnancy outcome.

Studies investigating non-infectious inflammatory disorders in pregnancy are of increasing interest in clinical and academic settings. Mothers experiencing reduced fetal movements (RFM) are at an increased risk of FGR and stillbirth that may be due to placental dysfunction (67,135,136). It has recently been demonstrated that circulating markers of inflammation

(cytokines and alarmins) are detectable in the serum of women reporting RFM (122). In a review of maternal cytokine profiles in FGR and SGA pregnancies, Mullins et al. detected a general pro-inflammatory shift in both maternal and placental cytokines (137). There is also growing evidence to suggest that maternal conditions such as diabetes and obesity stimulate placental macrophages to switch traits from tissue remodelling to pro-inflammatory (138,139).

With so many variables in pregnancy it is not surprising that different researchers focus on different contributory factors. Investigators state that more research is required into the role of inflammation in placental dysfunction (4). Searching the literature has highlighted that there has not been a systematic review of studies of VUE published since its discovery 40 years ago.

Immunohistochemical techniques have permitted the classification of some of the cells involved, yet there is still disagreement within the scientific community as to the proportions and origins of these cells. Labarrere et al. documented a major infiltration of macrophages and CD4+ cells in the villitis they found in normal term placentas (105). Conversely, Kim et al. describe the major maternal cellular infiltrate to be CD8+ T cells (87). It seems reasonable to propose that there may be different mechanisms producing a similar histological morphology, resulting in seemingly contradictory cell profiles. Though there is agreement that fetal macrophages are present during VUE, there has been little suggestion as to their role in the process. There is the possibility that the MHC II that they express on their cell surface is recognised as 'non-self' by maternal T cells or the fetal macrophages may be acting as APCs. Full classification of the cells involved in VUE would assist in the understanding of the mechanics of the process and whether or not those cells differ in placentas from healthy pregnancies or those ending in FGR (stillbirth and live births). Indeed, if different antigens or mechanisms are responsible, this may assist with the further clarification and sub-typing of VUE. Moreover, quantifying the cells in their location may offer a more objective definition of VUE. There would also be merit in comparing quantitative and qualitative approaches of diagnosis. The random focal nature of the lesions may mean that a purely numerical study is inappropriate and requires that histological examination is completed before quantification can take place.

Once a cell profile has been established, production of an *in vitro* model would allow observation of the way immune cells interact with placenta. Current knowledge is based on a snapshot of the placenta at a certain point in time that is always observed postpartum. A better understanding could be gleaned by observing living cells and tissue.

The contribution of VUE to stillbirth remains unclear as morphological studies do not enable observation of the dynamics of maternal cell interactions with placental tissue. There is a

reported occurrence of FGR in approximately 50% of stillbirths and with suggested links of VUE with FGR it may be logical to assume that VUE underlies some cases of stillbirth with FGR. This project aimed to identify and link VUE with placental insufficiency and therefore FGR and stillbirth. It is already known that altered placental vascularity and decreased nutrient transport detrimentally impact fetal growth. What is still unclear is whether VUE affects either of those factors, the endocrine function of the placenta or causes an imbalance in the rates of proliferation and apoptosis in the organ.

1.9 Hypothesis

VUE is an inflammatory immune response that causes FGR and stillbirth by detrimentally affecting placental function.

1.10 Aims

1 Determine whether VUE is associated with FGR and stillbirth

2.1 Characterise the cellular phenotype of VUE lesions in stillbirth and compare cell populations to FGR and healthy pregnancies

2.2 Develop a reliable, quantitative method of identification of VUE to reduce the subjectivity associated with a purely morphological approach

3 Establish an *in vitro* model of VUE

4 Identify the potential consequences of VUE that may lead to altered placental function

1.11 Objectives

1 To conduct a systematic review of the literature to determine if the suggested association of VUE with FGR and stillbirth is significant (Chapter 3).

2.1 To undertake a morphological study of VUE to identify the phenotype of the cells involved in inflammatory lesions (Chapter 4).

2.2 To use image analysis software to quantify the cells present in lesions and to identify pan-placental inflammatory changes in placentas from pregnancies with poor outcome (Chapter 4).

3 To develop an *in vitro* co-culture model of VUE using placental explants and maternal leukocytes (Chapter 5).

4 To use an *in vitro* model of VUE to investigate the consequences of placental inflammation (Chapter 6)

CHAPTER 2: MATERIALS AND METHODS

2.1 Systematic Review

A systematic review was conducted in accordance with the MOOSE guidelines (141). Searches were conducted using PubMed, MEDLINE (1946-2013) and EMBASE (1974-2013) databases using the search terms "villitis", "villitis unknown etiology", "stillbirth", "fetal growth restriction", "intrauterine growth restriction" and combinations thereof. The search strategy was as following:

1. Villitis.mp
2. VUE.mp
3. Stillbirth.mp
4. Intrauterine growth retardation /or fetal growth restriction.mp
5. 1 and 3
6. 1 and 4
7. 2 and 3
8. 2 and 4
9. 5 or 6
10. 5 and 6

Additional manuscripts were identified from the references lists of search-sourced literature. The search was not restricted to English language publications, however lack of translation services excluded full-texts of foreign language articles from the review (n=6).

One author (HD) used article title and abstract to identify potentially relevant studies from the database searches. A second investigator (AH) confirmed the relevance of the identified articles and their suitability for inclusion in the review. Studies that fulfilled the search criteria were obtained in full-text. Several groups of articles were included in the review: studies that reported the frequency of VUE in the placentas of infants with normal outcome, FGR/SGA or stillbirth. Various definitions of FGR or SGA were used and for the purposes of analysis these were grouped together. Manuscripts were excluded if they focussed on non-stillbirth deaths (neonatal deaths, pregnancy loss <20 weeks gestation), they were duplicate studies of the same study population, case reports, adverse neonatal outcomes or reported studies of villitis of infectious etiology (VIE). Reports detailing VUE in the presence of existing maternal or fetal conditions (e.g. pre-eclampsia, chromosomal anomalies) were also excluded.

The studies included in the systematic review were divided into groups based on the methodological approach applied. HD extracted the data from the studies, AH checked the suitability. Group 1 included studies that specifically investigated the incidence of VUE/villitis in pregnancies including appropriate for gestational age (AGA) birthweight, FGR/SGA and stillborn infants. Studies with a focus of placental pathologies associated with FGR/SGA, which included observations on villitis/VUE, were placed in Group 2. Group 3 consisted of studies investigating placental pathologies solely in stillbirth. Finally, group 4 is comprised of two studies examining the recurrence of VUE. Results from each paper were collated to produce tables for comparison. Systematic reviews in science are relatively new therefore there is not a standardised, robust quality assessment for observational studies. The format for the quality assessment in this review was adapted from a systematic review and meta-analysis published by Kattenburg et al. (142). As the original systematic review was completed in 2013, the searches were repeated in July 2015 (search dates January 2013 to July 2015) to update results for inclusion in this thesis.

2.2 Placental Tissue Collection and Processing for VUE Characterisation

Unless otherwise stated reagents were obtained from Sigma-Aldrich Chemical Company, Poole, UK.

Formalin-fixed paraffin embedded placental tissues from pregnancies ending in stillbirth were obtained from the Paediatric Histopathology departments of Manchester Royal Children's Hospital and Edinburgh Royal Infirmary. A contributory cause of death after post mortem examination was recorded as either VUE (n=12) or FGR (n=12). The post mortem reports of the stillborn infants with FGR specifically detailed no evidence of VUE lesions. Placentas from stillborn infants were retained and authorised for use in research with consent from parents obtained at the time of post mortem; their specific use in this project was approved by Humber Bridge Research Ethics Committee (Ref: 13/YH/0176). Placental tissue from live born infants was obtained from the biobank at the Maternal and Fetal Health Research Group (MFHRG), University of Manchester (North West REC 08/H1010/55). Live born FGR infants (n=11) were considered to be those with an individualised birthweight centile (IBC) $<5^{th}$ (29). Matched controls were placental samples from women with uncomplicated pregnancies (n=11) selected from the MFHRG biobank. All placentas were taken from mothers who showed no evidence of conditions such as pre-eclampsia or diabetes. Multiple pregnancies, abnormal fetal heartbeat (live birth only) and fetuses with congenital anomalies were excluded (143). In the group of placentas from cases of stillbirth 2-7 randomly selected samples per placenta were obtained for histological analysis. Three samples of placental villous tissue were taken from the placentas of the live born infants,

one each from the centre, middle and periphery of the tissue. Biopsies were fixed in neutral buffered formalin (NBF) for 24 hours before being processed and paraffin wax embedded.

2.3 Immunohistochemistry for VUE Characterisation

For immunohistochemistry, 5µm serial sections were cut and mounted onto poly-L-lysine coated slides. VUE lesions were initially identified by detecting clusters of leukocytes using a mouse monoclonal antibody (mAb) to CD45 (leukocyte common antigen), identified by immunoperoxidase staining. A VUE lesion was defined as an area of villous tissue with evidence of extensive CD45+ cell infiltrate. Subsequent immunostaining for specific immune cells was carried out to identify CD4+ T cells, CD8+ T cells, CD163+ macrophages and neutrophils. Further immunostaining for cytokines interleukin (IL)-2, IL-4, IL-10 and IL-12 was performed on representative sections from all samples. Finally, staining for IL-6 and transforming growth factor (TGF)-β was performed on selected stillbirth samples with VUE (n=6) and healthy controls (n=6). For all these analyses, our standardised laboratory protocol for immunohistochemistry with colorimetric detection was followed. Sections were dewaxed in HistoClear and rehydrated in graded alcohols. Heat-mediated antigen retrieval was performed for all antibodies except anti-neutrophil elastase and anti-IL-10 mAbs where manufacturers' protocol omitted this stage (Table 3). Endogenous peroxidases were quenched with 3% H_2O_2 for 10minutes at room temperature. Non-specific antibody binding was blocked using a non-immune block (NIB) prepared in house (10% goat or swine serum, 2% human serum (donated) 88% TBS-Tween 0.1%). Primary antibodies diluted in NIB were applied at the working concentrations detailed in Table 3 and incubated overnight at 4°C in a humidified chamber. Thorough washes with TBS and TBS-T 0.6% were carried out followed by application of biotinylated secondary antibodies (goat anti-mouse or swine anti-rabbit (Dako, Cambridgeshire, UK)) to all sections at 4µg/mL for 30 minutes at room temperature and washed with TBS and TBS-T 0.6%. Avidin peroxidase was applied at 5µg/mL for 30 minutes at room temperature and washed before the chromogen 3,3'diaminobenzidine (DAB) was applied to each section for up to 10 minutes until an appropriate intensity of colour developed. Sections were counterstained with Harris' haematoxylin for 3 minutes. Counterstained slides were dehydrated through graded alcohols and prepared for mounting in HistoClear. All coverslips were applied using DPX mounting medium (Raymond Lamb, London, UK).

2.4 Immunofluorescence for VUE Characterisation

The phenotype of the macrophages and T cells in the placentas was investigated by staining using dual staining immunofluorescence (IF). Sections were stained for combinations of immune cell markers CD4 and CD163 and cytokines IL-2, IL-12 or IL-4. Sections were fixed and processed as described previously. Autofluorescence was quenched by 1hr incubation in

0.25% ammonia in 70% ethanol. Primary antibodies were applied at the concentrations described in Table 3 at 4°C overnight. Secondary antibodies (goat anti-mouse Alexa 568 or goat anti-rabbit Alexa 488 (Life Technologies, Paisley, UK) were applied at 5µg/mL and incubated for 30 minutes at room temperature. DAPI-containing aqueous mounting medium was used to apply coverslips.

Table 3:Table of antibodies used for immunohistochemical staining.
NE: neutrophil elastase, TGF-β: transforming growth factor-β, CK7: cytokeratin 7, M mAb: mouse monoclonal antibody, R pAb: rabbit polyclonal antibody, PTLI: pre-term labour with infection

Ag	**Antigen Retrieval**	**Supplier**	**Working Conc**	**Ab Type**	**Control Tissue**
CD45	Sodium citrate	Dako	0.5µg/ml	M mAb	Tonsil
CD163	Sodium citrate	AbD Serotec	10µg/ml	M mAb	Tonsil
CD4	TRIS-EDTA or sodium citrate	Dako	4.6µg/ml	M mAb	Tonsil
CD8	TRIS-EDTA	Dako	1.6µg/ml	M mAb	Tonsil
NE	None	Dako	1.1µg/ml	M mAb	PTLI
IL-2	Sodium citrate	Novus Biologicals	4.5µg/ml	R pAb	Tonsil
IL-4	Sodium citrate	Thermo Scientific	2.5µg/ml	R pAb	Tonsil
IL-6	Sodium citrate	Novus Biologicals	9.6µg/ml	M mAb	Tonsil
IL-10	None	Abcam	5µg/ml	M mAb	Liver (Kupffer cells)
IL-12	Sodium citrate	Sigma-Aldrich	2.5µg/ml	R pAb	Trophoblast
TGF-β	Sodium citrate	Novus Biologicals	10µg/ml	M mAb	Tonsil
CK7	Sodium Citrate	Dako	0.9µg/ml	M mAb	Placenta
Ki67	Sodium Citrate	Dako	0.4µg/ml	M mAb	Tonsil
Casp-3	Sodium Citrate	Abcam	1µg/ml	R pAb	Tonsil
M30	Sodium Citrate	Roche	1µg/ml	M mAb	Tonsil

2.5 Image Analysis for VUE Characterisation

Images of peroxidase stained placental sections were captured using an Olympus BX41 light microscope (Essex, UK) and attached QIcam Fast 1394 camera (QImaging, BC, Canada). Image Pro Plus 6.0 (Media Cybernetics Inc., MD, USA) was used to view images. Serial images of 104 VUE lesions stained for CD45, CD163, CD4, CD8 and NE were captured using a x10 objective. In addition, 10 random images were taken with the x10 objective of each immune cell stain for each placental section from the stillbirth with VUE (n=12), stillbirth with FGR (n=12), live birth with FGR (n=11) and normal pregnancy (n=12) groups. All images were analysed using HistoQuest image analysis software (TissueGnostics, Vienna, Austria). HistoQuest image analysis converts colour images of stained sections into greyscale and assigns an arbitrary number (0-256) to the pixel intensity. It is able to identify and count DAB+ events, haematoxylin+ nuclei, area of staining and intensity of staining.

Fluorescent stained sections were visualised using a Zeiss fluorescence microscope, AxioCam MRn camera with Zen pro image processing software (Zeiss, Welwyn Garden City, UK).

2.6 Trypsinisation of Placental Tissue

Preliminary experiments were performed to ascertain the viability of using trypsin to 'lift' areas of syncytium. Villous tissue fragments were incubated with various concentrations of trypsin in sterile PBS (0.01-0.125%) for 5, 15 or 30 minutes at 37°C. After the incubation period trypsin was neutralised and fragments washed twice in RPMI culture medium. In the preliminary experiments, tissue fragment morphology was qualitatively assessed for evidence of syncytial lifting without complete denudation of villi. Syncytial lifting and shedding was visualised by immunoperoxidase staining for cytoskeletal protein cytokeratin (CK) 7. Images of villous tissue were evaluated by an assessor blinded to treatment groups. Further experiments considered the effect of trypsinising on placental function. After 4 days in culture the effect of trypsinisation was assessed: on syncytial integrity/regeneration by CK7 immunostaining, nutrient transport by system A uptake activity assay, hormone production by hCG ELISA and cell turnover by IHC for markers of proliferation (Ki67) and apoptosis (active caspase-3) (Table 3).

2.7 Placenta and CD4/CD8 T Cell Co-Culture

Term (37-40w) placentas were obtained within 30 minutes of delivery from women with uncomplicated pregnancies undergoing elective caesarean section. Tissue was donated with informed written consent in accordance with North West NHS REC approval (Ref: 08/H1010/55+5). Uncomplicated pregnancies were defined as maternal BMI $\leq$30 and no evidence of pre-eclampsia (PE), gestational diabetes mellitus (GDM) or FGR. Multiple pregnancies, fetal anomalies or abnormal fetal heart rate were excluded. Biopsies of full

thickness villous parenchyma were taken from four different areas of placenta. Villous tissue was further dissected into 3-4mm^2 (5mg) fragments, washed in Dulbecco's PBS (with calcium and magnesium) and incubated in 0.01% trypsin solution for 15 minutes at 37°C. Following neutralisation and two washes in RPMI culture medium fragments were transferred into 24-well plates (3 per well). Fragments were cultured for 24 hours in 1mL RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 5% fetal bovine serum (FBS), 1µg/mL insulin, 100µg/mL streptomycin sulphate, 100IU/mL penicillin G, 0.1µg/mL retinol acetate, 25µg/mL L-alanine, 200µg/mL L-cysteine and 50µg/mL ascorbic acid. Isolated CD4 and CD8 T cells (see Section 2.8) were applied to each fragment in 10µL suspensions (average 12 x 10^3 cells per fragment) and cultured for 24 hours at 37°C. RPMI culture medium alone was applied to control tissue (10µL per fragment). On day 2 an additional 300µL culture medium was added to each well and cultured for a further 24 hours. On day 3 of co-culture medium was replaced and incubated for a final 24 hours. Supernatants were collected for hCG analysis on days 1, 3 and 4 and stored at -20°C. Additional fragments were stored in RNA Later for 24 hours then frozen, frozen at -80°C for protein analysis, fixed in 4% neutral buffered formalin (NBF) then embedded in paraffin wax or frozen in optimal cutting temperature (OCT) compound. Nutrient transport was measured by system A uptake activity at 10, 30 and 60 minute time point determinations (Section 2.11).

2.8 CD4 and CD8 T Cell Isolation

CD4 and CD8 T cells were isolated from maternal blood samples with EasySep Human T Cell Isolation Kit (STEMCELL Technologies, Cambridge, UK) following the manufacturers' protocol. Briefly, peripheral blood mononuclear cells (PBMCs) were separated from whole blood by centrifugation and incubated with CD8 T cell positive isolation antibody cocktail for 10 minutes. The cell suspension was incubated for 10 minutes with magnetic nanoparticles and targeted cells selected with the EasySep magnet. Remaining cells in the supernatant were then incubated with CD4 T cell negative isolation antibody cocktail for 5 minutes and magnetic particles for 5 minutes. Unwanted tagged cells were selected from the suspension with the EasySep magnet. Isolated cells were incubated for 24 hours on anti-CD3 (BD Pharmingen, Oxford, UK) coated plates in RPMI 1640 medium supplemented with 1µg/mL anti-CD28 antibody (BD Pharmingen) and 10ng/mL recombinant IL-2 and IL-15 (Gibco).

2.9 CellTracker™ Fluorescence

Cultured CD4 and CD8 T cells were harvested by application of PBS (without Ca^{2+} and Mg^{2+}) and gentle scraping. The manufacturer's protocol for CellTracker™ fluorescence (Life Technologies) was followed. Briefly, harvested cells were incubated with 10mM CellTracker™ fluorescence (red for CD4, green for CD8 T cells) in RPMI culture medium for

45 minutes at 37°C, centrifuged then resuspended in pre-warmed culture medium for a further 30 minutes at 37°C. Cell suspensions were spun, washed in pre-warmed PBS (with Ca^{2+} and Mg^{2+}), centrifuged again and resuspended in RPMI culture medium ready for application to placental fragments. Intracellular fluorescence was verified by viewing cells with a fluorescence microscope (Zeiss, Welwyn Garden City, UK), AxioCam MRn camera with Zen pro image processing software. Cell number and viability was assessed by staining with trypan blue and counting using a haemocytometer.

2.10 Explant Culture with Cytokines

Placental tissue was obtained as per co-culture methodology with the same inclusion and exclusion criteria applied. Fragments of villous tissue 3-4mm^2 in size were prepared from the biopsies obtained. Three explants were placed into individual Costar Netwell (Corning, NY, USA) supports suspended in 1.5mL CMRL-1066 culture medium supplemented with 5% heat-inactivated FBS, 100 IU/ml penicillin, 100µg/ml streptomycin, 1µg/ml insulin, 0.1µg/ml hydrocortisone, and 0.1µg/ml retinyl acetate. Explants were incubated in CMRL medium before treatment began on day 4. Treatment of explants comprised 48 hour incubation of tissue fragments in reduced serum Opti-MEM® medium supplemented with 10ng/mL IL-2, IL-12, 1µg/mL IL-4 neutralising antibody, a combination of all three or Opti-MEM® alone (control). Supernatants were collected for hCG ELISA analysis on days 1, 2, 3, 4 and 6 and stored at -20°C. Additional fragments were stored in RNA Later for 24 hours then frozen, frozen at -80°C for protein analysis or fixed in 4% neutral buffered formalin (NBF) then embedded in paraffin wax. Nutrient transport was measured by system A uptake activity at a 60 minute time point determination.

2.11 Assessment of System A Activity

Placental explants cultured for either 4 days (with CD4 and CD8 T cells) or 6 days (with cytokines) were incubated with radiolabelled N-methylated aminoisobutyric acid (^{14}CmeAIB) solution at 0.5µCi/mL (8.5mM). Tyrode's buffer was either control or equimolar Na^+-free (NaCl replaced with 135mM/L choline chloride). Co-culture explants were incubated for 10, 30 or 60 minutes, explants cultured with cytokines were incubated for 60 minutes. Following incubation explants were lysed in dH_2O for 16-18 hours at room temperature then placed into 0.3M NaOH for >6 hours at 37°C. The radioisotope content of the lysed cells was determined using a β-scintillation counter. The protein content of the explants was ascertained by use of Bio-Rad protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). Radioactivity counts were corrected for protein content. System A is a sodium-dependent nutrient transporter therefore its activity was calculated by subtracting activity in Na^+-free Tyrode's from activity in control Tyrode's.

2.12 Assessment of hCG and PlGF Hormone Production

Undiluted culture medium was assayed for hCG and PlGF hormone secretions by ELISA or Duoset ELISA (hCG: DRG Diagnostics, Marburg, Germany, PlGF: R&D Systems, Oxford, UK) according to standard manufacturers' protocol. Reagent volumes were halved for use in Duoset ELISA. Plates were read on a FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK), values corrected for protein content and expressed as mIU/mL/mg protein (hCG) or pg/mL/mg protein (PlGF).

2.13 Fluorescence of Co-Culture Explants

Explants that had been co-cultured with maternal CD4 and CD8 T cells were either embedded in OCT or fixed in 4% NBF and embedded in OCT medium before being frozen at -80°C. Sections of 5µm were cut on a cryostat, mounted onto poly-L-lysine coated slides and a coverslip applied with aqueous DAPI-containing mountant. Slides were viewed with a fluorescence microscope (Zeiss, Welwyn Garden City, UK) to determine if there had been migration of cells into the villous stroma.

2.14 Immunohistochemistry to Assess Apoptosis and Cell Proliferation in Explants

Apoptosis and proliferation in explants was assessed using immunostaining for markers Ki67 (proliferation) and M30 (apoptosis). Syncytiotrophoblast regeneration was assessed by immunostaining for CK7. The standardised protocol for immunoperoxidase staining was followed, as described in Section 2.3. Antibody concentrations and positive control tissues are detailed in Table 3. Images of peroxidase stained explants were captured using an Olympus BX41 light microscope (Essex, UK) and attached QIcam Fast 1394 camera (QImaging, BC, Canada). Image Pro Plus 6.0 (Media Cybernetics Inc., MD, USA) was used to view images. Images were analysed using HistoQuest image analysis software (TissueGnostics, Vienna, Austria) (Section 2.5).

2.15 Cytokine Analysis in Tissue Lysates and Culture Supernatant (Bio-Plex Assay)

Lysates were prepared from frozen explant tissue using a Bio-Plex cell lysis kit (Bio-Rad Laboratories, Hemel Hempstead, UK). Defrosted tissue was homogenised in 400µL lysing solution (18.2mL cell lysis buffer, 73.6µL Factor 1, 36.8µL Factor 2, 73.6µL 500mM PMSF). Samples were frozen at -80°C, thawed then centrifuged at 4,500g for 4 minutes. Total protein content of lysates was determined with Bio-Rad protein assay. Lysate samples for assay were prepared for analysis as 10µg or 50µg protein per 50µL sample.

Tissue culture supernatants collected on days 3 and 4 (co-culture) and day 6 (cytokine culture) and tissue lysate samples were analysed for a panel of 10 inflammation-related cytokines using a Bio-Plex assay (Bio-Rad Laboratories, Hemel Hempstead, UK). The cytokines selected for assay were interleukins-1β, -1Ra, -2, -4, -6, -10, -12A (p70), -17, interferon (IFN)-γ and TNFα. The assay was carried out following manufacturer instructions. Briefly, plates were first coated with fluorescent beads/primary antibody and a fourfold standard dilution series for each cytokine was prepared. Standards and samples were added to each well (50µL) and incubated for 30 minutes. All incubations were carried out at RT on a shaker at 800rpm. Wash steps were completed using wash buffer and Bio-Plex handheld magnetic washer. Detection antibodies were added and incubated for a 30 minutes. Finally, 50µL streptavidin-PE solution was added to the wells and incubated for 10 minutes. Beads were resuspended in assay buffer prior to being read on a pre-calibrated Bio-Plex 200 array reader using Bio-Plex Manager™ software (Bio-Rad Laboratories, Hemel Hempstead, UK). Cytokine levels were extrapolated from fluorescence values based on supplied standard samples.

2.16 Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 6.0 software (San Diego, CA, USA). Non parametric categorical data were analysed using Fisher's Exact test, non-parametric continuous data with Mann Whitney U test or Kruskal-Wallis test with Dunn's post-hoc test. Comparisons between groups of non-parametric data were analysed using Wilcoxon signed rank test or Friedman test. A p value of 0.05 was considered statistically significant.

CHAPTER 3: Investigating the Association of Villitis of Unknown Etiology with Stillbirth and Fetal Growth Restriction - A Systematic Review

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A manuscript published by Placenta

Placenta (2013) 34:856-862

3.1 Abstract

Villitis of unknown etiology (VUE) is an inflammatory condition reported to occur in up to 15% of term placentas. It has been reported in association with fetal growth restriction and antepartum stillbirth. This study aimed to investigate the strength of these associations by completing a systematic review using established guidelines. 618 potentially relevant studies were identified. After exclusion of studies that were not relevant or of insufficient quality, a total of 24 case-control and cohort studies were included in the review. Studies were grouped according to whether their main focus was VUE, fetal growth restriction or stillbirth. A methodological quality assessment carried out for each group demonstrated significant heterogeneity in study design. VUE occurs more frequently in placentas of growth restricted infants. A significant link between VUE and stillbirth could not be reliably established because there were too few published studies. Further research into the pathological effects of VUE using robust protocols and reporting methods is required.

Keywords: villitis; villitis of unknown etiology; placental histopathology; stillbirth; fetal growth restriction

3.2 Introduction

Villitis of unknown etiology (VUE) is an inflammatory condition reported to occur in 5-15% of all third trimester placentas (88). It is characterised by a lymphohistiocytic infiltrate comprising maternal T cells and fetal macrophages into the villous stroma. VUE is focal, with surrounding parenchyma appearing as normal (Figure 6). The severity of the condition is highly variable, in some cases only one to two villi appear to be affected, whereas, in others large numbers of villi over several fields of view show VUE (31,82,90). Diagnosis of VUE is made by histological examination of the placenta, as there is no prenatal test for the condition. As its nomenclature suggests, a causative etiological agent has yet to be elucidated, despite many years of study. In 1975, Altshuler and Russell suggested an infectious etiology for all cases of villitis, including those identified as VUE (76). Current theories hypothesise that VUE is based upon a maternal immunological response to a semiallogeneic fetus (87,88,100,144). VUE is of interest as it is reported in association with fetal growth restriction (FGR), stillbirth and recurrent pregnancy loss. However, Pathak et al. recently questioned the association between VUE and pregnancies where the infant was small for gestational age (SGA) (95). We aimed to address uncertainty regarding the association of VUE with pregnancy complications by undertaking a systematic review of published literature to assess whether there is a significant link between VUE and stillbirth, FGR and SGA and to consider the quality of published studies.

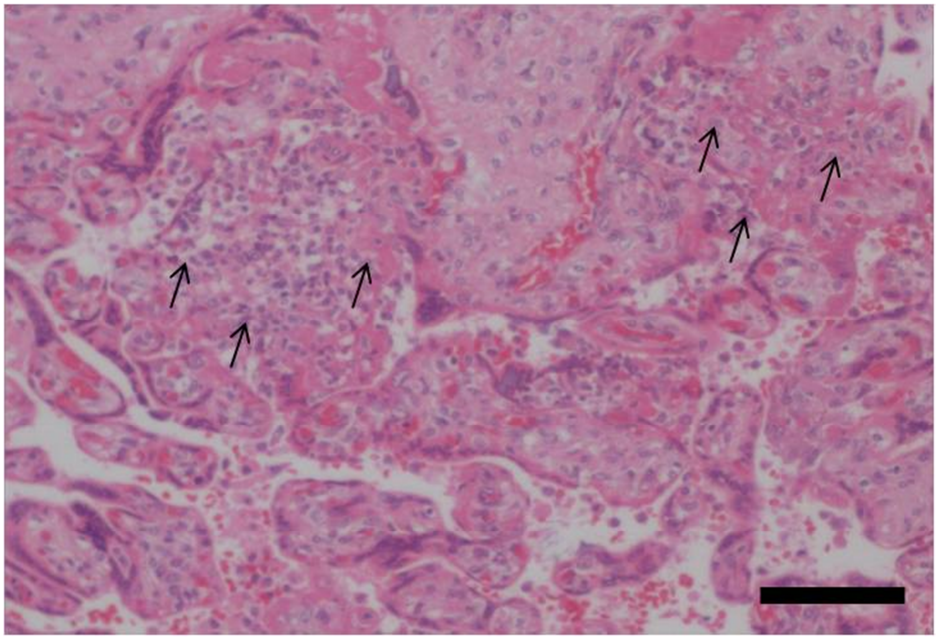


Figure 6: Photomicrograph of H&E stained placental tissue displaying VUE.
Arrows show areas of diffuse lymphohistioctic infiltrate within the villous stroma. Scale bar = 100µm (original magnification x100). Image courtesy of Dr. Gauri Batra, Dept of Paediatric Histopathology, Central Manchester University Hospitals NHS Foundation Trust.

3.3 Methods

A systematic review was conducted in accordance with the MOOSE guidelines (141). Searches were conducted using PubMed, MEDLINE and EMBASE databases using the search terms "villitis", "stillbirth", "fetal growth restriction" and combinations thereof. Additional manuscripts were identified from the references lists of search-sourced literature. Though the search was not restricted to English language publications, lack of translation services excluded these from the review (n=6).

One investigator (HD) conducted the database searches, using article title and abstract to identify potentially relevant material. Search results and study choices were confirmed by a second investigator (AH). The full text of relevant studies fulfilling set criteria was obtained. Studies included in the review were those that reported the frequency of VUE in the placentas of infants with normal outcome, FGR/SGA or stillbirth; as studies used various definitions of FGR or SGA these were grouped together for the purposes of analysis. Exclusion criteria were: non-stillbirth deaths (neonatal deaths, pregnancy loss <20 weeks gestation), duplicate studies (same study population), case reports, and villitis of infectious etiology (VIE). Reports detailing VUE in the presence of existing maternal or fetal conditions (e.g. pre-eclampsia, chromosomal anomalies) were also excluded.

Studies meeting the inclusion criteria were grouped according to their methodological approach. Studies that specifically investigated the incidence of VUE/villitis in populations including pregnancies with appropriate for gestational age (AGA) birthweight, FGR/SGA and stillbirth were described in Group 1. Studies describing placental pathologies associated with FGR/SGA, which included observations on villitis/VUE, were placed in Group 2. Studies examining placental pathologies solely in stillbirth were placed in Group 3. Lastly, Group 4 consists of two studies investigating recurrent VUE. Results from each paper were collated to produce tables for comparison.

3.4 Results

A PubMed search for the term "villitis" produced 254 results. Further searches including the terms "villitis unknown etiology", "small for gestational age villitis" and "villitis growth restriction" produced 65, 30 and 23 (duplicate) results respectively. An Embase search using "villitis" and "stillbirth" or "fetal growth restriction" or a combination of these terms gave 228 results and MEDLINE, using the same parameters, 136. This gave an overall total of 618 results across all three databases. Altering the search term to "intrauterine growth restriction" produced exactly the same number of results. A further 3 studies were identified from reference lists. Figure 7 illustrates the selection process for articles.

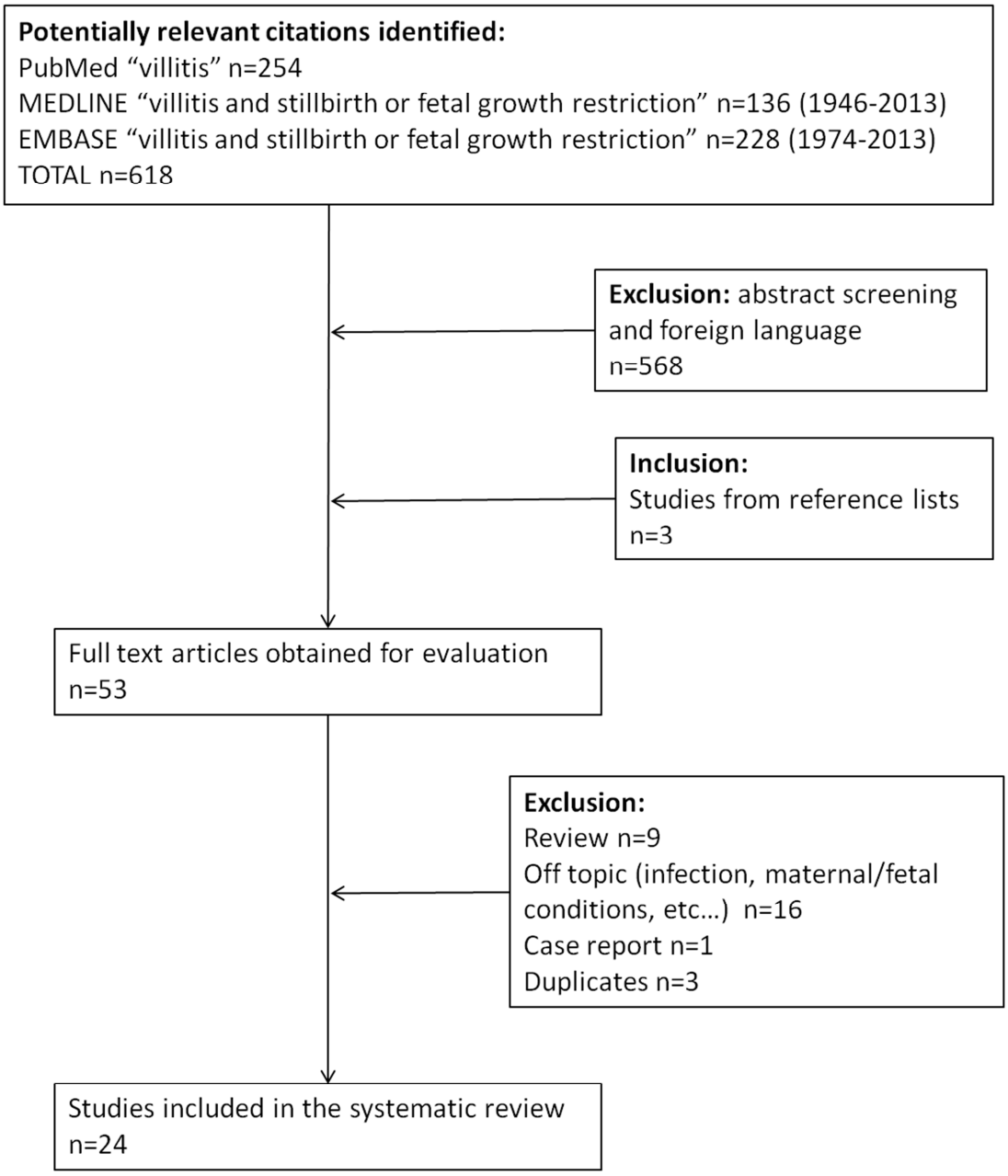


Figure 7: The selection process employed in this review

Abstract screening, foreign language citations and comparison of database results for duplicates excluded 568 articles, a large number of which were related to the epidemiology of and risk factors for stillbirth. A further 29 full texts were excluded because they were reviews, off-topic, case reports or duplicates. A total of 24 studies remained to be analysed in the systematic review.

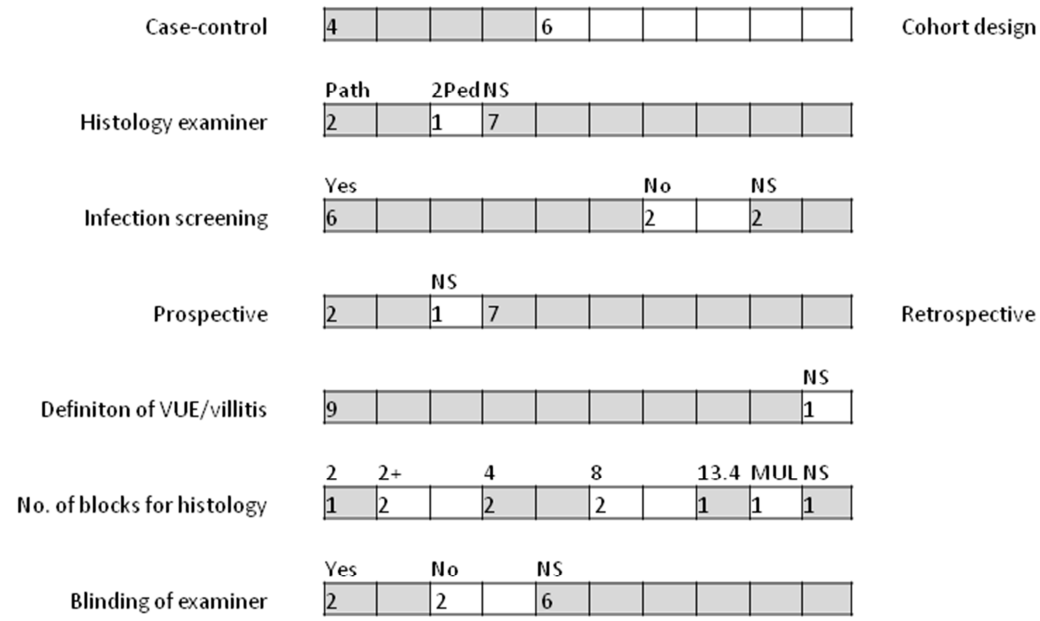
From the 24 studies selected for inclusion, different topics of focus were evident and were grouped accordingly. Ten studies specifically investigated the incidence of VUE (Group 1), 11 studies investigated causes of FGR (Group 2), and 4 studies focussed on stillbirth (Group 3), with one of these reporting on both FGR and stillbirth. One of the VUE studies (Group 1) focussed on pregnancy induced hypertension, but as normotensive controls were also

described it was included (145). A further 3 studies do not specify whether the villitis observed was of an infectious or unknown etiology (90,92,146). They were included as it is evident from their reports that VUE was the focus of the investigation. Of the 12 FGR studies, 7 were case-control design that included normal outcome infants in the study. Notably, few investigations (n=4) concentrate on a possible link between VUE and stillbirth. The most recent study relating to inflammation and stillbirth, by Hulthén Varli et al. (21), focussed on chorioamnionitis and did not differentiate between the incidence of VUE compared to VIE. However, it was included in the review because of its investigation of inflammation in relation to stillbirth.

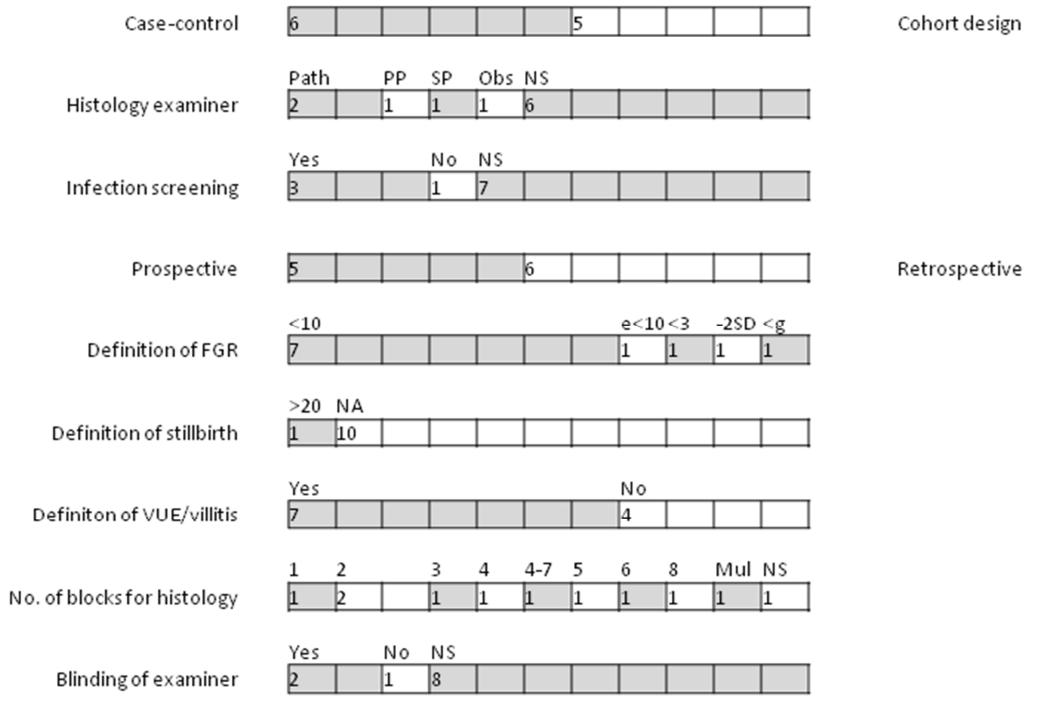
A methodological quality assessment was completed for each of the study groups (Figure 8). Studies were either case-control or cohort series and the majority were retrospective; the studies which described the incidence of VUE in FGR (Group 2) were more often case-control design. Where stated, examiners were pathologists and in three of the 24 studies included they were specifically described as paediatric pathologists. The number of specimen blocks taken for histological analysis ranged from 1 to an average of 13.4. Some researchers sampled only macroscopically normal placenta whereas others sampled macroscopically identifiable lesions. In the majority of cases it was not specified whether the examiner was blinded to clinical outcome.

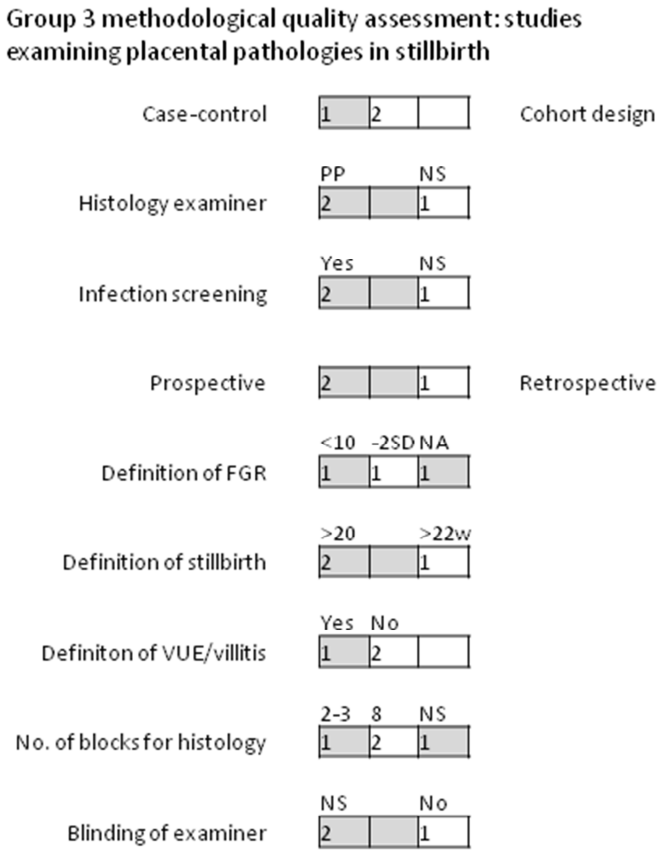
Analysis of studies that focussed on the incidence of VUE/villitis (Table 4) demonstrated an average incidence of 12.3% in the populations studied (which included a variety of pathologies); by performing a meta-analysis, the 95% confidence intervals for this figure lie between 11.8-12.7%, demonstrating a highly consistent finding. Pregnancies complicated by SGA/FGR and stillbirth are overrepresented in the cases with VUE/villitis (19.8% and 2.6% respectively), compared to their expected rates. These studies were mostly cohort series, though there were four case-control studies (31,95,146,147). The six cohort studies obtained samples either from selected populations or pathology archives, which could have introduced selection bias into the investigations. However, if the large, unselected population studies are analysed separately, the results/proportions are not affected (data not shown). The results of the studies that investigated the causes of FGR demonstrated a significant association with VUE (Table 5); villitis was present in 28.6% of FGR and 15.6% of AGA pregnancies (Fishers exact test, two-tailed P value <0.0001).

Group 1 methodological quality assessment: studies specifically investigating the incidence/aetiology of VUE/villitis



Group 2 methodological quality assessment: results of studies focussing on placental pathologies associated with FGR





Group 4 methodological quality assessment: studies investigating recurrent VUE

Author	N	Study design	Histology examiner	Infection screening	Prospective/ Retrospective	Definition of FGR	Definition of villitis	No. of blocks for histology	Blinding of examiner
Redline and Abramowsky	10 rVUE (41 pregnancies) 20 VUE (82 pregnancies)	Cohort design	Not stated	Yes	Retrospective	Not stated	Yes	Range 4-12 blocks	Not stated
Feeley and Mooney	19 2nd pregnancies (VUE in 1st pregnancy)	Cohort design	Not stated	Yes	Retrospective	Birthweight <3rd centile	Yes	Not stated	No stated

NS - not stated
Path – pathologist
Ped – paediatric pathologist
PP - perinatal pathologist
SP – surgical pathologist
Obs – observer/author

<10 - birthweight less than 10th centile
e<10 - estimated birthweight less than 10th centile
<3 - birthweight less than 3rd centile
-2SD - birthweight mean -2sd
<g - birthweight less than 2500g

Mul - multiple

Figure 8: Quality assessment of studies of VUE, IUGR and stillbirth included in this review. Bars represent 100% of studies in each group; numbers represent the actual number in each segment.

VUE was recorded in 5.1% of live births and 7.7% of stillbirths. However, there were not enough studies to definitively imply an association of VUE with stillbirth (Table 6). The three studies of stillbirth included one case-control study and two cohort studies (Figure 8). Examiners in two of the studies were expert perinatal pathologists and this is also implied in the third though not explicitly stated. Stillbirth was classified as death after the 20th week of pregnancy in two studies and after the 22nd week in one. All causes of fetal death were considered, including congenital and chromosomal anomalies, though the emphasis in each study was examination of the placenta for pathology. The number of blocks taken for

histology ranged from 2 to 8. In one study the examiner was aware of the clinical outcome, the other two do not specify.

Two studies investigated recurrent VUE (group 4) and showed increased incidence of both FGR and stillbirth in subsequent pregnancies following an index pregnancy with evidence of VUE. In their 1985 study, Redline and Abramowsky (98) examined placentas from 10 patients pregnancies where VUE recurred and compared them to placentas from 20 patients with a first occurrence of VUE. FGR was recorded in 44%, and stillbirth in 15%, of pregnancies with recurrent VUE. Feeley and Mooney (97) describe 19 women whose placentas were examined in a second pregnancy from 304 cases where VUE was identified in a previous pregnancy. Thirty two percent of first pregnancies with VUE resulted in an infant with FGR; stillbirth was experienced in 26% of first pregnancies in this study. Recurrent VUE was identified in seven of the nineteen placentas examined (37%). Two cases of recurrent VUE were SGA, but there were no stillbirths associated with recurrent VUE in the report. Though they are worthy of mention, meta-analysis has not been performed as the nature of their cohort design, the small number of participants, selection bias and the specificity of topic, as shown in the quality assessment, mean that they are not of a comparable level of evidence as other included reports (Figure 8).

3.5 Discussion

VUE is a recognised and well documented contributory factor in pregnancy pathology. Reviews in 2007 by Redline (88) and 2008 by Boog (89) have comprehensively described the pathophysiology of VUE. However, a robust systematic analysis of the literature was needed to collate and compare different reported rates of incidence that have been published over the past four decades. This systematic review has rigorously searched the literature and collated the findings of investigations into reported associations of VUE with FGR and stillbirth. We conclude that VUE is present in 15.6% of pregnancies with appropriately grown term infants and 28.6% of SGA/FGR infants. It is therefore reasonable to state that VUE has an association with FGR. However, it is prudent to note that most studies used a definition of FGR of birthweight $<10^{th}$ centile. Not all these infants would be considered growth restricted as some would be constitutionally small; approximately 30% of these infants have FGR compared to around 70% of infants $<3^{rd}$ centile (32). Importantly, of the twelve studies investigating FGR, only one defined FGR as a birthweight $<3^{rd}$ centile (96), with another using birthweight <2SD below the mean for gestational age (148). However, the incidence of VUE in these more tightly defined groups of FGR was not significantly different from studies using $<10^{th}$ centile, confirming an increased proportion of VUE compared to AGA infants.

Evidence for a demonstrable association of VUE with stillbirth remains scant. The three studies of sufficient quality to be included in this systematic review do not report a significantly increased incidence of VUE in stillbirth compared to AGA live births. This is surprising as FGR is the single most common cause of stillbirth (23), and we find that VUE is associated with FGR/SGA. This failure to identify an association may be a type 1 error, due to low numbers of studies. In addition, the focus of the articles included in this systematic review was the classification of cause of death, thus studies included stillbirths from all causes, including congenital and chromosomal anomalies and other non-placental causes. By excluding non-placental causes, the incidence of VUE in cases of stillbirth would be expected to increase as the denominator reduced. For example, Rayburn et al. report VUE in 4 out of 89 cases of stillbirth (4%). If congenital anomalies, hydrops, complications of multiple pregnancy and infection are excluded this proportion would become 4/61 cases (6.5%). Importantly, approaches that study stillbirths with FGR are more likely to state 'placental insufficiency' as a blanket term for the underlying cause of death, rather than specific pathologies such as VUE. We included a study by Gunyeli et al. (149) despite the main focus of the study being FGR, to increase the power of this review to determine whether stillbirth is associated with VUE. Importantly, this study identified that a high proportion of stillbirths with FGR had VUE. To address the uncertainty underpinning the relationship between VUE and stillbirth an adequately powered case-control study is required; assuming the incidence of VUE in non-anomalous stillbirth is similar to that of FGR, this comparison would require 137 stillbirths and matched controls. It is imperative that the stillbirths investigated in such a study should be non-anomalous stillbirths without another cause e.g. congenital anomaly, hydrops, placental abruption or overwhelming infection.

Analysis of studies of VUE was hampered by a number of factors. Primarily this related to the focus of the studies included; VUE rarely formed the primary topic of investigation, more often being considered a coincidental finding or one of several potential causes of FGR. In addition early investigations, such as those by Altshuler and Russell in 1975 (76) and Russell in 1980 (31), assumed that the etiology of the inflammation must be infectious, so greater effort was made trying to identify an infective agent rather than divide cases into VIE and VUE. Primary studies of VUE have largely occurred since it was demonstrated that VUE involves maternal cells infiltrating the villous stroma in a potential 'host-versus-graft' mechanism (87,88,100,102,144). Even then, the largest case-control study reporting placental inflammation in stillbirth primarily focussed on chorioamnionitis (21).

We identified other confounders that contributed to the heterogeneity of studies including varied definitions of FGR, VUE and gestational age of stillbirths. The methodological quality assessments clearly indicate this disparity, as well as the fact that many authors fail to define their criteria for VUE. However, in the case of VUE/FGR studies removing them from

the analysis does not affect the reported incidence (data not shown). In the stillbirth group, removal of studies that do not specify definition of VUE would leave only one investigation (21). Similarly, a description of the type of immune cells involved is not always recorded in studies. It is now accepted that VUE is characterised by a lymphohistiocytic infiltrate with few or absent plasma cells (83,88,101,102).

The gestation of samples included in the studies is important as Redline (2007) (88) states that VUE is a condition encountered in placentas from term or near term pregnancies, and is most strongly associated with the third trimester. Despite this, some FGR and VUE-specific studies have examined placentas from as early as 20 weeks gestation (31,80,82,97,98,149). According to Redline (88), villitis occurring at this timepoint is unlikely to be VUE. Therefore, the true incidence of VUE may have been misrepresented.

There is significant variation in the number of samples taken for histological examination. In 2003, Altemani et al. (86) stated that in order to diagnose 95% of villitis cases with a power of 0.85, 6 blocks should be examined. They suggest that examining 4 blocks from a placenta may result in considerable underdiagnosis of VUE. However, few subsequent studies have used more than 4 blocks, perhaps due to the findings of a pilot study by Knox and Fox in 1984 (82) which stated that four blocks was sufficient to accurately detect the majority of cases of villitis. It is also worth noting that those studies that took 8 samples of parenchyma neglected to sample the mid region of the placenta, opting to sample only the central and marginal areas (83,101,109,145).

The design of the studies also displays a degree of variability. Many of the studies were retrospective, following identification of a particular population, most often high-risk pregnancies. In several studies there were no matched controls, so design is that of a case-series rather than a case-control study. Often the investigators were already aware of the clinical outcome. In contrast to this Pathak et al. (2011) (95) designed their study to attempt to eliminate bias by blinding the assessors to clinical outcome during investigation. Theirs is the only study that demonstrates the same incidence of VUE in both their normal population and the SGA infants. In contrast to Pathak et al., Becroft, Thompson and Mitchell (91) were specifically and systematically searching for VUE. Their results showing incidence of VUE in FGR and AGA at 17% and 12% respectively is comparable to others in the field. From a population perspective, it appears that geographical location also influences the incidence of VUE. South American groups (Garcia, Labarrere, Altemani) (85,86,101) report higher incidence of VUE than European (Knox, Russell) groups (31,82). Whether or not this is related to a difference in the population or the examination practice is unclear.

Robust meta-analysis of VUE and stillbirth is currently unfeasible. There are too few studies and too much variation in study design to be able to accurately compare the results.

Standardisation of methods would improve reporting accuracy and reproducibility for future investigations, as would thorough and precise description of the number and location of samples, the expertise and blinding of the examiner and definitions of VUE. Thus far, diagnosis of VUE has been generally based on a qualitative assessment of H&E stained slides. It may prove useful to investigate whether there is a quantifiable cell population that could definitively characterise VUE.

The majority of research into FGR concentrates on hypoxia/metabolic insufficiency as a pathological mechanism. Inflammatory pathology is largely ignored. However, this systematic review has shown that inflammation, in this case VUE, remains an important yet elusive contributor to the pathogenesis of FGR. This echoes a previous systematic review of inflammatory subchorionic intervillositis which found increased incidence of FGR, and pregnancy loss rates of 46.4% (111). Research is needed to begin to answer questions related to placental inflammatory disorders, including: to determine which cells/pathways are involved in VUE; to determine the nature of the antigen in VUE; and to determine whether VUE is a significant underlying *causative* pathology in FGR infants. Any cited association of VUE with stillbirth remains inconclusive. This needs to be addressed by an appropriately powered study.

3.6 Funding

This review has been completed as part of a PhD studentship for HD funded by Tommy's – the baby charity. AH and RLJ are funded by Tommy's – the baby charity and AH additionally by the Holly Martin Stillbirth Research Fund.

3.7 Conflict of Interest

We declare that we have no conflict of interest

Table 4: Results of studies specifically investigating the incidence/aetiology of VUE/villitis (group 1).
**NB Percentages exceed 100% as some studies excluded SGA or stillbirths.*

			Where villitis was identified, proportions of outcomes recorded*:					
Author [Reference]	**Villitis**	**Total Samples**	**SGA**	**Total Villitis**	**AGA**	**Total Villitis**	**Stillbirths**	**Total Villitis**
Mortimer (90)	10	120	6	10	4	10	0	10
Labarrere, Faulk and McIntyre (92)	19	25	Excluded		19	19	Excluded	
Nordenvall and Sandstedt (146)	20	445	12	20	8	20	Not stated	
Altshuler and Russell (76)	No denominator given		14	63	45	63	4	63
Russell (31)	575	7505	47	575	514	575	14	575
Knox and Fox (82)	136	1000	18	136	116	136	2	136
Labarrere and Althabe (145)	18	72	Excluded		18	18	Not stated	
Labarrere, Althabe and Telenta (83)	43	63	38	43	5	43	Excluded	
Greer et al. (147)	1664	10207	366	1664	1298	1664	Excluded	
Pathak et al. (95)	42	1153	4	42	38	42	Excluded	
	2527	20590	505	2553	2065	2590	20	784
	12.3% (95% CI 11.8-12.7%)		19.8% (95% CI 18.3-21.4%)		79.7% (95% CI 78.1-81.2%)		2.6% (95% CI 1.7-3.9%)	

Table 5: Results of studies focussing on placental pathologies associated with FGR (group 2)

Author [Reference]	FGR/SGA + Villitis	Total Samples	AGA + Villitis	Total Samples
Salafia et al. (105)	38	128	34	179
Becroft, Thompson and Mitchell (91)	87	509	61	529
Aviram, Shental and Kidron (96)	11	65	2	38
Vedmedovska et al. (25)	13	50	3	50
Almasry et al. (35)	24	50	2	25
Gunyeli et al. (149)	18	26	8	26
Althabe and Labarrere (109)	79	105	39	106
Altshuler, Russell and Ermocilla (106)	15	63		
Garcia (85)	28	50		
Bjøro and Myhre (148)	31	108		
Kovo et al. (150)	15	99		
Total	359	1253	149	953
	28.6% (95% CI 26.2-31.2%)		15.6% (95% CI 13.5-18.1%)	

Table 6: Results of studies examining placental pathologies in stillbirth (group 3)

Author [Reference]	Stillbirth + Villitis	Total Samples	Livebirth + Villitis	Total Samples
Korteweg et al. (80)	15	750		
Rayburn et al. (13)	4	89		
Gunyeli et al. (149)	37	58		
Hulthén Varli et al. (21)	23	126	14	273
Total	79	1023	14	273
Percentage	7.7%		5.1%	

3.8 Systematic Review Update

The updated PubMed search for the term "villitis" produced 49 results. Further searches including the terms "villitis unknown etiology", "small for gestational age villitis" and "villitis growth restriction" produced 22, 5 and 11 (duplicate) results respectively. An Embase search using "villitis" and "stillbirth" or "fetal growth restriction" or a combination of these terms gave 22 results and MEDLINE, using the same parameters, 125. This gave an overall total of 234 results across all three databases. Altering the search term to "intrauterine growth restriction" produced exactly the same number of results. Abstract screening, foreign language citations and comparison of database results for duplicates excluded 228 articles. Similar to the original systematic review a large number of excluded reports were related to the epidemiology of and risk factors for fetal growth restriction and stillbirth. The remaining 6 studies were excluded because of the focus of the investigation: 3 featured co-existing maternal pathologies (including infections), one studied infants with thrombocytopaenia, one was a morphological study and one did not record VUE/VIE separately or detail the incidence of villitis in a control group. There were no further studies that required adding to the systematic review update.

CHAPTER 4: Characterising Villitis of Unknown Etiology and Inflammation in Stillbirth

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Margaret J. Evans, Alexander E.P. Heazell

A manuscript submitted to the *American Journal of Pathology*

4.1 Abstract

Villitis of unknown etiology (VUE) is an enigmatic inflammatory condition of the placenta associated with fetal growth restriction and stillbirth. Greater understanding of this condition is essential to understand how it contributes to adverse outcomes. We aimed to identify and quantify the cells in VUE in cases of stillbirth and to characterise immune responses specific to this condition. Immunohistochemistry was performed on placentas from stillborn infants whose contributory cause of death was recorded as VUE to identify: CD45+ leukocytes, CD163+ macrophages, CD4+ T-cells, CD8+ T-cells, neutrophils, and pro- and anti-inflammatory cytokines. Images were quantified using HistoQuest software. CD45+ leukocytes comprised 25% of cells in VUE lesions: macrophages (12%) and CD4 T-cells (11%) being the predominant cell types; CD8 T-cells were also observed in all lesions. Leukocytes and macrophages were increased throughout the placenta in stillbirths; pan-placental CD4+ and CD8+ T-cells outside VUE lesions were increased in stillbirth with VUE. There was increased IL-2 and IL-12, and reduced IL-4 immunostaining in VUE lesions. Our results suggest VUE in stillbirth has a similar immune cell profile to live birth. Pan-placental macrophages, CD4 and CD8 T-cells indicate a wider inflammatory response unrestricted to VUE lesions. The cytokine profile observed suggests a skew towards inappropriate Th1 immune responses. Full characterisation VUE lesion phenotype confirms its immunological origins and provides foundations to develop novel investigations.

4.2 Introduction

Stillbirth, the death of a baby after 20 weeks gestation, places a sustained economic and emotional burden on both maternity services and the wider community. Despite significant effort, the stillbirth rate has decreased at a slower rate than neonatal or infant death (151). In high income countries stillbirth rates are variable. The United States and UK perform relatively poorly with rates of 1 in 160 and 1 in 212 respectively, whereas in Finland 1 in 400 pregnancies result in a stillborn infant (8,15,152).

Placental dysfunction as a cause of stillbirth has increasingly become a focus of research effort with recognition that placental pathologies are the cause of death in up to 65% of stillbirths (4). However, the placental lesions associated with stillbirth are extremely varied, poorly defined and their relationship to pathological processes leading to stillbirth is difficult to assess (73,153). Placental lesions associated with stillbirth include those with genetic, environmental, infective, inflammatory, mechanical, metabolic and vascular origins (reviewed by Heazell et al.(73)).

Understanding of inflammatory pathologies of the placenta remains relatively limited in comparison to vascular lesions. Two main inflammatory conditions have been linked to placental dysfunction in the absence of infection: villitis of unknown etiology (VUE) and chronic histiocytic intervillositis (154). VUE is an inflammatory condition of the placenta reported to occur in up to 15% of term placentas, and more frequently in pregnancies resulting in poor outcome (88,155). The origin of VUE is unclear though it is proposed to be maternal immune rejection of a semi-allogeneic placenta (87,102,144). VUE is identified and characterised by the presence of elevated numbers of fetal macrophages (Hofbauer cells (HBC)) and an infiltrate of maternal T-lymphocytes in the villous stroma. Areas of the placenta not affected by VUE lesions display apparently normal morphology (88). The cell profile of VUE has previously been characterised in both appropriately grown and growth restricted live born infants (87,100,156,157). Conflicting reports state either CD4+ or CD8+ T-cells are the majority lymphocyte type in VUE lesions (87,89,93,156). The subtype of CD4+ cells (Th1/Th2) has not been examined therefore the type of immune response in VUE remains to be elucidated.

A thorough characterisation of VUE has yet to be completed in stillbirth; it is not understood why it may cause fetal demise. In addition, the cytokine profile in VUE has not been described in either live births or stillbirths. Characterisation of the cell types present and their cytokine profile will provide a greater understanding of the pathophysiology of VUE. We hypothesised that the immune cells in VUE lesions in stillbirth would be comparable to those described in live birth and that evidence of widespread inflammatory changes would be detectable in the placentas of stillborn infants with VUE. In this immunomorphological

study we aimed: to accurately quantify the type and number of immune cells present in VUE lesions, to assess leukocyte infiltration across the placenta and to analyse the expression of a range of pro- and anti-inflammatory cytokines using immunohistochemical approaches, coupled with an unbiased sensitive image analysis method. We compared leukocyte infiltration and cytokine expression across the placenta to ascertain whether this was altered in dysfunctional placentas from stillbirth with and without VUE and fetal growth restriction (FGR) compared to healthy controls.

4.3 Methods

4.3.1 Study Population

Placental tissues from pregnancies resulting in stillbirth where a contributory cause of death was recorded as VUE (n=12) or FGR (n=12) were obtained from the Paediatric Histopathology departments of Manchester Royal Children's Hospital and Edinburgh Royal Infirmary. Samples from stillborn growth restricted infants had no evidence of VUE lesions recorded on post-mortem examination. Consent for tissue use in research was obtained at the time of consent for post mortem; their use in this project was approved by Humber Bridge Research Ethics Committee (Ref: 13/YH/0176). Live born infants (n=11) with an individualised birthweight centile (IBC) $<5^{th}$ were considered FGR infants; placental tissue was obtained from the Maternal and Fetal Health Research Group (MFHRG) Biobank, University of Manchester (North West REC 08/H1010/55). Matched placental samples from women with uncomplicated pregnancies (n=12) were selected from the MFHRG Biobank and used as controls. All placentas were taken from mothers who showed no evidence of conditions such as pre-eclampsia or diabetes. Multiple pregnancies, abnormal fetal heartbeat (live birth only) and fetuses with anomalies were excluded.

4.3.2 Sample Collection

Placentas from cases of stillbirth had 2-7 randomly selected samples per placenta obtained at the time of post mortem for histological analysis. For live births three samples of placental villous tissue were taken, one each from the centre, middle and periphery of the tissue (67). Biopsies were fixed in neutral buffered formalin (NBF) for 24 hours before being processed and paraffin wax embedded.

4.3.3 Immunohistochemistry

For immunohistochemistry, 5µm serial sections were cut and mounted onto poly-L-lysine (Sigma, Poole, Dorset) coated slides. Immunostaining with colorimetric detection was performed as described by Hamilton et al. (158). VUE lesions were initially identified by detecting clusters of leukocytes using a mouse monoclonal antibody (mAb) to CD45 (leukocyte common antigen, LCA). A VUE lesion was defined as an area of villous tissue with

evidence of an extensive CD45+ cell infiltrate that displayed the morphological properties of macrophages and lymphocytes (Figure 9A). Subsequent immunostaining for specific immune cells was carried out to identify CD4+ T-cells, CD8+ T-cells, CD163+ macrophages and neutrophils. Further immunostaining for cytokines interleukin (IL)-2, IL-4, IL-10 and IL-12 was performed on representative sections from all samples. Finally, staining for IL-6 and transforming growth factor (TGF)-β was performed on selected stillbirth samples with VUE (n=6) and healthy pregnancies (n=6). Heat-mediated antigen retrieval was performed for all antibodies except anti-neutrophil elastase and anti-IL-10 mAbs. Primary antibodies were applied at the working concentrations detailed in Table 7. Each tissue section had a corresponding negative control on which mouse or rabbit IgG (Sigma Aldrich, Dorset, UK) was applied at the same concentration as the primary antibody. Each staining run included a positive control tissue as described in Table 7.

4.3.4 Immunofluorescence

The phenotype of the macrophages and T-cells in the placentas/lesions was investigated by dual immunofluorescence (IF) with combinations of immune cell markers CD4 and CD163 and cytokines IL-2, IL-12 or IL-4. Sections were fixed and processed as described above. Autofluorescence was quenched by 1hr incubation in 0.25% ammonia (Sigma-Aldrich) in 70% ethanol. Primary antibodies were applied at the concentrations described in Table 7 at 4°C overnight. Secondary antibodies (goat anti-mouse Alexa 568 or goat anti-rabbit Alexa 488 (Life Technologies, Paisley, UK)) were applied at 5μg/mL and incubated for 30 minutes at room temperature. DAPI-containing aqueous mounting medium was used to apply coverslips.

4.3.5 Image Capture and Analysis

Tissue sections were visualised using the x10 objective of an Olympus BX41 light microscope (Southend-on-Sea, UK). Images were captured with QIcam Fast 1394 camera (QImaging, BC, Canada) and Image Pro Plus 6.0 (Media Cybernetics Inc., MD, USA). Quantitative, unbiased image analysis was completed using HistoQuest image analysis software (TissueGnostics, Vienna, Austria). The pixels of images viewed in this software are converted to greyscale (0-256 scale) and assigned an arbitrary number that correlates to staining intensity. The image analysis software allows differentiation of haematoxylin+ and DAB+ cells or areas. Analysis parameters were such that the number of DAB+ events and haematoxylin+ events were counted (Figure 10).

Two analyses were performed. Firstly, for characterisation of VUE lesions in stillbirth, inflammatory foci were identified in CD45 stained sections then the remaining immunostains matched and captured in serial sections (Figure 9). In tissue sections with

widespread/numerous inflammatory foci, representative lesions were selected (n=2-5 per section).

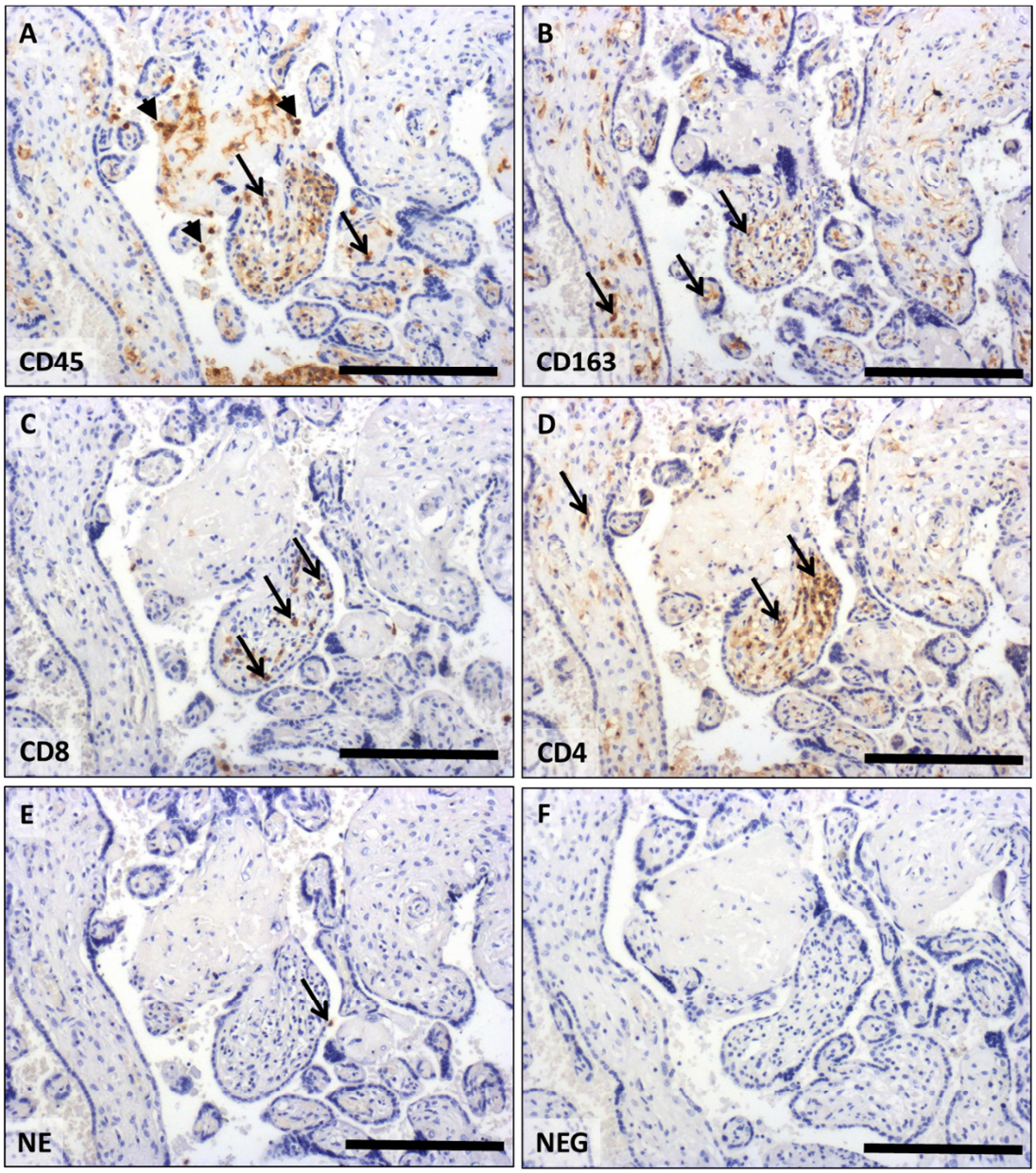


Figure 9: Serial images of a representative VUE lesion immunostained for immune cell markers. (A) CD45, (B) CD163+ macrophages, (C) CD4+ T helper cells, (D) CD8 cytotoxic T cells, (E) neutrophil elastase, (F) negative. Arrows highlight examples of positively stained cells. Scale bar represents 50µm. Original magnification x100.

To assess pan-placental inflammation in tissue, ten random images (excluding lesions) were taken for each placental slide from all groups (stillbirth with VUE, stillbirth with FGR, live birth with FGR and controls), immunostained for CD45, CD163 macrophage, CD4 T cell and CD8 T cell markers. DAB+ cells and nuclei were quantified to ascertain whether there were any differences in leukocyte numbers between the study groups.

Table 7: Antibodies and positive control tissues used for identifying immune cell populations and cytokines in placental villous tissue.
Ag: antigen, Conc: concentration, Ab: antibody, PTLI: pre-term labour with infection, M mAb: mouse monoclonal antibody, R pAb: rabbit polyclonal antibody.

Ag	Ag Retrieval	Supplier	Working Conc	Ab Type	Control Tissue
CD45	Sodium citrate	Dako	0.5µg/ml	M mAb	Tonsil
CD163	Sodium citrate	AbD Serotec	10µg/ml	M mAb	Tonsil
CD4	TRIS-EDTA or sodium citrate	Dako	4.6µg/ml	M mAb	Tonsil
CD8	TRIS-EDTA	Dako	1.6µg/ml	M mAb	Tonsil
NE	None	Dako	1.1µg/ml	M mAb	PTLI
IL-2	Sodium citrate	Novus Biologicals	4.5µg/ml	R pAb	Tonsil
IL-4	Sodium citrate	Thermo Scientific	2.5µg/ml	R pAb	Tonsil
IL-6	Sodium citrate	Novus Biologicals	9.6µg/ml	M mAb	Tonsil
IL-10	None	Abcam	5µg/ml	M mAb	Liver (Kupffer cells)
IL-12	Sodium citrate	Sigma-Aldrich	2.5µg/ml	R pAb	Trophoblast
TGF-β	Sodium citrate	Novus Biologicals	10µg/ml	M mAb	Tonsil

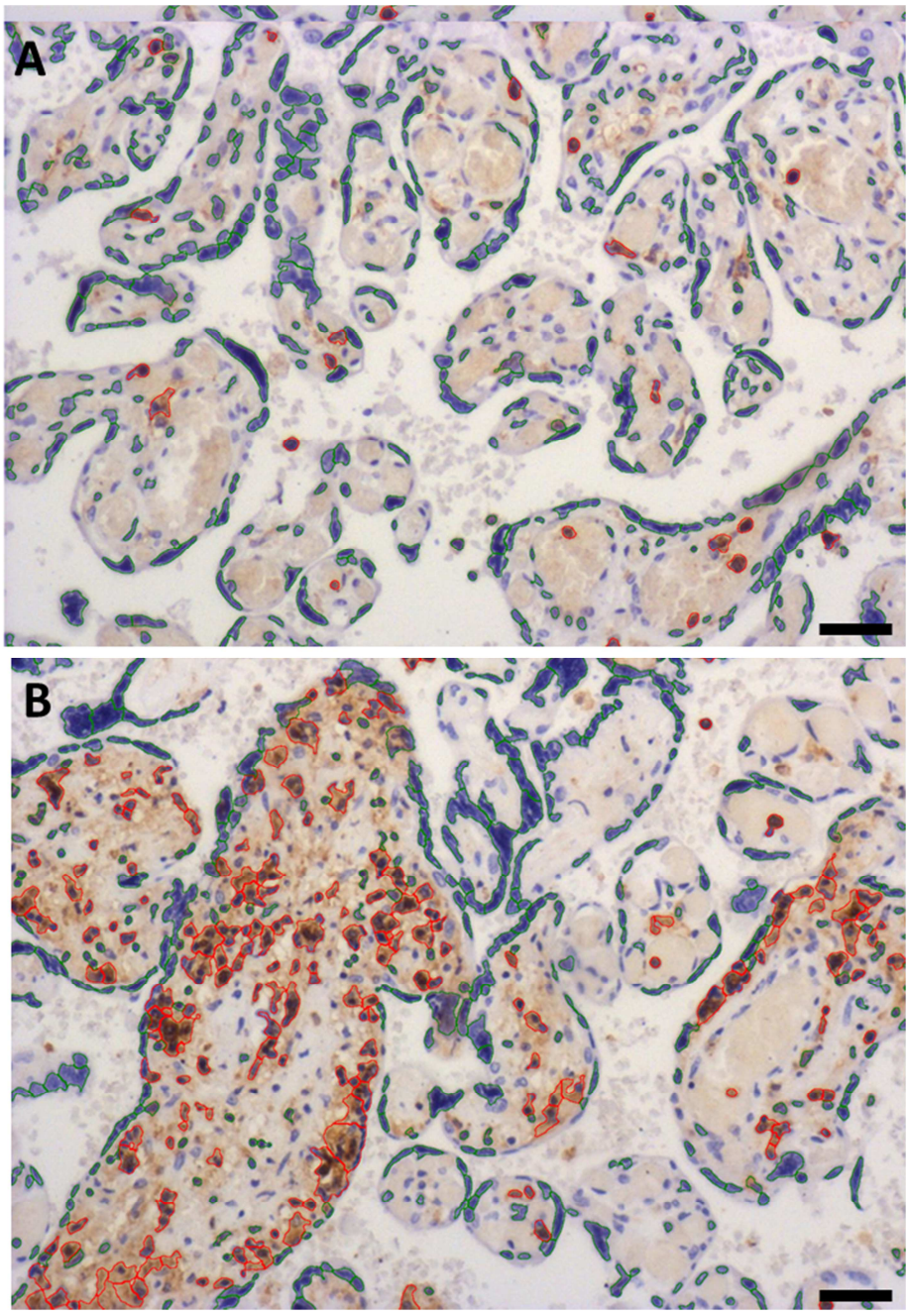
The presence of cytokines was determined by immunostaining for IL-2, IL-4, IL-6, IL-10, IL-12 and TGF-β. Images were analysed quantitatively in sections of tissue from VUE lesions, areas of stillbirth with VUE placentas that were devoid of lesions, stillbirth with FGR placentas, live birth with FGR placentas and controls (n=10 images per placenta). Area and

intensity of staining was recorded for each placental sample and quantitative analysis completed using HistoQuest image analysis software.

Fluorescent stained sections were visualised using a Zeiss fluorescence microscope, AxioCam MRn camera with Zen pro image processing software (Zeiss, Welwyn Garden City, UK).

4.3.6 Statistical Analysis

We powered our analysis to determine the number of CD45+ and CD163+ cells in placentas from stillborn infants assuming similar increases in these cells as seen in placentas from poor outcome pregnancies experiencing reduced fetal movements (122). No similar studies have been conducted with CD4 and CD8 T cells, so we were not able to take direct comparisons. For 80% power with an α value of 0.05 the sample size was calculated as 12. All statistical analysis was carried out using GraphPad Prism 6.0 software (San Diego, CA, USA). Non parametric categorical data was analysed using Fisher's Exact test, non-parametric continuous data with Mann Whitney U test or Kruskal-Wallis test with Dunn's post-hoc test.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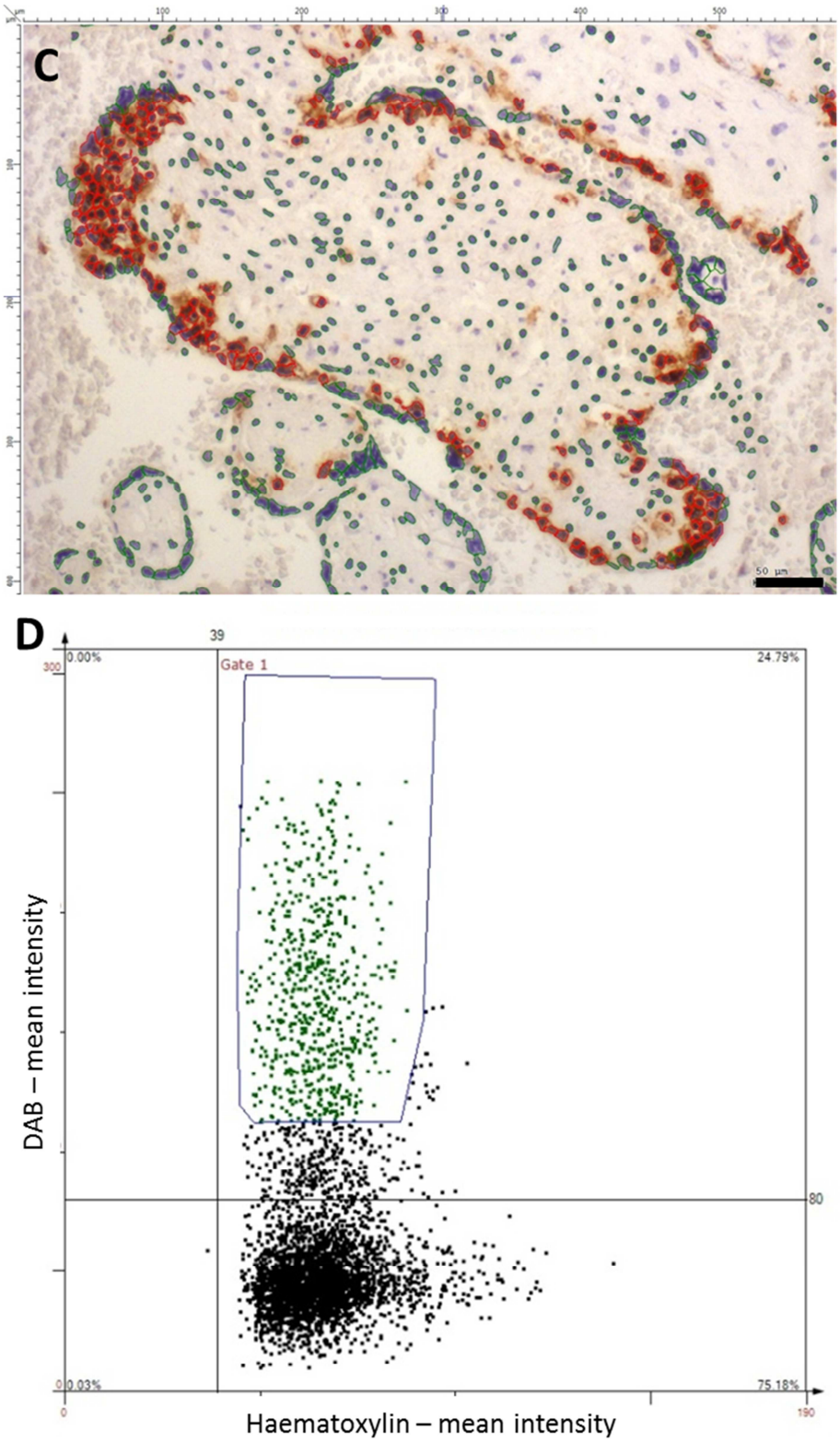


Figure 10: Screenshots from HistoQuest image analysis software.
The selection of DAB+ events is shown in sections stained for A, B) CD45 leukocyte common antigen and C) cytotoxic T lymphocyte marker CD8. Positive events are highlighted in red; nuclear events encircled in green indicate haematoxylin positive/DAB negative events. Figure 1D shows an example scattergram from which staining intensity parameters were ascertained. Scale bar = 50µm in all images, original magnification x100.

4.4 Results

4.4.1 Participant Demographics

Table 8 shows the demographic data for the 48 women included in the study. There was a significant difference in maternal age between stillbirth with FGR and normal pregnancy groups (p=0.02). By experimental design birthweight and IBC differences between stillbirth with FGR, live birth with FGR and normal pregnancies were highly significant (p<0.001 and p<0.01 respectively).

Table 8: Demographic information for the women included in the study.
*Results are presented as median (range). NA: data not available, ♆birthweight centile not individualised birthweight centile (IBC). *p<0.05, **p<0.01, ****p<0.0001 compared to normal pregnancy.*

	Stillbirth with VUE (n=12)	**Stillbirth with FGR (n=12)**	**Live birth with FGR (n=11)**	**Normal pregnancy (n=12)**	**p value**
Maternal age	32 (21-36)	23.5 (19-42) *	32 (16-35)	35 (25-42)	0.0192
Gestational age	39 (20-41)	35.21 (28-38)	37.8 (27.7-42.1)	33.5 (30.0-41.7)	0.0983
Gravidity	3 (1-7) (n=7)	1 (1-4)	1 (1-9)	2 (1-18)	0.2984
Parity	1 (0-3) (n=7)	0 (0-2)	0 (0-3)	1 (0-3)	0.1182
Birthweight (g)	NA	1061 (385-2000)****	2246 (747-3060)**	3430 (2900-3840)	<0.0001
IBC	23 (3-90) (n=7)♆	NA	3 (0-10)****	45 (12-93)	<0.0001

4.4.2 Characterisation of Focal Inflammatory Lesions

VUE lesions were identified in all placentas from the stillbirth with VUE cohort; a representative lesion is shown in Figure 9. In total 104 VUE lesions from 12 placentas were photographed and analysed. The number of CD45+ cells in the lesions contributed 25% (median, range 8 - 48%) to the total number of nuclei. Macrophages and CD4+ T lymphocytes were the most prevalent immune cells in the lesions accounting for 12% (9 - 23%) and 11% (3 - 14%) of the total number of nuclei respectively. CD8+ T lymphocytes were also present in VUE lesions, contributing 4% (0.3 - 11%) to the total number of nuclei. Neutrophil levels were low in all samples, accounting for 1% (0.37 - 2%) of total number of nuclei.

4.4.3 Quantification of Pan-Placental Immune Cells

Significant increases were detected in the number of pan-placental CD45+ leukocytes in all placentas from pregnancies from stillborn infants (with or without VUE) when compared to normal pregnancies ($p<0.001$) (Figure 11A). Significant elevation in macrophage numbers was detected in the placentas from stillborn infants with VUE ($p<0.01$), stillborn infants with FGR ($p<0.001$) and live born infants with FGR ($p<0.01$) compared to normal pregnancy (Figure 11B). Placentas from the stillbirth with VUE group showed significantly more pan-placental CD4 and CD8 cells compared to all other groups ($p<0.001$) and were observed in every field of view, excluding lesions (Figure 11C-D).

4.4.4 Cytokines

In order to fully characterise the immune response in VUE, immunostaining of characteristic Th1 (IL-2, IL-12) and Th2 (IL-4) cytokines was performed (Figure 12). Staining of IL-2 and IL-12 was localised to cells within the stromal core and STB, as previously shown (159) (Figure 13). Area of IL-12 staining was significantly increased in VUE lesions and live born FGR placentas compared to normal pregnancy ($p<0.001$). A significant increase in area of IL-2 staining was observed in VUE lesions ($p<0.05$) (Figure 14). IL-4 staining was localised to both stromal and trophoblast cells (160) (Figure 13). There was a significant reduction in the area of IL-4 immunostaining in VUE lesions and areas devoid of lesions compared to controls ($p<0.05$ and $p<0.01$, respectively). A significant reduction in IL-4 staining area was also observed in the stillbirth with FGR group compared to live birth with FGR and controls ($p<0.05$ and $p<0.001$, respectively). IL-10 appeared significantly increased overall in areas of VUE placentas devoid of lesions ($p<0.001$), but individual comparisons failed to reach statistical significance (Figure 12, Figure 14 and Supplementary Figure 1). Staining intensity for all cytokines was significantly different between samples (Supplementary Figure 2). Intensity of IL-2 was significantly reduced (stillbirth FGR $p=0.006$, live birth FGR $p=0.0004$) and IL-12 increased (stillbirth FGR $p=0.0002$, live birth FGR $p<0.001$) in FGR samples compared to control. IL-4 intensity was significantly reduced in VUE lesions compared to control ($p=0.02$). IL-10 staining intensity was significantly increased in VUE placentas compared to stillbirth with FGR (VUE lesion $p=0.03$, VUE non-lesion $p=0.01$). Immunostaining for cytokines IL-6, and TGF-β was not detected in VUE lesions (Supplementary Figure 1).

4.4.5 Macrophage and T cell Phenotype

Dual immunofluorescence was performed to identify immune cell sources of cytokines of interest. IL-2 co-localised with CD4+ staining, CD163+ with IL-12 staining and IL-4 with CD4+ (Figure 15). There is evidence of faint IL-4 fluorescence in the syncytiotrophoblast layer as observed in the images of chromogen staining (Figure 12 and Figure 15).

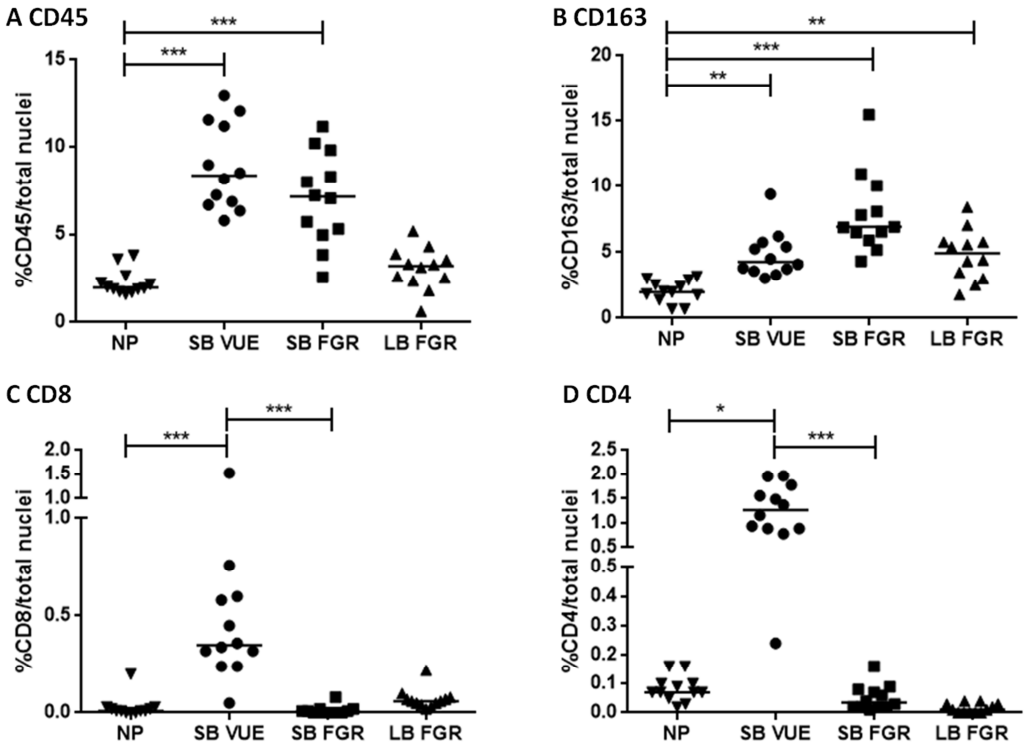


Figure 11: Quantification of pan-placental immune cells in normal and pathological pregnancies. (A*) CD45, (B) macrophages (CD163), (C) cytotoxic T cells (CD8), (D) T helper cells (CD4). p<0.0001. SB VUE: stillbirth with VUE, SB FGR: stillbirth with fetal growth restriction, LB FGR: live birth with fetal growth restriction, NP: normal pregnancy. Kruskal-Wallis test with Dunn's post-hoc, lines represent median. *p<0.05, **p<0.01, ***p<0.001.*

4.5 Discussion

This study has fully characterised VUE in placentas from stillborn infants by quantifying the immune cells found in the lesions using unbiased image analysis software, following immunohistochemistry to detect specific immune cells types. We have also demonstrated specific pan-placental inflammatory changes in stillbirths (with and without VUE), indicating a more widespread immune reaction occurring out with localised VUE lesions. Furthermore, our cytokine analyses indicate that the immune response in the lesions is directed towards Th1-type response.

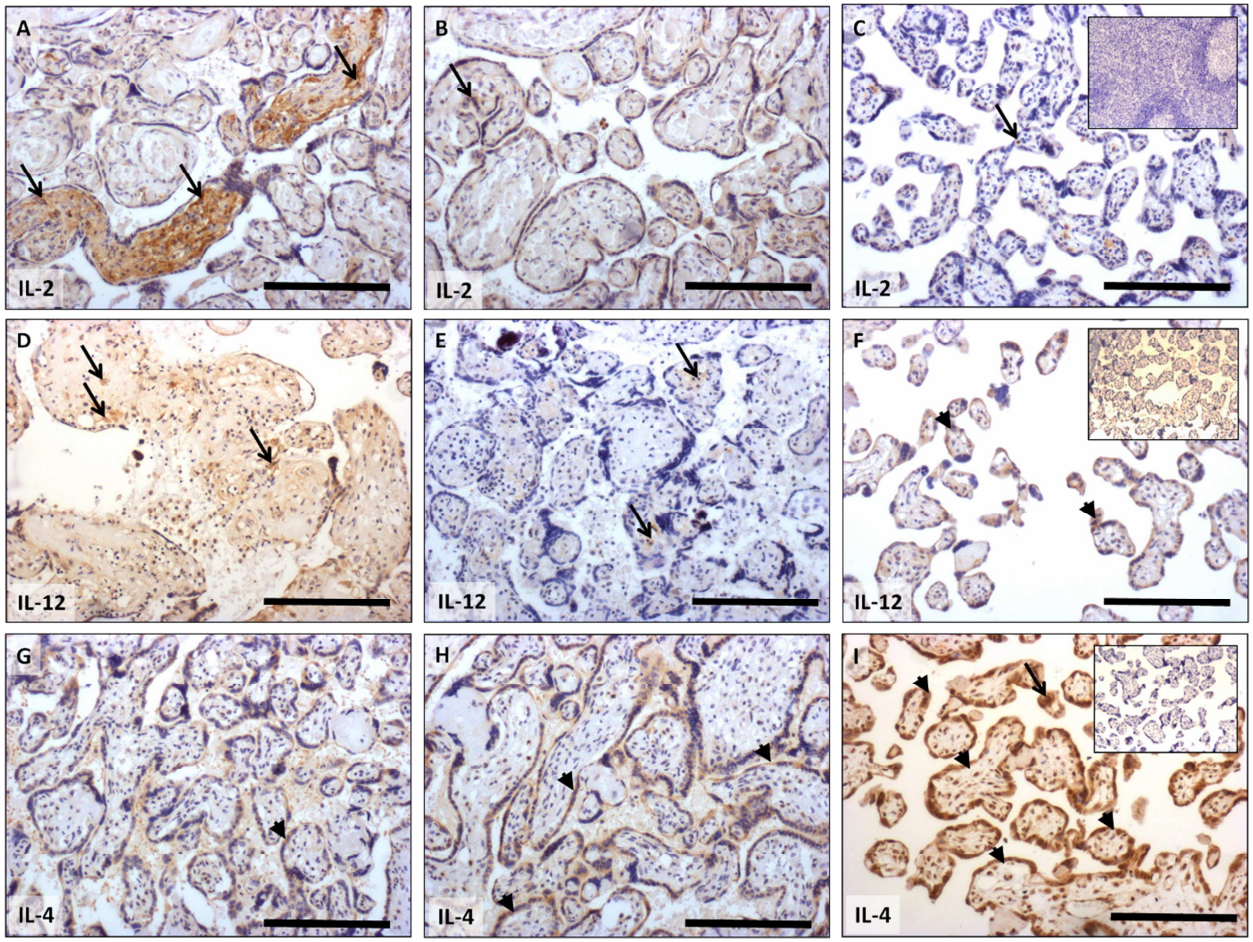


Figure 12: Representative images of placental sections immunostained for pro- and anti-inflammatory cytokines.
VUE lesions stained for: (A) Interleukin (IL)-2, (D) IL-12 and (G) IL-4. Cytokine staining in areas of placenta unaffected by lesions from stillborn infants with VUE stained for: (B) IL-2, (E) IL-12 and (H) IL-4. Representative sections of placenta from pregnancies with a normal outcome stained for: (C) IL-2, (F) IL-12 and (I) IL-4 (insets: negatives). Arrows highlight staining associated with cells, arrowheads highlight staining associated with trophoblast. Scale bar represents 50µm. Original magnification x100.

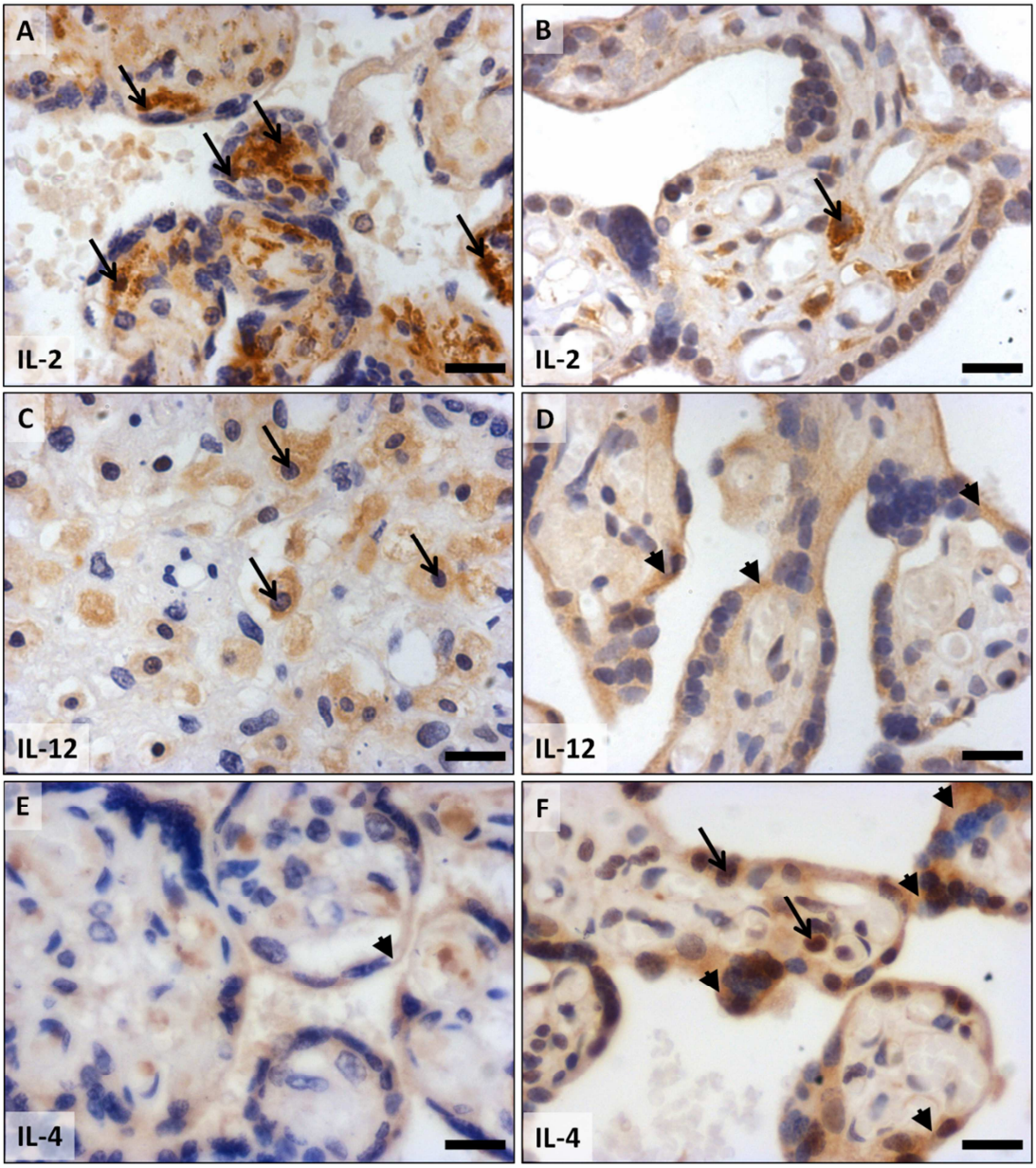


Figure 13: Representative high power images of placental sections following immunostaining for pro- and anti-inflammatory cytokines.
VUE lesions stained for: (A) Interleukin (IL)-2, (C) IL-12 and (E) IL-4. Representative sections of placenta from pregnancies with a normal outcome stained for: (B) IL-2, (D) IL-12 and (F) IL-4. Arrows highlight staining of cells in the stromal core, arrowheads highlight staining associated with syncytiotrophoblast. Scale bar represents 20μm. Original magnification x400.

Previous studies of VUE in live birth have described lesions comprising fetal macrophages (161) and maternal CD4+ (92,93) or CD8+ (87,156) T-lymphocytes. Our results support these descriptions: in cases of stillbirth, macrophages and CD4+ T-cells predominate, although elevated numbers of CD8 cells are also present. A key strength of the current study is the use of immunostaining to detect specific immune cell types. Pathologists examining H&E stained sections would not be able to identify individual leukocyte subsets, which are clearly visible following immunostaining for leukocyte, macrophage, CD4 and CD8 cell markers. VUE has been likened to graft rejection or graft-versus-host disease

(87,102,144). The phenotype that we observed, with CD4+ lymphocytes outnumbering CD8+ lymphocytes, supports this hypothesis as the T-helper cells would assume responsibility for directing the immune response towards a Th1-type response (123,162). The presence of cytotoxic CD8 T-cells in the lesions may contribute to both the fetal placental vasculopathy often observed in VUE and the recently described elevated apoptosis of intravillous cells (88,89,97,163).

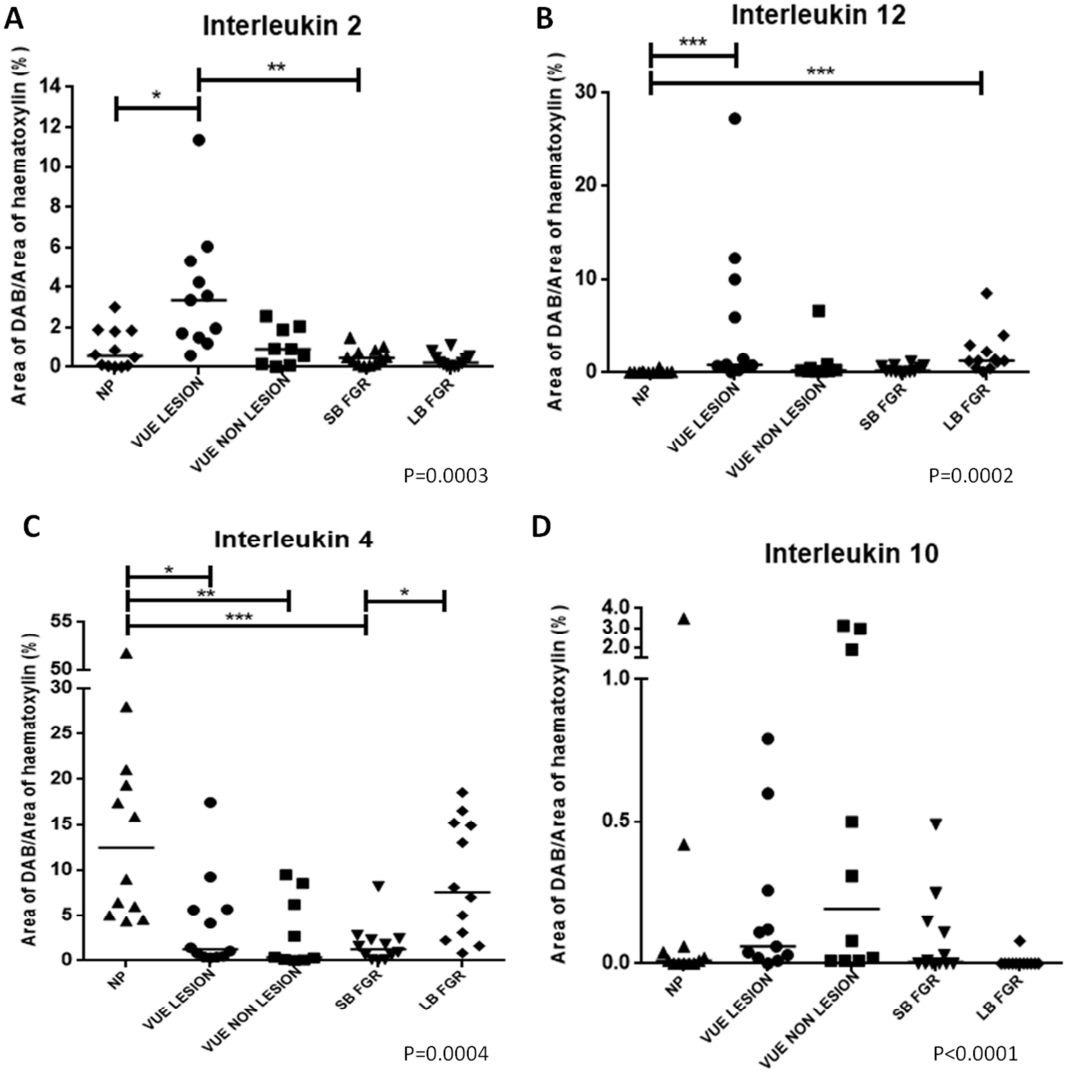


Figure 14: Quantification of cytokine staining area using HistoQuest image analysis software.
*(A) Interleukin (IL)-2, (B) IL-12, (C) IL-4, (D) IL-10. NP: normal pregnancy, SB FGR: stillbirth with fetal growth restriction, LB FGR: live birth with fetal growth restriction. Kruskal-Wallis test with Dunn's post-hoc, lines represent median. *p<0.05, **p<0.01, ****p<0.0001.*

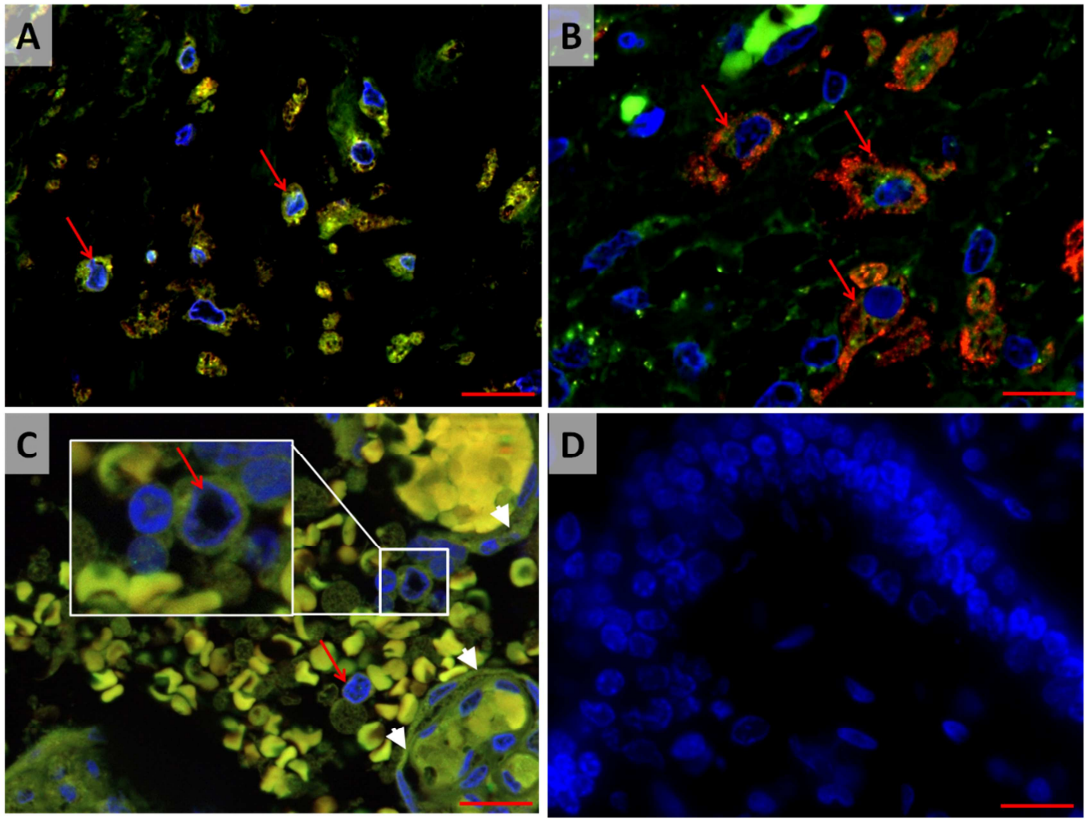


Figure 15: Representative high power images of placental sections following dual immunofluorescence for pro- and anti-inflammatory cytokines and cell markers.
VUE lesions stained for (A) CD4 (red), interleukin (IL)-2 (green), co-localisation (yellow), (B) CD163 (red) and IL-12 (green) and (C) CD4 (red) and IL-4 (green), co-localisation (yellow). (D) Mouse and rabbit IgG isotype control. Red arrows highlight co-localised immunofluorescence (yellow), white arrowheads highlight trophoblast localised IL-4. Scale bar represents 20μm. Original magnification x630.

Since Medawar first described the immunological paradox of pregnancy (126), much has been published on the shift from Th1 to Th2 responses in pregnancy (132,164–167). Suppression of anti-fetal cell-mediated responses and a skew towards antibody-mediated immunity appears to be advantageous in the maintenance of a successful pregnancy (167,168). The cytokine profile differs between Th1 and Th2 responses; IL-2, IL-6 and IL-12 are associated with Th1 responses, whereas anti-inflammatory cytokines IL-4, IL-10 and TGF-β predominate in Th2-type responses. Our findings of increased levels of IL-2 and IL-12 in VUE lesions, suggest there may be an inappropriate shift towards cell-mediated immunity in VUE cases associated with stillbirth. Staining intensity of IL-2 and IL-12 was not significantly different between VUE samples and controls implying that more cells are secreting the cytokine but the expression of it is not different. Co-localised immunofluorescent staining of CD163/IL-12 and CD4/IL-2 is indicative of pro-inflammatory macrophage and Th1 CD4 T cell phenotypes. We have also detected a significant reduction in levels of anti-inflammatory IL-4 in VUE placentas from cases of stillbirth. The reduction in IL-4 levels was mirrored by a significant reduction in expression in VUE lesions. The placental anti-inflammatory cytokine environment characterised by IL-4, IL-10 and TGF-β,

which induces maternal tolerance of the fetus, is critical in maintaining healthy pregnancy (164,168,169). Conversely, levels of pro-inflammatory IL-12 remain low throughout successful pregnancy (168). Significant decreases in IL-4 in stillbirth placentas may be a contributory factor in permitting a shift from Th2-type to Th1-type immunity. These data support a reduction in Th2 and a Th1 bias in VUE lesions, which may lead to unresolved cell mediated inflammation. Therefore the critical balance appears to be disrupted in VUE lesions in cases ending in stillbirth.

It has been stated that macrophages and their cytokines are fundamental in establishing and maintaining the placenta at the start of pregnancy (55,170). It is therefore reasonable to suggest that an excess of immune cells and pro-inflammatory cytokines in late pregnancy may have a detrimental effect on placental function, which is then manifested as FGR or stillbirth. These effects would be in addition to the cytotoxic effects of any immune response mounted. Interestingly, systematic reviews suggest a general shift towards a pro-inflammatory cytokine profile in maternal serum and placentas from FGR and SGA infants, confirming a role for a pro-inflammatory state in FGR (137,171). Conflicting reports of M2 placental macrophages potentially acting as either pro-inflammatory (172) or immunosuppressive (163) cells only serves to compound the mystery underlying VUE. Our investigation has shown placental macrophages expressing the M2 marker CD163, yet producing the pro-inflammatory cytokine IL-12, further enhancing the evidence of the plasticity of these cells and their potential role in pregnancy complications.

Pan-placental increases in leukocyte numbers in VUE are a novel finding. Significantly elevated numbers of leukocytes and macrophages were detected in all stillbirths compared to controls. The elevation in macrophages in live born FGR suggests that this is not a post mortem artefact. These data are consistent with our recent findings of elevated placental macrophage numbers in pregnancies at high risk of stillbirth (122). Staining intensity of cytokines in both live born and stillborn FGR were consistent throughout, further confirming that the morphology observed is not related to post mortem tissue changes. The placentas from these pregnancies also exhibited a pro-inflammatory bias in relation to cytokine mRNA and protein expression (elevated IL-1 family members, reduced IL-10 and IL-4). Together, these data are highly suggestive of placental inflammation being an underlying factor in pregnancies that result in FGR and stillbirth.

Previous descriptions of VUE have stated the occurrence of focal villous lesions with adjacent unaffected tissue displaying a normal morphology (88). It has been suggested that the occurrence of VUE may have been underestimated because sampling of the placenta was not extensive enough to find evidence of lesions (86); Altemani et al. reported that at least 6 blocks are needed to achieve an 85% chance of diagnosing 95% of cases (30). In our study, CD4 and CD8 T-cell numbers were increased in stillbirths with VUE compared to

all other group samples and these increases were not confined to VUE lesions. This finding is a strength of the specificity of the IHC in our study. A sensitive and unbiased image analysis tool that can accurately detect immunostained cells using a relatively low magnification (x100) also contributes to the strength of the study. Our data suggest that a pan-placental increase in CD4 and CD8 T-cells should stimulate the pathologist to suspect an inflammatory condition, such as VUE, without relying on locating a specific lesion. This means that using IHC and sensitive image analysis software such as HistoQuest, the incidence of VUE could potentially be identified and accurately reported using fewer tissue samples. More sophisticated placental pathology investigations may improve diagnosis of VUE (as was the case with oestrogen receptor expression in breast cancer (173)), and thus provide much sought after explanations for the cause of death.

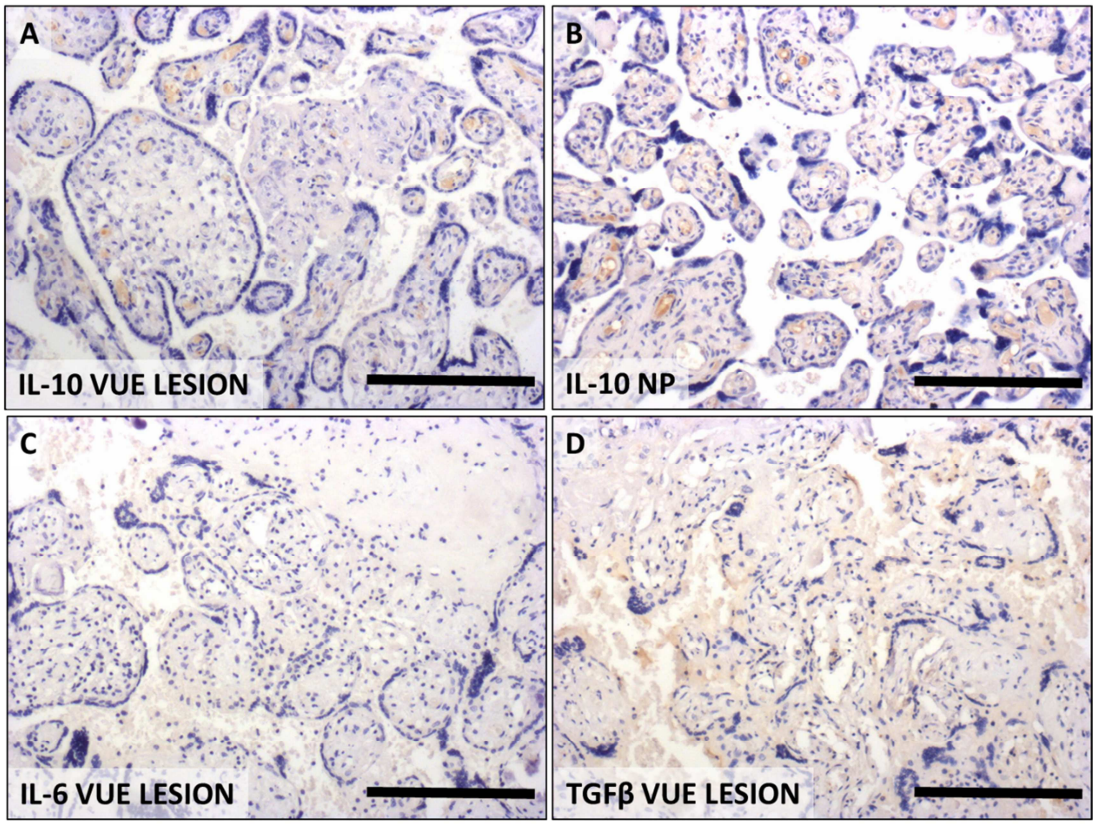
By examining placental tissue after delivery, immunohistochemistry can only provide a snapshot of VUE, rather than a temporal profile of the cell migration and its associated effects. This could explain why descriptions of the proportions of T-cells in VUE differ between research studies as they sample different periods in the evolution of lesions (87,89,93,156). In addition, the pan-placental presence of CD4 and CD8 T-cells may represent 'pre-lesions' that, given time, would expand within the villous stroma, potentially explaining why VUE is considered a third trimester phenomenon (88). This dynamic process cannot be surmised from the current method of studying placental tissue. The results of this study would be further enhanced by including samples of placenta from live born infants with evidence of VUE. At the time the study was conducted, these were not available.

A greater understanding of the immune responses associated with VUE may lead to the development of screening tests to enable antenatal detection of VUE. Our laboratory recently published data indicating alterations in concentrations of maternal serum cytokines in pregnancies characterised by placental inflammation (122). This is supported by the observation of elevated maternal plasma chemokines (CXCL9, 10 and 11) in cases of VUE (144). It may therefore be possible to identify placental inflammation antenatally which would allow increased antenatal surveillance and potential anti-inflammatory therapies to prevent stillbirth.

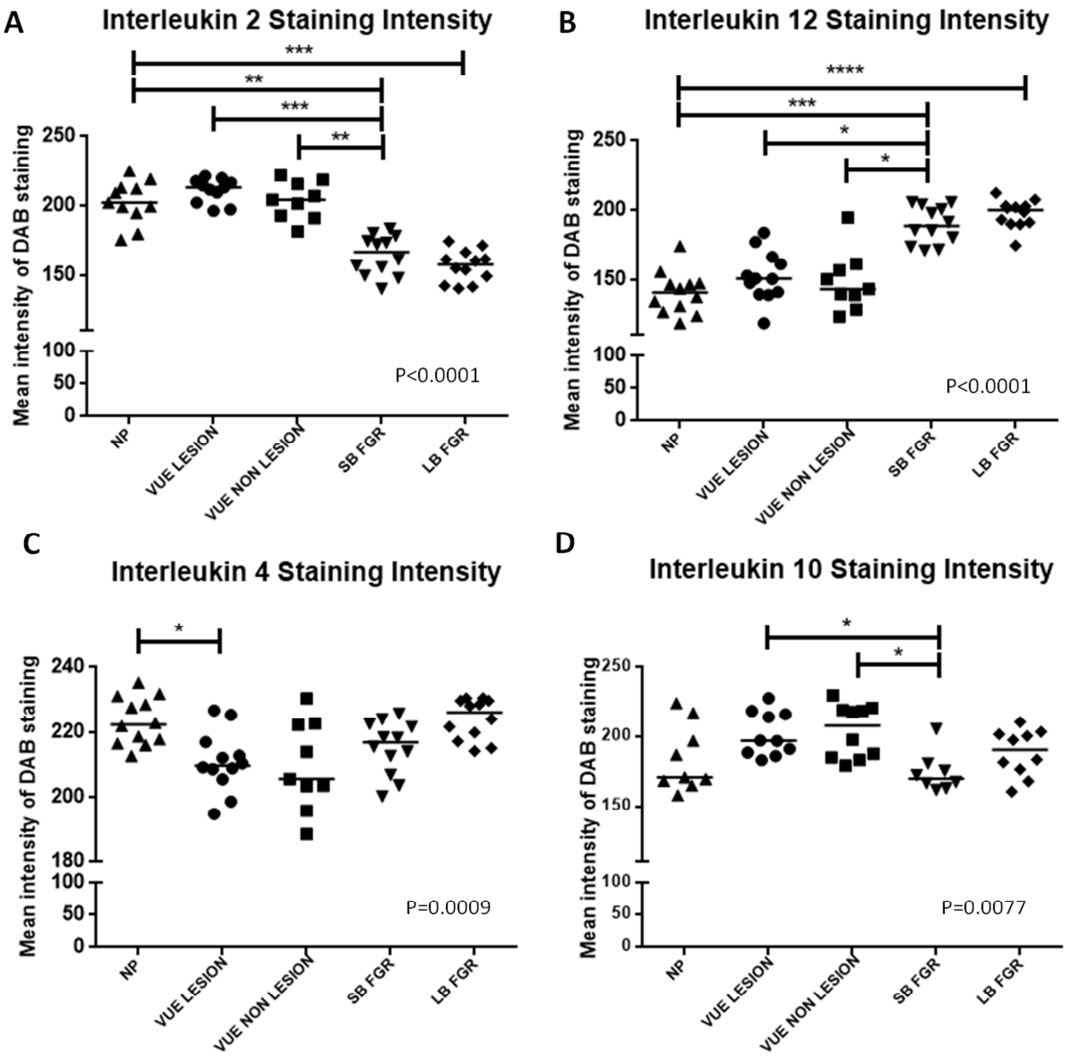
In summary, we have fully characterised VUE in stillbirths using immunohistochemistry to identify specific immune cell types and have detected evidence of a Th1-type cytokine response. These data present novel insights into the pathogenesis of VUE and provide solid foundations for functional studies to determine the impact of inflammation on placental function.

4.6 Acknowledgements

We wish to express our gratitude to the mothers who give consent for us to use the placentas that we studied, the recruiters who consent the participants and Dr Mark Wareing who co-ordinates the MFHRG Biobank. Thank you to Dr Sylvie Girard for allowing us to use some of her immunostained sections from healthy pregnancies and Dr Rachael Wright who dedicated a lot of time optimising antibodies for IHC. Thanks also to Dr Joanne Pennock for her immunological advice. This work forms part of a PhD project for HD funded by Tommy's – The Baby Charity.



Supplementary Figure 1: Representative images of placental sections immunostained for cytokines. VUE lesions stained for (A) Interleukin (IL)-10, (C) IL-6 and (D) transforming growth factor β. Cytokine staining of a placental section from normal pregnancy (NP) immunostained for (B) IL-10. Scale bar represents 50μm. Original magnification x100.



Supplementary Figure 2: Quantification of cytokine staining intensity using HistoQuest image analysis software.
*(A) Interleukin (IL)-2, (B) IL-12, (C) IL-4, (D) IL-10. NP: normal pregnancy, SB FGR: stillbirth with fetal growth restriction, LB FGR: live birth with fetal growth restriction. Kruskal-Wallis test with Dunn's post-hoc, lines represent median. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

CHAPTER 5: Co-culture of placental explants with isolated maternal CD4 and CD8 T cells

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A manuscript submitted to the *Journal of Biological Methods*

5.1 Abstract

Appropriate placental function is essential for successful pregnancy and placental dysfunction is associated with fetal growth restriction (FGR) and stillbirth. Villitis of unknown etiology (VUE) and chronic intervillositis of unknown etiology (CIUE) are immune-mediated conditions characterised by placental infiltrates of macrophages, CD4 and CD8 T cells. VUE and CIUE occur more frequently in the placentas of pregnancies complicated by FGR. The mechanisms by which this inflammation induces placental dysfunction are yet to be defined. We aimed to develop an *in vitro* model of placental inflammation to investigate functional consequences of immune cells in the placental environment. Fragments of placental tissue were co-cultured with CD4 and CD8 T cells isolated from whole blood. CellTracker™ fluorescence was used to identify T cells in cultured explants. Tissue histology, endocrine and nutrient transport function was assessed using established methods. This novel preparation will enable future investigations into immune cell interactions with placenta.

Keywords: inflammation, placenta, T lymphocytes, villitis, VUE

5.2 Background

The placenta is critical to successful pregnancy, forming the maternal-fetal interface that supplies nutrients and oxygen to support fetal growth and removes waste products of fetal metabolism whilst maintaining a distinct barrier between mother and fetus (39). If the placenta fails to function adequately there are serious health implications for the fetus, including an increased risk of fetal growth restriction (FGR) and stillbirth (34,65,137,174). There is increasing evidence that sterile inflammation has a role in the placental dysfunction underlying these pregnancy complications. Villitis of unknown etiology (VUE) and chronic intervillositis of unknown etiology (CIUE) are specific inflammatory conditions of the placenta associated with FGR that may also be linked to stillbirth (155). In the majority of cases of VUE and CIUE, an underlying clinical infection cannot be detected (97,100). VUE is characterised by migration of CD4 and CD8 T cells from maternal blood into the stroma of the villi, the functional units of the human placenta and has been well characterised in morphological studies (113,156,161,175,176). In CIUE maternal macrophages, CD4 and CD8 T cells accumulate in the intervillous space (111). In addition to a characteristic cellular phenotype of VUE lesions, increases in pro-inflammatory cytokines interleukin (IL)-2 and IL-12 and a decrease in the anti-inflammatory cytokine IL-4 have been described in lesions (176). Upregulation of pro-inflammatory chemokine mRNA has also been reported in VUE lesions (144). It is probable that migrating T cells alter intravillous cytokine levels which may dysregulate placental development or function leading to increased risk of fetal harm. However, these effects have not been explored using human placenta; to do so requires an

in vitro model of maternal immune cell infiltration into placental tissue in order to examine the functional consequences. Previous models of placental inflammation have utilised lipopolysaccharide (LPS) as a stimulator of the immune response, which mimics infectious etiology, as opposed to the sterile inflammation of VUE and CIUE (177).

Therefore, we aimed to develop an *in vitro* model of VUE/CIUE to enable investigations into the effect of inflammation on placental function. There is an established method of placental explant culture in which villous tissue fragments are dissected from term placental tissue and maintained in culture for 7-11 days (178). The morphological, endocrine and functional characteristics of these cultures are well established and explants have been used extensively since 2001 to investigate the effects of long term (days) interventions on placental cell turnover, endocrinology and nutrient transport function (61,65,179,180). T cells can be isolated from whole blood (181) and intracellular fluorescence can be introduced to enable cell tracking (182). Therefore, we explored the possibility that T cells isolated from maternal blood could be co-cultured with placental explants and would migrate into the villous stroma mimicking the lesions in VUE. A unique feature of placental explant culture is that the syncytiotrophoblast (STB), the continuous layer of highly specialised nutrient transporting epithelium that covers the villous tissue, partially detaches in the first 1-2 days of culture and then regenerates to form a new STB thereafter (178). An intact STB layer acts as a barrier to cells migrating from the intervillous space to the villous tissue. Here we tested whether mild trypsin digestion of term placental fragments (STB) before the start of culture encouraged the passage of T cells into the tissue. A previous study that trypsinised first trimester placental tissue reported no effect on the viability of the tissue in culture (180). We also examined whether the function of the explants was influenced by culturing them in the presence of cytokines required to maintain T cells *in vitro*. Here we describe the results of experiments that have established a novel protocol for placental explant co-culture with isolated, traceable maternal CD4 and CD8 T cells (Figure 16).

5.3 Materials

5.3.1 Placentas and Blood

Term (37-42 week) placentas were obtained within 30 minutes of delivery from women with uncomplicated pregnancies undergoing elective caesarean section. Tissue was donated with informed written consent in accordance with North West NHS REC approval (Ref: 08/H1010/55+5). Uncomplicated pregnancies were defined as maternal BMI $\leq$30 and no evidence of pre-eclampsia, gestational diabetes mellitus or fetal growth restriction. Multiple pregnancies, fetal anomalies or abnormal fetal heart rate were also excluded. Preliminary experiments utilised blood samples from non-pregnant donors (10mL) collected in lithium heparin tubes on day 0 of co-culture.

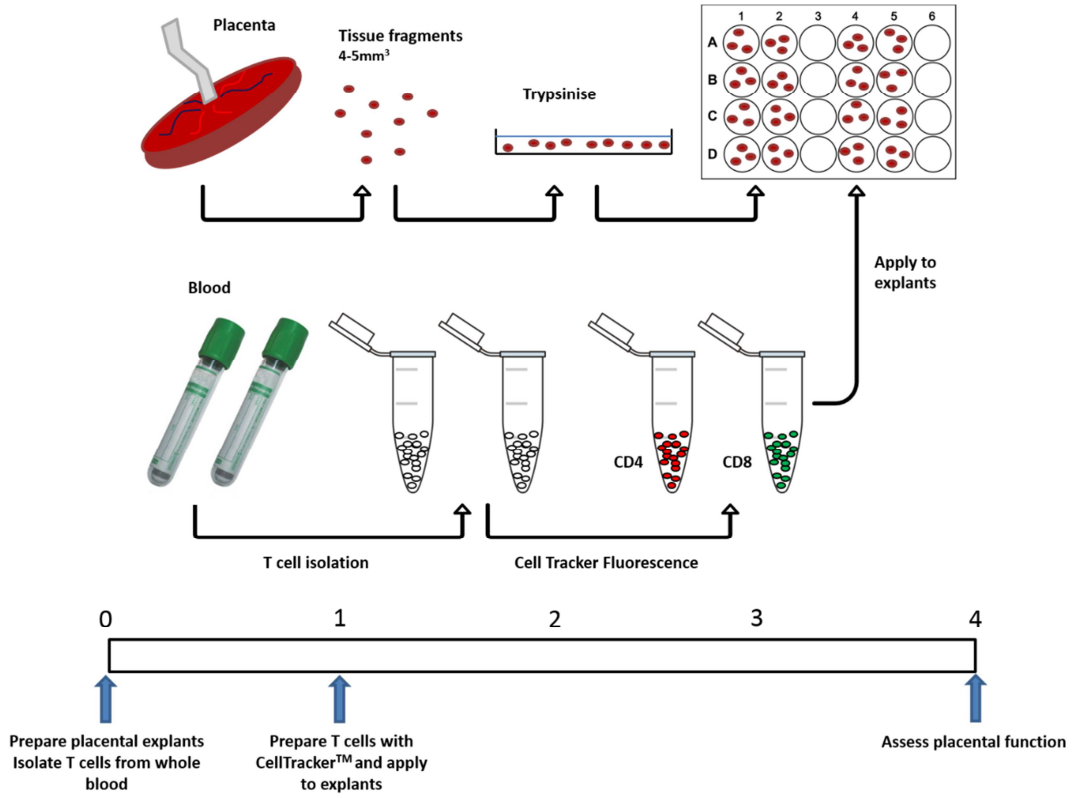

Figure 16: Schematic representation of the steps involved in the process of explant and T cell co-culture.

5.3.2 Chemicals/reagents/other

All reagents supplied by Sigma-Aldrich (Dorset, UK) unless otherwise stated.

- RPMI-1640 medium (Cat# 21875, Gibco, UK) supplemented with:
  - 5% Fetal bovine serum (FBS) (Gibco, UK)
  - Insulin solution 1µg/mL
  - Streptomycin sulphate 100µg/mL
  - Penicillin G 100IU/mL
  - Retinol acetate 0.1µg/mL
  - Calcium chloride
  - L-alanine 25µg/mL
  - L-cysteine 200µg/mL
  - Ascorbic acid 50µg/mL
- Sterile PBS with Ca^{2+} and Mg^{2+}
- Sterile PBS without Ca^{2+} and Mg^{2+}
- 70% ethanol
- 0.01% trypsin in sterile PBS
- Lymphoprep™ (Stem Cell Technologies, UK)

- EasySep™ buffer (Stem Cell Technologies, UK)
- EasySep™ CD8 T cell isolation kit, positive selection (Cat# 18013, Stem Cell Technologies, UK)
- EasySep™ CD4 T cell enrichment kit, negative selection (Cat# 19012, Stem Cell Technologies, UK)
- Recombinant human (rh) interleukin (IL)-2 10ng/mL (Life Technologies, UK)
- RhIL-15 10ng/mL (Life Technologies, UK)
- Anti-CD3 antibody 5µg/mL (BD Pharmingen, UK)
- Anti-CD28 antibody 1µg/mL (BD Pharmingen, UK)
- CellTracker™ CMPTX red dye (Life Technologies, UK)
- CellTracker™ CMFDA green dye (Life Technologies, UK)

5.3.3 Equipment

- Sterile Petri dishes
- 24-well culture plates
- 12-well culture plates
- Sterile Pasteur pipettes
- SepMate centrifugation tubes (Stem Cell Technologies, UK)
- 15mL and 50mL centrifuge tubes
- 5mL Eppendorf tubes
- 5mL, 10mL and 25mL sterile stripettes
- 5mL round-bottomed polystyrene falcon tubes
- Sterile dissecting scissors and forceps
- Centrifuge (Beckman Coulter)
- EasySep™ magnet (Stem Cell Technologies, UK)
- Humidified incubator 37°C, 21% O_2, 5% CO_2
- Laminar air flow
- Inverted microscope (Olympus CK2, Olympus, Southend-on-Sea, UK)
- Fluorescent microscope (Zeiss Observer Z1 AX10, Zeiss, Welwyn Garden City, UK)
- Flow cytometer (BD Accuri C6, BD Biosciences, Oxford, UK)

5.4 Procedure

DAY 0 – preparation of anti-CD3 coated plates for cell culture

1. Prepare 4mL sterile PBS supplemented with 5µg/mL anti-CD3 antibody.
2. Add 1mL antibody solution to 4 wells of a 12-well plate.
3. Incubate for two hours at 37°C.
4. Wash twice with 1mL sterile PBS.

NOTE: Coated plates can be prepared the day before they are required and stored at 4°C overnight instead of a two hour incubation at 37°C on the day of isolation.

Day 0 – production of placental fragments for culture

5. Prepare 1L RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS), 1µg/mL insulin, 100µg/mL streptomycin sulphate, 100IU/mL penicillin G, 0.1µg/mL retinol acetate, 25µg/mL L-alanine, 200µg/mL L-cysteine and 50µg/mL ascorbic acid. Filter prepared medium through 0.2µm bottle top vacuum filter (Corning, Germany).
6. Receive placenta within 30 minutes of delivery. Take four biopsies of approximately 1cm^3 from random areas of parenchyma, place in pre-warmed sterile PBS. All subsequent steps take place in the laminar flow hood.
7. Cut approximately 30 smaller fragments (~4-5mm^3) of villous tissue from each of the large biopsies, taking care to avoid fetal chorionic plate, decidua and large blood vessels. Transfer to sterile petri dishes with 10mL pre-warmed sterile PBS.
8. Wash twice in sterile PBS.
9. Transfer tissue fragments to an empty petri dish. Add 10mL 0.01% trypsin, incubate at 37°C for 15 minutes.
10. Remove trypsin solution then neutralise with two changes of RPMI medium.
11. Add 1mL RPMI medium to 16 wells of a 24 well culture dish and place three fragments in each
12. Culture for 24 hours in a humidified incubator at 37°C, 21% O_2.

Day 0 – T cell isolation from 10mL whole blood

13. Add 15mL Lymphoprep to a SepMate tube.
14. In a separate centrifuge tube, mix blood with an equal volume of EasySep buffer. Carefully transfer the blood/buffer mix to the SepMate tube ensuring the tube is kept vertical.
15. Centrifuge at 1200g for 10 minutes at room temperature with the brake on.
16. Pour off the supernatant (which contains the peripheral blood mononuclear cells) into a new centrifuge tube; do not invert the SepMate tube for longer than two seconds. NOTE the tube that the supernatant is poured into does not have to be a SepMate tube.
17. Centrifuge for 8 minutes at 300g at RT.
18. Remove supernatant with a stripette taking care not to disturb the pellet.
19. Add ~10mL EasySep buffer and re-suspend the cells. Centrifuge for 8 minutes at 300g at RT.
20. Repeat steps 18 and 19.

21. Remove supernatant and re-suspend cells in 4mL EasySep buffer. Pour into a 5mL round-bottomed falcon tube.
22. Centrifuge for 8 minutes at 300g at RT, remove supernatant.
23. Re-suspend pellet with 100µL EasySep buffer. Add 10µL EasySep CD8 positive selection cocktail. Mix well and incubate for 15 minutes at RT.
24. Mix the magnetic nanoparticles by vigorous pipetting (do not vortex). Add 10µL of magnetic nanoparticles. Mix well, incubate for 10 minutes at RT.
25. Bring the cell suspension to a total of 2500µL using EasySep buffer. Mix by gentle pipetting. Place the tube in the magnet for 5 minutes ensuring that that tube does not have the cap on.
26. Whilst the tube is still in the magnet invert it, pouring the supernatant into a new 5mL falcon tube. Leave inverted for 2-3 seconds but do not shake or blot any drops. Save the pour off for the isolation of CD4 T cells.
27. Take the tube out of the magnet, add ~2500µL EasySep buffer. Mix by gentle pipetting, place the tube back in the magnet and set aside for 5 minutes.
28. Whilst the tube is still in the magnet invert it, pouring off the supernatant. (There is no need to save this supernatant.)
29. Repeat steps 27 and 28.
30. Re-suspend in 2500µL EasySep buffer, centrifuge for 8 minutes at 300g at RT.
31. Remove supernatant, re-suspend in 2mL RPMI medium with 1µg/mL anti-CD28, 10ng/mL rhIL-2 and 10ng/mL rhIL-12.
32. Use the supernatant/first pour off from the CD8 selection, centrifuge for 8 minutes at 300g at RT.
33. Remove the supernatant, re-suspend cells in 250µL EasySep buffer.
34. Add 12.5µL EasySep Human CD4 T cell enrichment cocktail. Mix well and incubate for 10 minutes at RT.
35. Vortex the D magnetic particles for 30 seconds. Add 25µL magnetic particles, mix well and incubate for 5 minutes at RT.
36. Bring the cell suspension to 2500µL by adding EasySep buffer (~2200µL) and mix gently.
37. Place the tube, without cap, into the magnet for 5 minutes.
38. With the tube in the magnet, invert, pouring the supernatant into a new 5mL tube. The cells are in the pour off.
39. Centrifuge for 8 minutes at 300g at RT.
40. Remove supernatant, re-suspend in 2mL RPMI medium with 1µg/mL anti-CD28 to activate T cells, 10ng/mL rhIL-2 and 10ng/mL rhIL-15 to stimulate proliferation of T cells.
41. Plate cells into the coated wells of a 12-well plate.

42. Incubate overnight in a humidified incubator at 37°C, 21% O_2.

Day 1 – Introduction of CellTracker™ fluorescence

43. Remove medium from wells, transferring into two 5mL Eppendorfs (one for CD4 cells, one for CD8 cells).
44. Add 500µL ice-cold sterile PBS (without Ca^{2+} and Mg^{2+}), gently scrape the bottom of the wells to release the cells. Transfer to the Eppendorfs containing the medium.
45. Examine the plates on an inverted microscope to check how many cells remain attached.
46. Repeat step 44.
47. Centrifuge at 1300rpm for 5 minutes at RT.
48. Dilute 1µL CellTracker red in 1mL pre-warmed RPMI medium and 1µL CellTracker green in 1mL pre-warmed RPMI medium.
49. After centrifugation, remove supernatant, re-suspend cells in pre-warmed medium with CellTracker (we used red for CD4 cells and green for CD8 cells). Incubate at 37°C for 45 minutes.
50. Centrifuge at 1300rpm for 5 minutes at RT.
51. Re-suspend in 1ml pre-warmed medium. Incubate at 37°C for 30 minutes.
52. Centrifuge at 1300rpm for 5 minutes at RT.
53. Re-suspend in 1ml pre-warmed sterile PBS (with Ca^{2+} and Mg^{2+}). Centrifuge at 1300rpm for 5 minutes at RT.
54. Re-suspend in 480µL medium supplemented with rhIL-2 and rhIL-15.

Day 1 – Application of cell suspension to placental explants

55. Collect medium from 2 wells for analysis
56. Aspirate medium from other wells.
57. Add 10µL cell suspension (~16,000 cells (range 4,000-42,000) to each explant.
58. Incubate for 24 hours in a humidified incubator at 37°C, 21% O_2.

Day 2 – Add medium

59. Add 300µL RPMI medium with rhIL-2 and rhIL-15 to each well.
60. Incubate for 24 hours in a humidified incubator at 37°C, 21% O_2.

Day 3 – Change medium

61. Collect medium from 2 wells for analysis
62. Aspirate medium from other wells.
63. Add 1mL RPMI medium with rhIL-2 and rhIL-15 to each well.
64. Incubate for 24 hours at 37°C, 21% O_2, 95% humidity.

Day 4 – Harvest explants and process for assessment of placental function

65. Collect medium from two wells for analysis

5.5 Anticipated Results

5.5.1 Trypsinisation

In explant culture, placental fragments shed STB which regenerates over the course of 5-7 days (178). We aimed to accelerate this process in order to conform to a culture period of 4 days; the optimal time that T cells can be sustained *in vitro* (183). We proposed that mild trypsinisation would promote STB detachment without complete denudation of the underlying cytotrophoblasts and stromal tissue. To assess this, different concentrations of trypsin (0.01%-0.125%) were trialled over 5-30 minutes. The effect of trypsinisation on STB detachment was confirmed by examination of immunohistochemistry (IHC) to detect cytokeratin 7, a cytoskeletal protein expressed by STB. Incubation in 0.01% trypsin for 15 minutes was found to be sufficient to accelerate the shedding process without affecting the integrity of the villous stroma (Figure 17).

The effect of villous fragment trypsinisation on placental nutrient transport, endocrine secretion and cell kinetics was determined after 4 days of culture to assess whether encouraging the STB to detach before commencing culture had detrimental effects on STB regeneration and function in the longer term. We assessed various facets of placental function by determining system A activity, hCG secretion, STB regeneration, and levels of apoptosis and proliferation using standard techniques.

5.5.2 System A Activity

System A is a well characterised sodium-dependent placental amino acid transporter whose activity can be recorded by examination of its transport of radiolabelled amino acid analogue ^{14}CmeAIB (59,179). System A activity in placental villous explants has been measured previously (179) and is known to be reduced in FGR (184). To determine whether trypsinisation and culture in RPMI medium with cytokines affected nutrient transport, system A activity was examined as the sodium dependent uptake of ^{14}CMeAIB into the explants after 4 days of culture. Figure 18A-B shows that system A activity over a 60 minute period remained linear and was not affected by trypsinisation (n=3) or culture in medium containing cytokines (n=5) compared to untreated explants (controls).

5.5.3 Hormone Secretion

Differentiated STB produces human chorionic gonadotropin (hCG), a hormone that promotes trophoblast cell fusion and stimulates progesterone production (39,70). hCG is used as a marker of syncytialisation and STB viability (185). Its production is maximal in early

pregnancy, but rises again in late pregnancy and is predominantly secreted into the maternal circulation (39). Endocrine function of the STB was determined by measuring hCG secretion into culture medium. hCG secretion was not affected by pre-culture exposure of tissue fragments to trypsin (n=4, Figure 18C) or by the addition of cytokines to culture medium (n=2, data not shown).

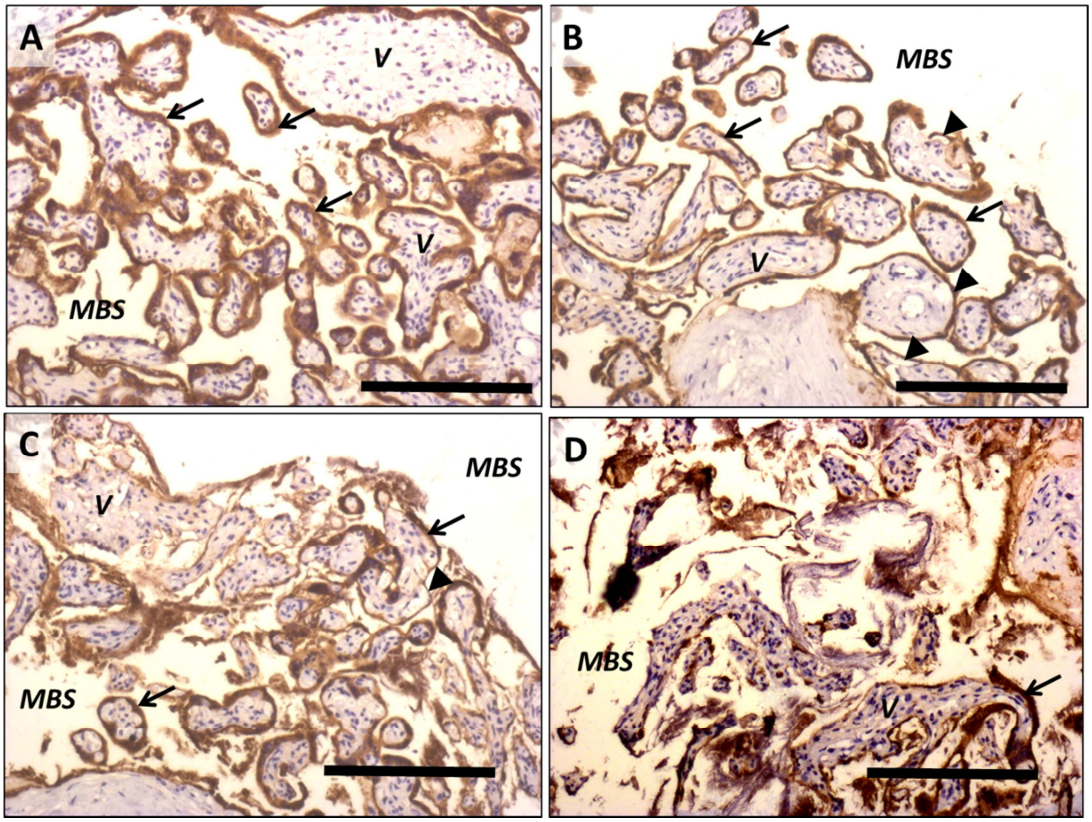

Figure 17: Representative images of placental tissue fragments stained with CK7 following incubation in trypsin (0.01-0.125%) for 5-30min.
When intact, STB staining positive for CK7 can be observed surrounding the villous tissue. A) Fragments incubated with 0.01% trypsin for 5 minutes. Arrows show areas where the STB and villous stroma remain completely intact. B) 0.01% trypsin for 15 minutes. Arrows show areas where the STB layer is intact but has started to lift from the villous stroma. C) 0.05% trypsin for 15 minutes. Arrows point to areas where STB has completely come away from the villous tissue and has started to disintegrate. D) 0.125% trypsin for 30 minutes. Large areas of complete denudation, degeneration and vacuolation of STB and villous stroma. V: villous stroma, MBS; maternal blood space. Scale bars represent 100µm, original magnification x100.

5.5.4 Proliferation and Apoptosis

Cell proliferation and apoptosis in explants after 4 days of culture (n=3) was assessed using IHC with antibodies against Ki67 (proliferation) and active caspase-3 (apoptosis) as previously described (186). HistoQuest (TissueGnostics, Austria), unbiased image analysis software, was used to quanitfy DAB staining. DAB+ areas or events were expressed as a percentage of total haematoxylin+ areas or events. There was no significant difference in levels of proliferation or apoptosis in the trypsinised explants compared to controls (Figure 18D-E) or the explants cultured in medium supplemented with cytokines compared to those cultured in medium alone (data not shown).

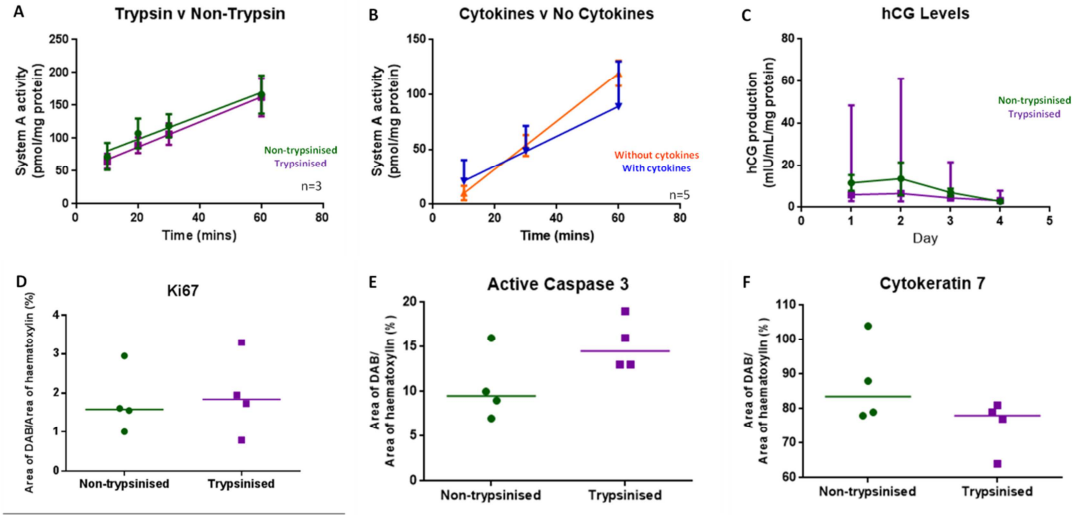

Figure 18: Assessment of placental function following pre-culture trypsinisation and culture with cytokines interleukin (IL)-2 and IL-15 at day 4 of culture.
A) system A activity, measured as the sodium-dependent tissue accumulation of the radiolabelled substrate ^{14}C meAIB over time, was unaffected by pre-culture exposure to trypsin (mean±SE of 3 placentas), B) system A activity was unaffected by the presence of cytokines in the culture medium (mean±/+SE of 5 placentas), C) hCG secretion was not altered by pre-culture exposure to trypsin (mean±SE of4 placentas). There was no significant difference in immunostaining for D) proliferation (Ki67), E) apoptosis (caspase-3), and F) STB regeneration (cytokeratin 7) all expressed as DAB+ area as a percentage of haematoxylin+ area between trypsinised and non-trypsinised explants.

5.5.5 Isolated T cells

Flow cytometric analysis of the CD4 and CD8 T cells demonstrated that ~80-90% purity had been achieved. After 24 hours in culture an average cell yield of 1.7 x 10^6 CD4 T cells (range 5.1 x 10^5 - 4.1 x 10^6) and 1.5 x 10^6 CD8 T cells (4.2 x 10^5 - 3.8 x 10^6) was achieved. It is normal to encounter different proportions of CD4 and CD8 T cells between individuals. In our samples CD4 T cells accounted for 85-95% of those isolated and CD8 T cells 5-15%.

5.5.6 Isolated Cells in Explants

After the introduction of Cell Tracker fluorescence, samples of cell suspension were observed on a fluorescence microscope to confirm that the fluorescent marker had been incorporated into the cells (Figure 19A-B). In further pilot studies, fluorescence imaging was employed to ascertain whether isolated CD4 and CD8 T cells could be located in the vicinity of villous tissue. This confirmed that Cell Tracker persisted for the 3 days that the cells were required to be in co-culture and localised maternal cells to the areas of the placenta associated with VUE. Figure 19C-D show representative images of placental explants with fluorescing CD4 (red) and CD8 (green) T cells in the explant. Qualitative analysis of the explants that were co-cultured with T cells showed evidence of sparse cell infiltration; T cell presence was not observed in every field of view. Trypsinised explants presented areas of tissue with slightly more extensive T cell presence (Figure 19C-D).

The developmental work completed to create this model of VUE/placental inflammation has demonstrated that CD4 and CD8 T cells can be isolated, purified and fluorescently labelled, that their viability is maintained for 4 days in culture and that they migrate into the stroma of pre-trypsinised placental villous tissue over this time frame. The pilot data confirm that exposure of placental tissue to trypsin prior to culture does not compromise nutrient transport function, endocrine secretion or cellular kinetics. This provides a working model on which to base co-culture studies to explore the mechanisms by which T cell infiltration could cause placental dysfunction in VUE. However, the use of this model could be advocated for a broader spectrum of experimental conditions including CIUE, diabetes-induced inflammation and autoimmune conditions such as systemic lupus erythematosus. The role of a range of specific leukocytes in inflammatory conditions could be examined as could the mechanism by which tolerance of a pregnancy is achieved.

TROUBLESHOOTING

1. The preliminary studies carried out to design this protocol used blood from non-pregnant donors. For experiments to examine the mechanics of VUE, placenta and maternal blood from the same pregnancy would be used. On day 0 if the placenta and blood samples arrive at the laboratory at the same time, processing of the placenta should take priority as its functional capacity depletes in a shorter timeframe.
2. If Cell Tracker cytoplasmic fluorescence fails, ensure that the vials have been protected from light during storage.

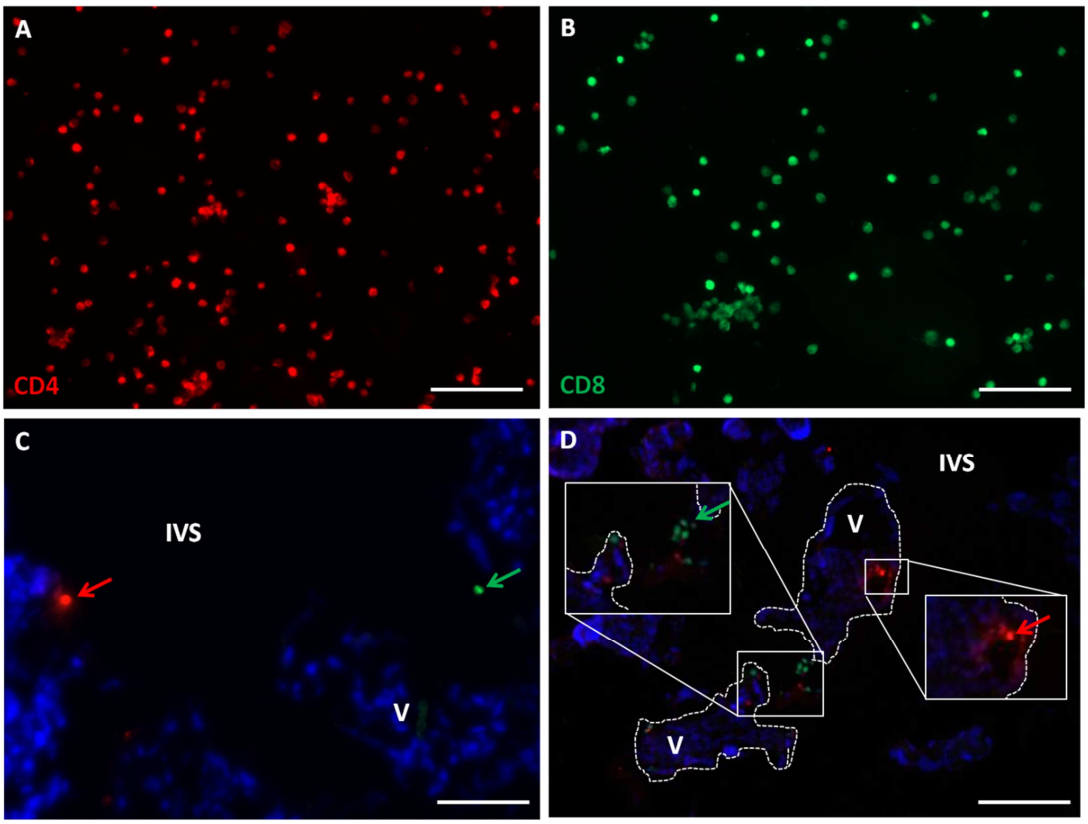

Figure 19: Representative images of isolated CD4 and CD8 T cells.
A) CD4 T cells fluorescing red after the addition of Cell Tracker CMPTX. B) CD8 T cells fluorescing green after the addition of Cell Tracker CMFDA green. C-D) CD4 T cells (red arrows) and CD8 T cells (green arrows) in placental explant tissue at day 4 of co-culture. Nuclei are stained with DAPI. V: villous stroma, IVS: intervillous space. Dotted lines demarcate STB. Scale bars represent 20µm, original magnification x200.

CHAPTER 6: An *In Vitro* Model of Villitis of Unknown Etiology Demonstrates Altered Placental Hormone and Cytokine Profile

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A manuscript in preparation for the *Journal of Immunology*

6.1 Abstract

Human placental dysfunction is linked to pregnancy complications, being present in up to 65% of cases of stillbirth. Villitis of unknown etiology (VUE), an inflammatory condition of pregnancy not attributable to an infectious agent, is characterised by macrophage and T cell infiltrates in the placental villous stroma with an associated dysregulation of inflammatory cytokines. VUE is diagnosed post-partum by histological examination of the placenta and is found more frequently in pregnancy complications. We hypothesised that maternal T-cells in the villous stroma impair placental function in VUE. An *in vitro* model of placental inflammation was developed to explore mechanisms through which VUE might impair the function of syncytiotrophoblast, the primary site of nutrient transport and hormone production within the placenta. Placental tissue and matched maternal bloods were obtained from uncomplicated pregnancies. Tissue explants were co-cultured with isolated maternal CD4 and CD8 T-cells or cytokines IL-2, IL-12 and/or anti-IL-4 neutralising antibody. Syncytiotrophoblast integrity/function was assessed by measuring hormone and cytokine secretion, nutrient transport activity and cell turnover. Compared to controls, human chorionic gonadotrophin secretion was significantly lower in explants co-cultured with T-cells ($p=0.015$) or treated with cytokines ($p=0.03$). Significantly elevated protein concentrations of IL-1β ($p<0.01$), IL-1Ra ($p=0.05$), IL-6 ($p<0.01$), IL-10 ($p<0.05$) and IFN-γ ($p<0.05$) were observed in tissue, and conditioned medium, of explants co-cultured with T cells compared to controls. This novel *in vitro* model of placental inflammation supports the hypothesis that maternal T-cells in the placental environment detrimentally affect placental function and could contribute to pregnancy pathologies.

6.2 Introduction

Placental dysfunction is cited as a cause of death in up to 65% of stillbirths (187,188). Until recently, investigations of the origins of placental dysfunction have generally concentrated on the implications of failure of spiral artery remodelling as seen in early onset pre-eclampsia and fetal growth restriction (FGR) (72). However, interest has grown in the consequences of placental inflammation and its contribution as a cause of stillbirth and FGR. Infectious agents such as cytomegalovirus or toxoplasmosis can lead to placental inflammation, though such cases are usually in the minority (88). More frequently, placental inflammation occurs in the absence of infection (122). Villitis of unknown etiology (VUE) and chronic intervillositis of unknown etiology (CIUE) are such inflammatory conditions of the villous tissue and intervillous space respectively. As their names suggest, the origin of these conditions is unknown but it is not attributable to infectious agents.

VUE occurs in approximately 15% of term placentas with a higher incidence in FGR (155). Increased severity of inflammation has been linked to stillbirth (88); CIUE is rarer than VUE and is associated with very poor obstetric outcome, including late miscarriage and stillbirth (111). Both conditions are characterised by infiltrates of mononuclear cells (monocytes/macrophages, CD4 and CD8 T-cells) in either the villous stroma (VUE) or intervillous space (CIUE). The T-cells in both VUE and CIUE lesions are maternal in origin, however, the origin of the monocytes differs. In VUE, macrophages are mainly fetal in origin whereas in CIUE they are almost exclusively maternal (87,189). These conditions are not detectable prenatally and are diagnosed on histological examination of the placenta after delivery. The mechanisms by which these inflammatory infiltrates negatively affect placental function and therefore play a causative role in pregnancy pathologies are, as yet, unknown.

We demonstrated a decrease in levels of immunoreactive anti-inflammatory cytokine IL-4 and increases in pro-inflammatory IL-2 and IL-12 protein levels within specific VUE lesions (176) in pregnancies ending in stillbirth. We hypothesised that the presence of maternal CD4 and CD8 T-cells and/or their related cytokines in the villous stroma would damage trophoblast, the key functional cell layer, leading to impaired placental function. VUE is not identifiable before birth thus it is not possible to assess the effect of inflammation on the placenta by a traditional case-control or cohort study design. Therefore, an *in vitro* model is needed to recreate VUE and examine the functional consequences. To address our hypothesis we developed *in vitro* models of placental inflammation mimicking that observed in VUE and performed a range of established placental analyses to assess the impact on placental function (including cell turnover, nutrient transport and endocrine function) (65,179,180,186). Our objectives were to: a) assess the direct effect of cytokines altered in VUE on placental function, integrity and viability and b) to determine the effect of

introducing maternal peripheral T-cells into the villous environment on placental function, integrity and viability.

6.3 Materials and Methods

6.3.1 Sample Collection

Term (37-42w) placentas (n=14) and matched maternal blood samples (n=8) were obtained within 30 minutes of delivery from women undergoing elective caesarean section. Tissue was donated with informed written consent in accordance with North West NHS REC approval (Ref: 08/H1010/55+5). Placental tissue was taken from uncomplicated pregnancies with maternal body mass index $\leq$30 and no evidence of pre-eclampsia, gestational diabetes mellitus or fetal growth restriction. Multiple pregnancies, fetal anomalies or abnormal fetal heart rate were excluded.

6.3.2 Explant Culture with Cytokines

Full thickness villous parenchymal biopsies of ~1cm^3 were taken from four different areas of placenta. Villous tissue was further dissected into 4-5mm^2 fragments and washed in Dulbecco's PBS. Three explants were placed into individual Costar Netwell (Corning, NY, USA) supports suspended in 1.5mL CMRL-1066 culture medium (Gibco, Paisley, UK) supplemented with 5% heat-inactivated FBS, 100 IU/ml penicillin, 100µg/ml streptomycin, 1µg/ml insulin, 0.1µg/ml hydrocortisone, and 0.1µg/ml retinyl acetate (Sigma Aldrich, Dorset, UK). An established method of placental explant culture was employed in which villous tissue fragments are maintained in culture for 7-11 days (178). The morphological, endocrine and functional characteristics of these cultures are well established and explants have been used extensively since 2001 to investigate the effects of long term (days) interventions on placental cell turnover, endocrinology and nutrient transport function (61,65,179,180). Explants were incubated in CMRL before treatment began on day 4. Treatment of explants comprised 48 hour incubation of tissue fragments in reduced serum Opti-MEM® medium (Gibco, Paisley, UK) supplemented with recombinant human IL-2 (rhIL-2, 10ng/mL), recombinant human IL-12 (rhIL-12, 10ng/mL) (Life Technologies, Paisley, UK) or IL-4 neutralising antibody (1µg/mL, R&D Systems, Oxford, UK) individually, all three in combination or Opti-MEM® alone as a control. Conditioned culture medium was collected after 4 and 6 days of culture and stored at -20°C for later analysis of human chorionic gonadotrophin (hCG) by ELISA and quantification of cytokines by Bioplex 10-plex assay (Bio-Rad laboratories, Hertfordshire, UK). Additional explants were frozen at -80°C for protein analysis or fixed in 4% neutral buffered formalin (NBF) then embedded in paraffin wax prior to immunohistochemical staining to assess apoptosis, proliferation and syncytiotrophoblast (STB) regeneration using antibodies against M30, Ki67 and CK7 respectively. System A amino acid transporter activity was assessed in placental explants after 6 days of culture.

6.3.3 Placental Explant Co-Culture

Villous tissue explants were prepared as described in the previous section. In order to enhance the accessibility of villous stroma for T cells we used mild trypsin treatment to lift areas of STB. Trypsin treatment has been previously used to denude STB in first trimester placentas (180). In preliminary experiments, tissue explants were incubated in different concentrations of trypsin solution (0.01%-0.125%) for up to 30 minutes at 37°C then washed with serum-containing RPMI medium to neutralise the trypsin (180). Preliminary histological and functional assessment demonstrated that trypsinisation in 0.01% solution for 15 minutes was sufficient to lift areas of syncytium without compromising tissue integrity and function (Derricott et al., submitted for publication). All tissue fragments used in co-culture experiments were trypsinised using this protocol. Following trypsinisation, fragments were transferred into 24-well plates (3 per well). Fragments were cultured for 24 hours in 1mL RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 5% fetal bovine serum (FBS), 1µg/mL insulin, 100µg/mL streptomycin sulphate, 100IU/mL penicillin G, 0.1µg/mL retinol acetate, 25µg/mL L-alanine, 200µg/mL L-cysteine and 50µg/mL ascorbic acid. After 24 hrs, RPMI culture medium was aspirated and isolated CD4 and CD8 T-cells were applied to each fragment in 10µL suspensions (approximately 10^4 cells per fragment) and cultured for 24 hours at 37°C. RPMI medium alone was applied to control tissue (10µL per fragment). On day 2, 300µL medium was added to each well and cultured for a further 24 hours. On day 3 of co-culture medium was replaced and incubated for a final 24 hours. Conditioned medium was collected on days 3 and 4 for measurement of hCG by ELISA and quantification of cytokines by Bioplex analysis and stored at -20°C. On day 4 explants were processed for analyses of placental function as detailed below.

6.3.4 CD4 and CD8 T Cell Isolation

CD4 and CD8 T-cells were isolated from maternal blood samples with EasySep Human T Cell Isolation Kit (STEMCELL Technologies, Cambridge, UK) following the manufacturers' protocol. Briefly, peripheral blood mononuclear cells (PBMCs) were separated from whole blood by centrifugation and incubated with CD8 T cell positive isolation antibody cocktail for 10 minutes. Isolated cell suspension was incubated for 10 minutes with magnetic nanoparticles and targeted cells selected with the EasySep magnet. Remaining cells in the supernatant were incubated with CD4 T cell negative isolation antibody cocktail for 5 minutes and magnetic particles for 5 minutes. Unwanted tagged cells were selected from the suspension with the EasySep magnet. Isolated CD4 and CD8 T-cells were incubated for 24 hours on anti-CD3 (BD Pharmingen, Oxford, UK) coated 12-well plates in RPMI 1640 medium with 1µg/mL anti-CD28 antibody (BD Pharmingen) and 10ng/mL recombinant IL-2 and IL-15 (Gibco, Paisley, UK) to activate and stimulate proliferation of T cells.

6.3.5 CellTracker™ Fluorescence

Cultured CD4 and CD8 T-cells were harvested after 24 hours by application of ice-cold Dulbecco's PBS (without Ca^{2+} and Mg^{2+}) and gentle scraping. The manufacturer's protocol for CellTracker™ fluorescence (Life Technologies, Paisley, UK) was followed. Briefly, harvested cells were incubated with 10mM CellTracker™ fluorescence (red for CD4 T-cells, green for CD8 T-cells) in RPMI for 45 minutes at 37°C, centrifuged and resuspended in pre-warmed medium for a further 30 minutes at 37°C. Cell suspensions were spun, washed in pre-warmed PBS (with Ca^{2+} and Mg^{2+}), centrifuged and resuspended in RPMI supplemented with rhIL-2 and rhIL-15 before application to placental fragments. Intracellular fluorescence was verified by viewing cells with a fluorescence microscope. Cell number and viability was assessed by counting using a haemocytometer and staining with Trypan Blue (Sigma Aldrich, Poole, UK).

6.3.6 Assessment of System A Amino Acid Transporter Activity

Placental explants cultured for either 4 days (with CD4 and CD8 T-cells (n=8)) or 6 days (with cytokines (n=6)) were incubated with radiolabelled amino acid analogue N-methylated aminoisobutyric acid (^{14}C-meAIB) solution at 0.5µCi/mL (8.5µM) as previously described (179). The incubation buffer was either control Tyrode's or Na^+-free Tyrode's (NaCl replaced with equimolar (135mM/L) choline chloride). Co-culture explants were incubated for 10, 30 or 60 minutes, explants cultured with cytokines were incubated for 60 minutes only. Following incubation, explants were lysed in dH_2O for 16-18 hours at room temperature then denatured in 0.3M NaOH for >6 hours at 37°C for later protein assay. The radioactivity of the explant lysate was determined using a β-scintillation counter. The protein content of the explants was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). System A is a Na^+-dependent nutrient transporter and its activity was calculated by subtracting ^{14}C-meAIB uptake in Na^+-free Tyrode's from uptake in control Tyrode's buffer. System A activity was expressed per mg explant protein.

6.3.7 hCG and PlGF Hormone Secretion

Conditioned culture medium was collected on days 3 and 4 (co-culture) or days 4 and 6 (cytokine cultures). Undiluted culture medium was assayed for hCG and PlGF hormone secretions by ELISA or Duoset ELISA (hCG: DRG Diagnostics, Marburg, Germany, PlGF: R&D Systems, Oxford, UK) according to standard manufacturers' protocol. Reagent volumes were halved for use in Duoset ELISA. Plates were read on a FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK), values corrected for protein content and expressed as mIU/mL/mg protein (hCG) or pg/mL/mg protein (PlGF).

6.3.8 Cell Migration

Explants that had been co-cultured with maternal CD4 and CD8 T-cells were embedded in OCT with or without fixation in 4% NBF and frozen at -80°C. Sections of 10µm were cut on a cryostat, mounted onto poly-L-lysine coated slides and a coverslip applied with aqueous DAPI-containing mountant. Slides were viewed with a Zeiss fluorescence microscope (Zeiss, Welwyn Garden City, UK) to determine the presence of labelled CD4 and CD8 T-cells in the villous stroma.

6.3.9 Proliferation, Apoptosis, hCG, STB Regeneration and Leukocytes in Explants

Proliferation and apoptosis were assessed in formalin-fixed explants using immunostaining and quantification of markers of proliferation (Ki67; Dako, Cambridge, UK) and apoptosis (M30, Roche, West Sussex, UK). STB regeneration and hCG in STB were investigated by assessing area of immunostaining for cytokeratin 7 (CK7, Dako) and hCG (Dako). The presence of leukocytes in explants was determined by immunostaining for the cell surface marker CD45 (leukocyte common antigen, (Dako)). A standardised laboratory protocol for immunostaining with colorimetric detection was followed, as described by Hamilton et al. (158). Heat-mediated antigen retrieval with citrate buffer was performed for all antibodies. Primary antibodies were applied at the following concentrations: CK7 0.9µg/mL, Ki67 0.4µg/mL, M30 0.01µg/mL, hCG 2 µg/mL and CD45 0.46 µg/mL. Each tissue section had a corresponding negative control on which mouse IgG (Sigma Aldrich, Dorset, UK) was applied at the same concentration as the primary antibody.

6.3.10 Image Capture and Analysis

Explant sections were visualised using the x10 objective of an Olympus BX41 light microscope (Southend-on-Sea, UK). Images were captured with QIcam Fast 1394 camera (QImaging, BC, Canada) and Image Pro Plus 6.0 (Media Cybernetics Inc., MD, USA). Quantitative, unbiased quantification of staining area was completed using HistoQuest image analysis software (TissueGnostics, Vienna, Austria) (190). Image pixels, when viewed in this software, are converted to greyscale (0-256 scale) and assigned an arbitrary number following detection of DAB and haematoxylin and optimisation of staining intensity ranges. Results are presented as histograms or dot plot scattergrams to which staining area and/or intensity cut-offs can be applied to differentiate between cell populations.

6.3.11 Cytokine Analysis in Culture Supernatant and Tissue Lysates (Bio-Plex Assay)

Tissue lysates were prepared using a Bio-Plex cell lysis kit (Bio-Rad Laboratories, Hemel Hempstead, UK). Tissue culture medium collected on days 3 and 4 (co-culture) and day 6 (cytokine culture) and tissue lysate samples were analysed for a panel of 10 inflammation-

related cytokines using a Bio-Plex assay (Bio-Rad Laboratories, Hemel Hempstead, UK) following manufacturers' instructions. The cytokines selected for assay were IL-1β, IL-1Ra, IL-2, IL-4, IL-6, IL-10, IL-12A (p70), IL-17, IFN-γ and TNF-α. Cytokine levels were extrapolated from fluorescence values based on supplied standards.

6.3.12 Statistical Analysis

We powered our analysis to determine hCG expression, system A activity and cytokine production and secretion assuming similar changes of these processes in culture with cytokines/inflammatory cells as previous states of culture in hypoxic conditions or with dexamethasone (80% power, α=0.05). Statistical analysis was carried out using GraphPad Prism 6.0 software (San Diego, CA, USA). Non-parametric continuous data were analysed with Mann Whitney U-test or Kruskal-Wallis test with Dunn's post-hoc test. Comparisons between groups of non-parametric data were analysed using Wilcoxon signed rank test or Friedman test. Due to considerable variability in the basal values of control placentas results are presented as fold change from control (179).

6.4 Results

6.4.1 Demographics

Six placentas were used to explore the effects of cytokines on villous explant function and a further 8 used for co-culture with T-cells isolated from matched maternal blood samples. Table 9 shows the demographic information of the study participants.

6.4.2 Cell Isolation and Fluorescence

Flow cytometry carried out during pilot studies of the cell isolation protocol demonstrated that positive (CD8) and negative (CD4) antibody selection techniques yielded populations with ~91% and ~83% purity respectively. CD4 and CD8 cell isolation and Cell Tracker fluorescence was successful in all experiments (Supplementary Figure 3). The volume of blood received was variable and this influenced the T cell yield. An average of ~12,000 cells (range 800-24,500) was applied to each villous tissue fragment. Isolated cells had an average viability of 97.9% (range 95.2-99.6%). T cell populations comprised 85-95% CD4 T-cells and 5-15% CD8 T-cells.

6.4.3 Explant Culture with VUE-Associated Cytokines Alters Placental Function

There was a significant decrease in hCG in conditioned culture medium at day 6 in the explants cultured with all treatments (rhIL-2, rhIL-12 and IL-4 neutralising antibody) compared to control (p=0.03) (Figure 20A). There was a trend towards decreased PlGF in conditioned culture medium at day 3 in the explants cultured with all treatments, but this

did not reach statistical significance (p=0.16) (Figure 20B). Treatment with individual cytokines did not produce any significant effect on hCG production.

Placental system A activity was not significantly affected by independent or combined rhIL-2, rhIL-12 and IL-4 neutralising antibody treatment compared to control (Figure 20C). A significant reduction in proliferation (Ki67 staining) was detected in the explants treated with 10ng/mL IL-2 ($p<0.05$). No significant differences were seen in apoptosis, STB regeneration/area or area of hCG staining in any of the experimental conditions compared to control (Figure 20D-G). No significant differences were observed in cytokine levels in explant lysates or conditioned culture medium in any of the treatment conditions (Supplementary Figure 4).

Table 9: Demographic information of the 14 study participants. Results are median (range).

	Explant Co-Culture (n=8)	**Explant Culture with Cytokines (n=6)**
Maternal Age	36.5 (34-45)	32.5 (26-38)
Gestational Age	39^{+1} (37^{+4}-39^{+2})	38^{+6} (38^{+0}-39^{+0})
Maternal BMI	25.59 (19.49-28.48)	26.11 (20.31-28.58)
Gravidity	3 (2-4)	2 (1-3)
Parity	1+0 (1+0-3+0)	1+0 (1+0-3+1)
Birthweight	3450 (2590-4000)	3292.5 (2840-3630)
IBR	52.55 (6.3-98.2)	55.15 (10.8-66.3)
Gender		
Male	6	3
Female	2	3

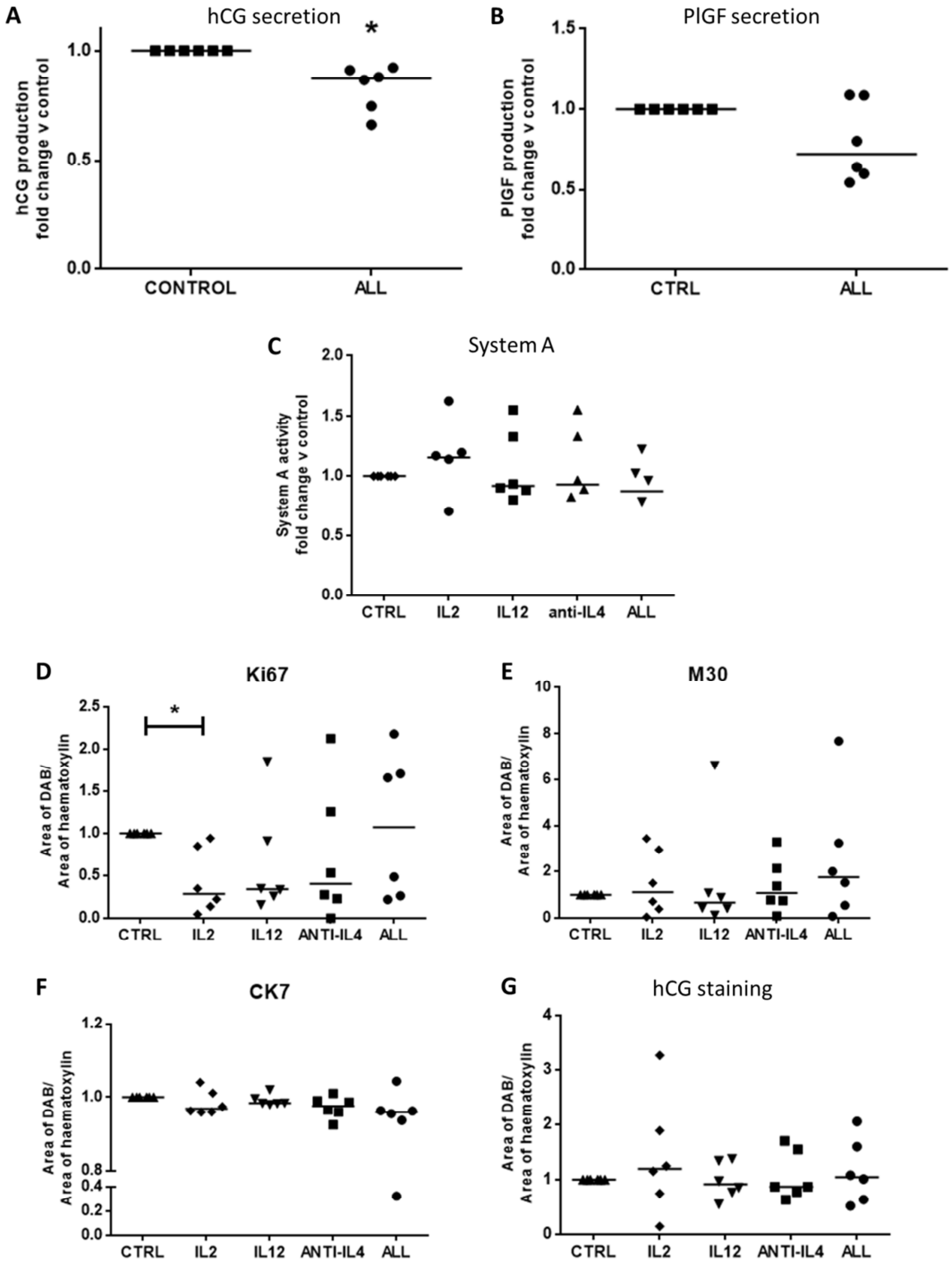


Figure 20: The effects of placental explant culture with recombinant cytokines and/or neutralising antibodies on placental function and cell turnover.
*(A) hCG concentration in culture medium, (B) PlGF concentration in culture medium, (C) System A activity (amino acid transporter). DAB+ staining area presented as fold change from control for: (D) Ki67 (proliferation), (E) M30 (apoptosis), (F) cytokeratin 7 (syncytiotrophoblast) and (G) human chorionic gonadotropin. CTRL: no treatment, IL-2: treatment with 10ng/mL recombinant human IL-2, IL-12: treatment with 10ng/mL recombinant human interleukin-12, ANTI-IL4: treatment with 1mg/mL anti-IL4 neutralising antibody, ALL: treatment with 10ng/mL IL-2, IL-12 and 1mg/mL anti-IL4. Lines represent median. (A-B) Wilcoxon signed rank test, (C) Kruskal-Wallis test, (D-F) Wilcoxon signed rank test, *p<0.05, n=6.*

6.4.4 An *In Vitro* Model of VUE Shows Altered Placental Function

Representative images of the co-culture explants illustrate CD4 and CD8 T-cells in the intervillous space, the equivalent of the maternal blood space, and in the villous stroma (Figure 21). IHC for CD45 was carried out to quantify leukocytes in the explants. There was a significant increase in CD45+ staining in explants cultured with CD4/CD8 T-cells (p=0.05) compared to controls (Figure 22A).

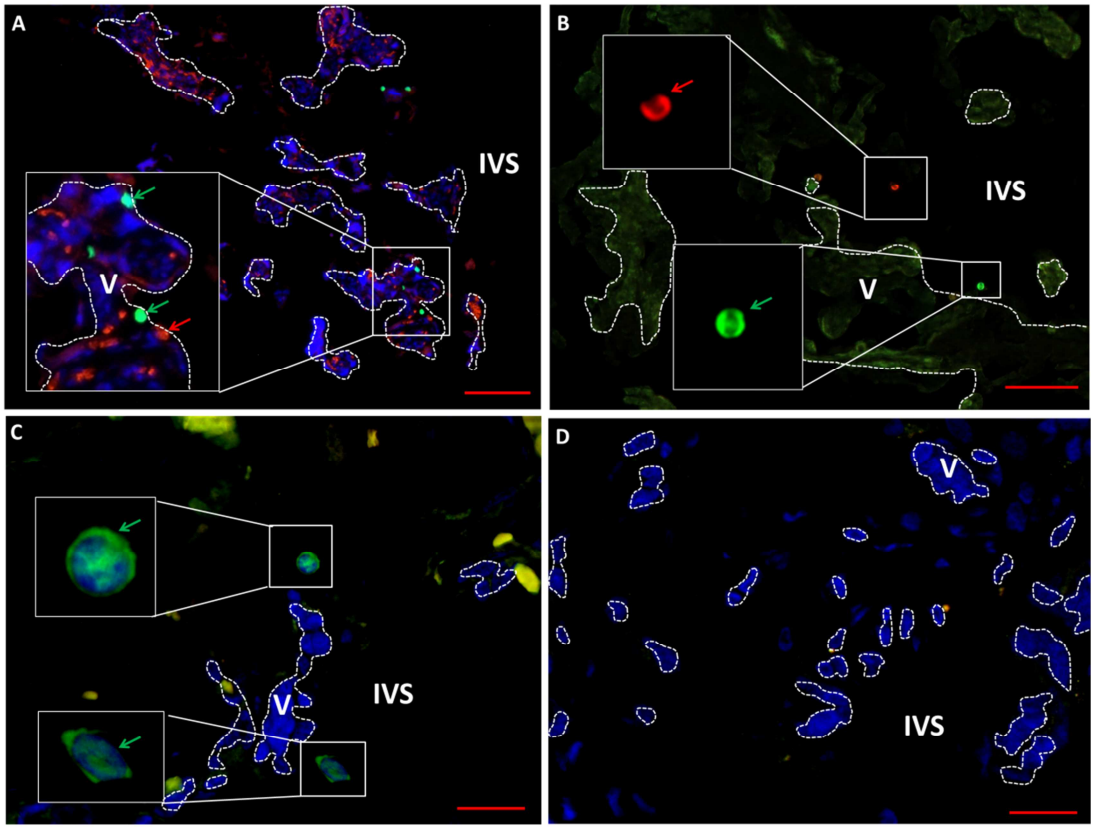


Figure 21: Representative images of explants at day 4 after culture with isolated T-cells.
Red arrows highlight CD4 T-cells, green arrows highlight CD8 cells, A, C and D) nuclei stained blue with DAPI. A-C) Isolated maternal T-cells are visible in the villous stroma (V) and intervillous space (IVS). A-B) Scale bar represents 100µm, original magnification x200, C) Scale bar represents 20µm, original magnification x630 D) explant cultured without isolated cells, scale bar represents 20µm, original magnification x630.

There was a significant decrease in hCG concentration in conditioned culture medium at day 3 in the explants cultured with CD4/CD8 T-cells compared to those cultured in medium alone (p=0.015) (Figure 22B). PlGF followed the same pattern as hCG decrease, however, in this group there was a limited sample size (n=3) so statistical analysis could not be performed (Figure 3C). No significant differences were recorded in hCG concentrations at day 4.

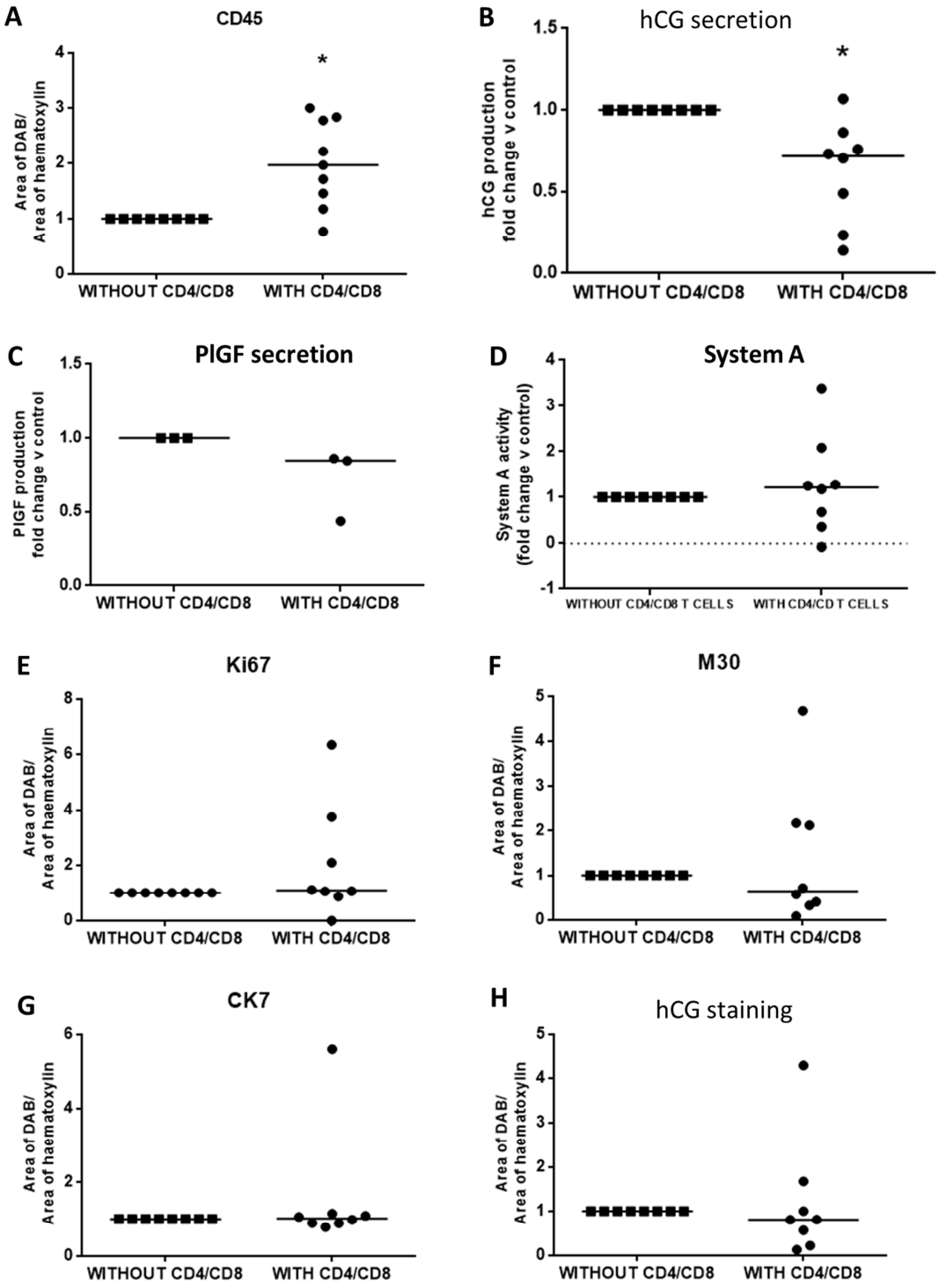


Figure 22: The effects of placental explant co-culture with matched maternal T cells on placental function and cell turnover.
*(A) area of DAB+ staining for CD45 leukocytes presented as fold change compared to control ($p<0.05$), (B) hCG concentration in culture medium, (C) PlGF concentration in culture medium (n=3), (D) system A activity (amino acid transporter). DAB+ staining area presented as fold change compared to control of (E) Ki67 (proliferation), (F) M30 (apoptosis), (G) cytokeratin 7 (syncytiotrophoblast) and (H) human chorionic gonadotropin. Lines represent median. Wilcoxon signed rank test, *$p<0.05$, n=8 unless otherwise stated.*

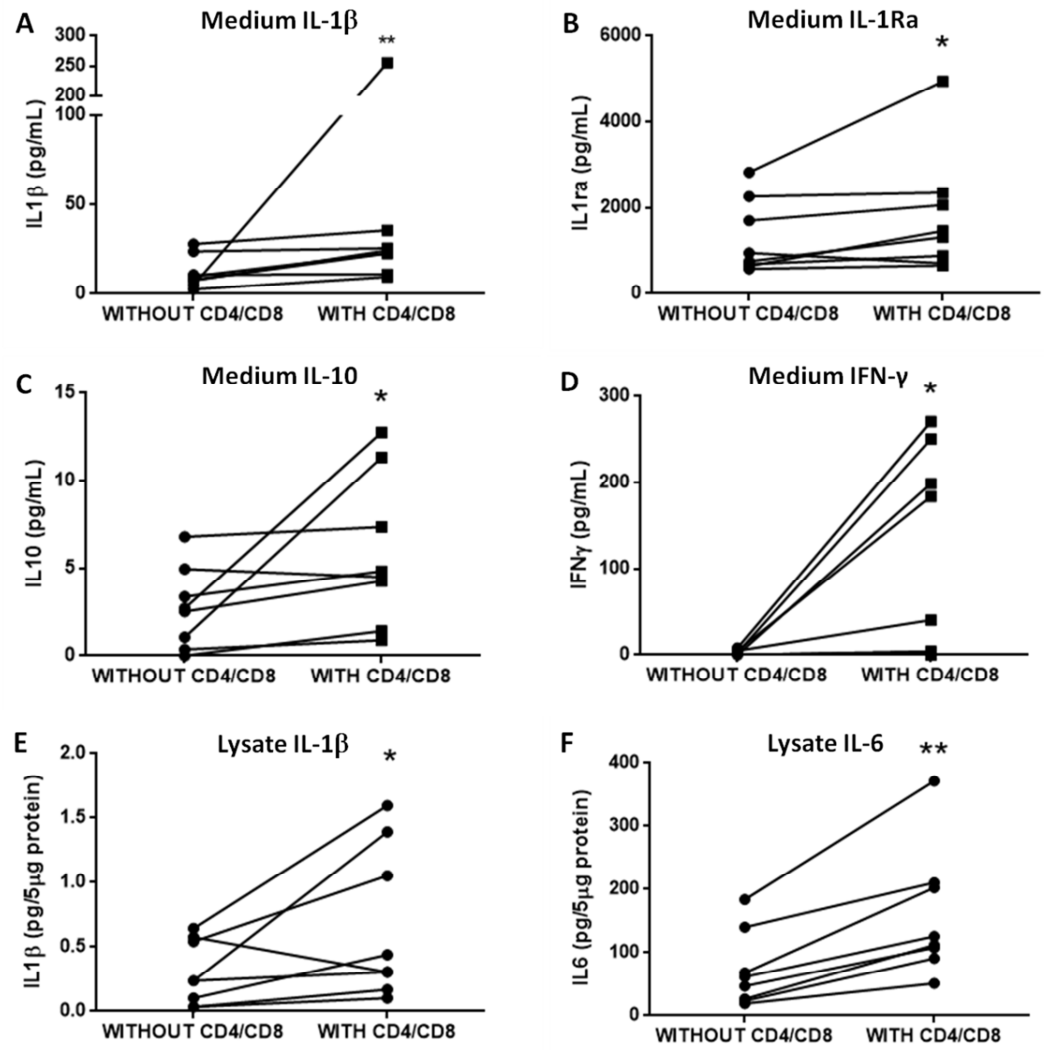


Figure 23: Effect of explant co-culture with matched maternal CD4 and CD8 T-cells on cytokine release and tissue cytokines.
*Levels of IL-1β (A), IL-1Ra (B), IL10 (C) and IFNγ (D) in culture medium were significantly elevated in the presence of CD4/CD8 T-cells compared to control. IL-1β (E) and IL6 (F) were significantly higher in the tissue lysates of explants co-cultured with T-cells compared to control. Wilcoxon signed rank test, *p<0.05, **p<0.01, n=8.*

Significant increases were recorded in the concentrations of IL-1β (p=0.008), IL-10 (p=0.02), IFNγ (p=0.02) and IL-1Ra (p=0.05) in day 3 culture medium from explants cultured with CD4/CD8 T-cells compared to controls. These differences were diminished by day 4 of culture. Significantly greater concentrations of IL-6 (p=0.008) and IL-1β (p=0.02) were detected in the lysates of placental explants cultured with T-cells compared to controls after four days of culture (Figure 23). No significant differences were seen in IL-2, -4, -12, -17 or TNFα in either culture supernatant or tissue lysate samples.

6.5 Discussion

VUE is a well-documented inflammatory condition of the placenta in late pregnancy and its association with poor pregnancy outcome (particularly FGR) is accepted (25,96,155).

Despite these associations, little is known about the mechanisms by which VUE may cause placental insufficiency and adverse pregnancy outcome. Here we present new insights into the possible functional consequences of immune cell interaction with the placenta and the subsequent release of cytokines on the placental environment. We developed a novel *in vitro* model of placental inflammation using placentas with matched T-cells obtained from maternal peripheral blood samples and have also investigated the effects of treating explants with cytokines identified as altered in VUE in a previous study (176). We have demonstrated that a pro-inflammatory response can be induced by the application of maternal immune cells to normal placental tissue. Furthermore, inflammatory mediators have the potential to detrimentally affect placental function as identified by the significant reductions in hCG secretion in explants co-cultured with CD4/CD8 T-cells or treated with exogenous cytokines (IL-2, IL-12 and anti-IL-4 in combination). Correlations of decreased levels of hCG with intrauterine fetal demise (191), FGR (192) and in adverse outcome in pregnancies with reduced fetal movements (RFM), a risk factor for stillbirth (135), have been reported. These findings support the hypothesis that VUE adversely affects placental function, thereby increasing susceptibility to FGR and/or stillbirth.

Significant increases were observed in the concentrations of the pro-inflammatory cytokine IL-1β in both tissue lysates and culture medium of explants co-cultured with T-cells. Significantly increased IL-1Ra was detected in the culture medium of co-culture explants, demonstrating that IL-1R has been activated, indicating that IL-1 is acting on the placenta. With an extended culture period it would be expected that IL-1Ra levels would continue to rise as the explants respond to increased IL-1β levels (193). In addition, the significantly increased levels of IL-6 in co-culture lysates are likely to have been stimulated by IL-1β (194,195).

Inflammation may prove to be a key contributor to the placental dysfunction of pathological pregnancies seen in clinical practice. Identification of high risk pregnancies using biomarkers detectable in the antenatal period has the potential to significantly impact upon obstetric care. Recently published work from our laboratory reported a pro-inflammatory cytokine profile in serum from mothers with a high risk of placental dysfunction (122). This profile reflected increased levels of IL-1β and IL-1Ra and decreased IL-10 proteins in villous tissue. Furthermore, increased mRNA expression of pro-inflammatory members of the IL-1 family, including IL-1β and IL-18, and decreased IL-10 and IL-4 mRNA expression in villous tissue was reported. These inflammatory mediators have been shown to reduce trophoblast viability and fusion during syncytialisation, which may negatively impact upon hCG/PlGF production (196). Further reports of elevated levels of chemokines CXCL9, CXCL10 and CXCL11 in maternal serum in cases of VUE (144) and a shift towards a pro-inflammatory cytokine phenotype in maternal serum in pregnancies resulting in FGR (137) provide

supportive evidence of the contribution of placental inflammation to poor pregnancy outcome. In contrast to many cytokines, there is a notable absence of IL-2 production by the placenta (197). This is perhaps related to the specificity of its action stimulating T-cells and driving a Th1-type immune response. A 1997 study of the effects of lymphokines on murine placental cells showed that IL-2 did not stimulate placental growth *in vitro* (198) however, very little is known about the effect of IL-2 on human non-immune tissue.

Representative images of isolated T-cells in co-culture confirm that the cells were largely residing in the intervillous space. Some cells were found in the villous stroma suggesting that they have the ability to migrate across the STB and into the placenta *in vitro.* T-cells in the intervillous space, T-cells in the villous stroma and culture with cytokines represent three different, but clinically relevant scenarios. The former promotes interaction within the villous stroma, whereas the latter two are more representative of exogenous interaction. The responses to these situations may vary depending on cell-cell interaction or cytokine receptor localisation on STB, stromal cells, endothelium, monocytes and macrophages. In VUE there is potential for interaction of maternal T-cells with fetal cells, including villous macrophages (Hofbauer cells, HBCs). VUE has been likened to immune rejection. Either the mother rejects the semiallogeneic placenta (host-versus-graft) (88,102) or maternal T-cells infiltrate the placenta acting in a graft-versus-host manner (21,134). What remains to be elucidated is the manner of maternal-fetal cell interaction. It is not yet known whether T-cells are activated by HBCs or whether they recognise fetal MHC as non-self.

It is perhaps unsurprising that the results of the Bio-Plex assay in the cytokine culture experiments did not mirror those of the co-culture experiments. Culturing explants with cytokines alone would not necessarily reflect a complex inflammatory phenomenon such as VUE but rather an exogenous insult. It is highly likely that the cell and cytokine milieu is ultimately responsible for a cascade of events resulting in placental dysfunction.

It should be noted that all of the placentas used in this study were from normal, healthy pregnancies in which it is likely that maternal tolerance of the feto-placental unit was well established. This may be a reason why experimental lesions were not as extensive as those seen in the high grade villitis described in the literature (31,88). Other factors that may have influenced the extent of T cell infiltration include the duration of the culture experiments compared to the duration of a pregnancy and the lack of blood flow in the *in vitro* model. The study was limited by the length of time the co-culture could be sustained. A culture period of 4 days was chosen in order that the viability of the isolated T-cells could be maintained. The cytokine culture experiments could be extended by using a cytotrophoblast culture model rather than an explant culture model although this would be more representative of the effect of generalised inflammation on trophoblast rather than VUE on the placenta.

If chronic disruption of the STB had occurred, then a change in system A activity might have been expected. That there was no significant alteration in system A activity was potentially because the treatments carried out here represent a relatively short exposure to cells/cytokines when compared to that *in utero*. There are also many other nutrient transporter systems that could have been affected that might provide the basis for further investigations. In this study, system A was chosen because of its pivotal role in supporting fetal growth; placental system A activity is reduced in FGR pregnancies compared to normal (184) and in pregnancy associated with RFM (67).

This study has demonstrated that it is possible to produce an *in vitro* model of placental inflammation in order to study the functional consequences of a maternal immune reaction to the fetus. As VUE is a post-partum histopathological diagnosis, previous investigations have been limited to descriptive studies thus their scope for functional interpretation has been limited. The co-culture of placental villous tissue with T-cells demonstrates that inflammation adversely affects placental function. This *in vitro* model has the potential to increase opportunities to determine the mechanisms underlying associations between VUE and poor pregnancy outcome and to identify targets for therapeutic intervention.

6.6 Authorship

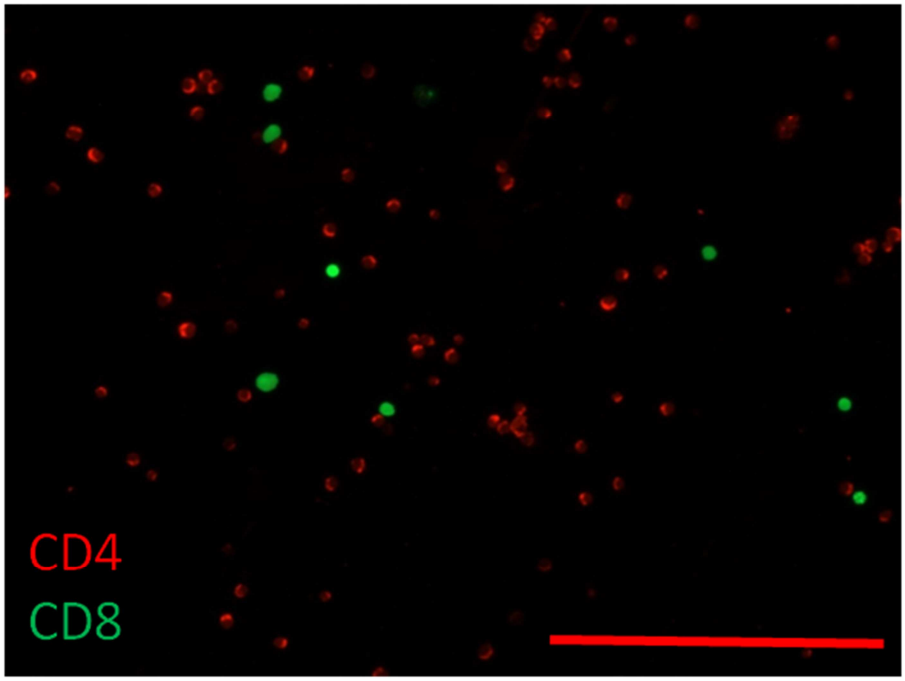
HD, RLJ, SLG and AEPH conceived and designed the experimental concept. HD and SLG carried out the preliminary studies, HD carried out the experimental work. HD compiled the manuscript, RLJ, SLG and AEPH provided editorial advice.

6.7 Acknowledgements

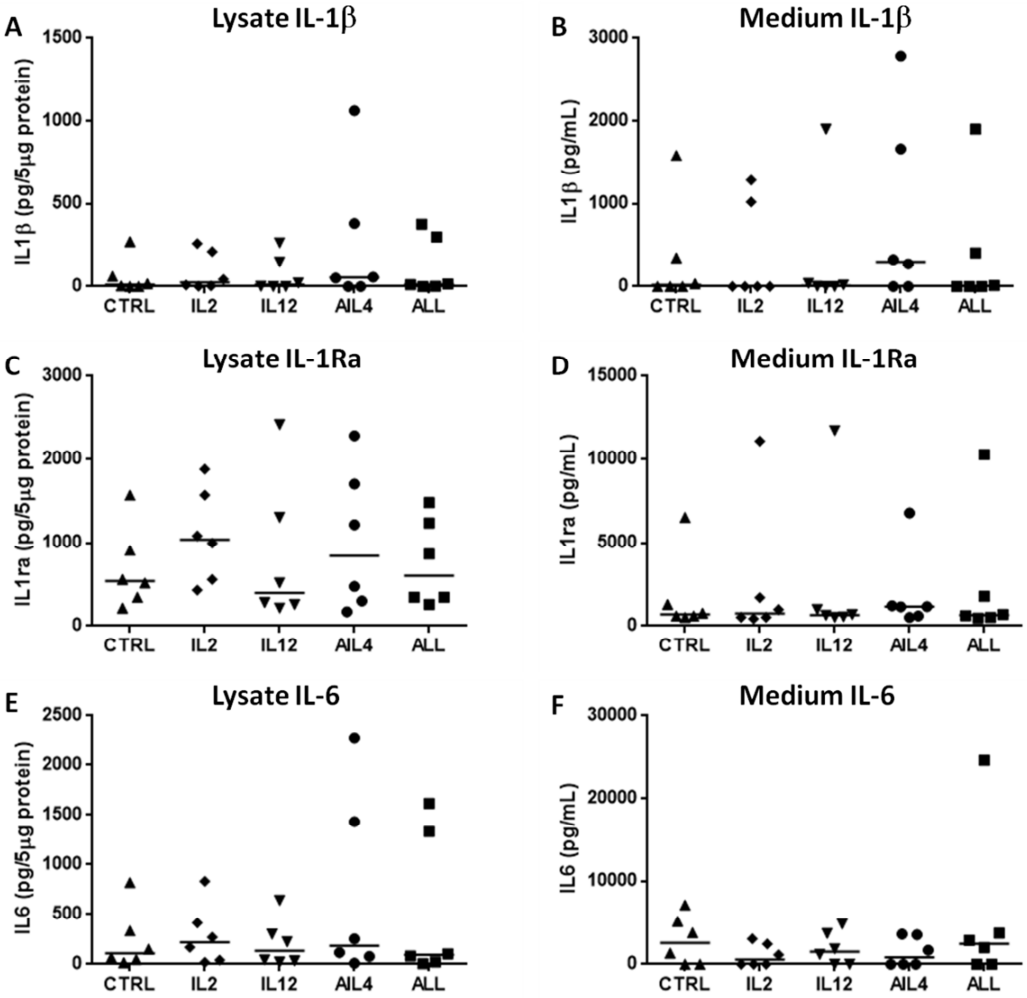
We wish to express our gratitude to the mothers who donated their blood and gave consent for us to use the placentas that we studied, the recruiters who consent the participants and Dr Mark Wareing who co-ordinates the MFHRG Biobank.

6.8 Conflict of Interest

The authors declare no conflict of interest.



Supplementary Figure 3: Representative image of isolated T-cells after introduction of Cell Tracker fluorescence.
CD4 T-cells fluoresce red, CD8 cells green. Scale bar represents 50µm, original magnification x100.



Supplementary Figure 4: The effects of placental explant culture with recombinant cytokines and/or neutralising antibodies on cytokine concentrations in tissue lysate and culture supernatant samples as measured by Bio-Plex assay.
No significant differences were observed in levels of (A-B) IL-1β in lysates or culture medium, (C-D) IL-1Ra in lysates or medium, (E-F) IL-6 in lysates or medium. CTRL: no treatment, IL-2: treatment with 10ng/mL recombinant human IL-2, IL-12: treatment with 10ng/mL recombinant human interleukin-12, ANTI-IL4: treatment with 1mg/mL anti-IL4 neutralising antibody, ALL: treatment with 10ng/mL IL-2, IL-12 and 1mg/mL anti-IL4. Friedman test, n=6.

CHAPTER 7: Discussion and Further Work

7.1 General Discussion

The work presented in this thesis addressed the hypothesis that VUE is an inflammatory immune response that causes FGR and stillbirth by detrimentally affecting placental function. A systematic review established the association between VUE and FGR. A lack of robust studies meant that a definitive link between VUE and stillbirth could not be verified; however, given the high frequency of FGR in stillbirth, this association would be logical. Detailed characterisation of VUE lesions in stillbirth, demonstrated that macrophages, CD4 and CD8 T cells are all participants in the lesions. Furthermore, analysis of pan-placental immune cells indicated wider inflammatory changes in VUE placentas that have not previously been reported. Identification of increased pro-inflammatory and decreased anti-inflammatory cytokines in lesions suggested that VUE represents a Th1-type immune response. Placental explant culture and T cell isolation were modified to develop a novel *in vitro* co-culture technique to enable investigation of the functional effects of inflammation on placental function. This demonstrated that culture in a pro-inflammatory cytokine milieu or with maternal T cells affects placental hormone production and induces secretion of inflammatory cytokines.

The rest of this chapter will discuss the findings of these studies and how they enhance our current understanding of inflammatory placental conditions and their relationship to adverse pregnancy outcome. A certain amount of speculation about the mechanisms of cell-cell interactions that might underlie VUE is required to begin to understand the complexities of pregnancy immunology. The clinical relevance of these conditions and potential therapies will also be discussed. The final part of this chapter will introduce ways in which this work could be advanced in order to confirm or refute the hypotheses generated from these studies.

7.1.1 Characterisation of VUE Lesions in the Placentas of Stillborn Infants

The 2011 Lancet Stillbirth Series urged for worldwide focus on the prevention of stillbirth. In high income countries, the majority of stillbirths could be preventable with appropriate intervention, even though currently this is restricted to delivery of the infant. The drive to reduce stillbirth rates has been hampered by a lack of recognition of the problem in worldwide data collection and WHO Millennium Development Goals (199) and difficulty in identifying important risk factors for stillbirth (187). Women who have previously experienced stillbirth and are known to be high-risk are monitored more closely in subsequent pregnancies which may contribute to improved outcome in the cohort

(200,201). However, such measures are not feasible for the population as a whole. Antenatal detection of fetuses at risk is therefore an important tool as an eight-fold increased rate of stillbirth is recorded in pregnancies with FGR; this rate is reduced if the growth restriction is detected before birth (202). It is reported that up to 60% of stillbirths are attributable to placental insufficiency (4).

Inflammation as a cause of placental dysfunction and adverse pregnancy outcome is gaining momentum as a research topic. Previously, efforts have largely concentrated on the effects of inadequate spiral artery remodelling in pre-eclampsia and early-onset FGR (46,65). Recently, however, attention has turned to phenomena such as non-infectious or sterile inflammation, the result of an immune response to endogenous danger signals (damage associated molecular patterns, DAMPs) (120,122,203). VUE and subchorionic intervillositis potentially arise as a maternal response to the fetal allograft (78,97).

Whilst an association of VUE with stillbirth and recurrent pregnancy failure is often cited, there is sparse tangible evidence to confirm this (31,98,100–102). Our systematic review published in 2013 found few published studies where the focus was the incidence of VUE in stillbirth (155). Furthermore, there was little investigation into the morphological characteristics of VUE in stillbirth and whether the lesions were similar in live born and stillborn infants. Reports of more severe inflammation and increased adverse outcomes during recurrence of VUE serves to highlight the nature and importance of this condition (97,98). It was therefore surprising that VUE, reported to be "an important pattern of placental injury" and "an important cause of IUGR and recurrent reproductive loss" (88), had not been as extensively studied as its reputation may have merited.

The characterisation studies yielded some novel and exciting findings that may assist with the future detection and reporting of inflammatory pathologies. It is often questioned how lesions affecting <10% of villous parenchyma can have such a devastating effect on fetal growth and survival (89). The finding that there are specific increases in CD4 and CD8 T cells across the placenta in cases of VUE, is suggestive of a wider, pan-placental inflammatory response. VUE lesions may represent obvious areas of immune cell accumulation, destructive tissue damage, fibrin deposition and vasculopathy, but it is possible that more subtle changes in maternal cell infiltration into the villous stroma have previously been overlooked. These pan-placental changes could contribute to a global impairment of placental function yet are unlikely to be detectable using standard histopathological techniques. Diagnosis of VUE by a Paediatric or Perinatal Pathologist would involve the detection of VUE lesions in H&E sections, which may not always be observed in the parenchymal biopsies obtained. It has been stated that the incidence of VUE may have been historically underestimated, in part because an area of placenta was sampled that did not contain any lesions (86). The presence of CD4 and CD8 T cells in areas of placenta

seemingly unaffected by lesions may direct the Pathologist to consider VUE as an underlying pathology. The reports of increased risk of VUE in subsequent pregnancies and increased severity of inflammation when it does recur (97,98,101) should give the clinician the incentive to investigate contributors to increased risk of poor outcome.

7.1.2 Cytokine Immunostaining in VUE Lesions

The differences in placental immunoreactive cytokines between VUE lesions, areas devoid of lesions, growth restricted infants and healthy pregnancies is intriguing. Significantly increased immunostained area of pro-inflammatory cytokines IL-2 and IL-12 and significantly decreased anti-inflammatory IL-4 was observed. Many authors state that a successful pregnancy relies on a skew towards Th2-type antibody-mediated immunity (119,127,130,132,166). This is achieved, in part, by the anti-inflammatory cytokine IL-4. IL-4 is produced by CD4 T cells that have differentiated down the Th2 pathway. However, IL-4 is also produced by the STB of placenta in normal pregnancy (160). It seems plausible that, in normal pregnancy, IL-4 represses pro-inflammatory reactions, preventing uncontrolled cell-mediated inflammation. Decreased IL-4 levels in VUE lesions may be the catalyst that permits the switch from a predominantly Th2- to a Th1-type immune response. When IL-4 levels are reduced the pro-inflammatory cytokine IL-12, produced by activated macrophages and also STB, could potentially exert its influence, further supporting a drive towards a Th1 response. That IL-2 (a Th1 cytokine) can be localised, by immunofluorescence, to CD4 cells further confirms the phenotype of the inflammatory cells. VUE may therefore represent the combination of a 'double hit' of reduced inflammatory repression and increased inflammatory stimulation.

There remains the question as to what constitutes the initial stimulus for VUE. A loss of STB integrity might expose villous stroma, and therefore fetal antigen, to maternal immune cells. Alternatively, placentally-induced imbalance of cytokines may drive the switch from Th2 to Th1-type responses, favouring T cell infiltration into the villous stroma.

7.1.3 Inflammatory Cytokines in STB Function

Cytokines have been shown to influence STB turnover and regeneration (204–207). In the current study placental production and secretion of IL-1β and IL-6 was significantly elevated in explants co-cultured with maternal CD4 and CD8 T cells compared to control. Recently published data show that treatment of isolated trophoblasts with DAMPs (uric acid and HMGB1) induced inflammation, evidenced by increased secretion of IL-1β, IL-6, IFN-γ, RANTES and MIP-1α. Culture with DAMPs and IL-1β also increased trophoblast apoptosis and diminished syncytialisation (196). It has been suggested that TNF-α induces focal areas of STB damage by stimulating apoptosis (208,209). As the STB effects placental hormone production and secretion, pro-inflammatory events could detrimentally affect STB integrity

and/or turnover thereby reducing hormone secretion, as seen in the co-culture experiments. This may, in turn, further reduce CTB differentiation into STB (210). A damaged or incomplete STB layer potentially unmasks fetal antigen from the villous stroma to the maternal immune system and would have diminished endocrine and nutrient transport capacity.

7.1.4 Macrophages in Placental Pathologies

Much of the available data about VUE notes the significant presence of Hofbauer cells (HBCs) in lesions (50,87,93,161). The current study consolidates these observations; in VUE lesions CD163+ macrophages and CD4 T cells were the predominant cell types. There were elevated numbers of placental macrophages in all other poor outcome groups, both stillborn and live born with FGR compared to controls. A similar elevation of HBC numbers has been reported in pregnancies with diabetes (211), with evidence that they acquire a pro-inflammatory phenotype (138). An increase in macrophage number conflicts with reports that the HBC population declines in the third trimester of pregnancy (211,212). This supports a placental/fetal immune response and suggests that inflammation occurs in several pregnancy pathologies.

Tissue resident macrophages are known to have numerous critical roles in tissue homeostasis and immunity. Traditional descriptions of macrophages denote two classifications. Classically-activated M1 macrophages release the pro-inflammatory cytokine IL-12, driving an immune response towards a Th1-type. Alternative activation drives the differentiation of M2 macrophages and their production of IL-10, the result being tissue repair and promotion of antibody-mediated immunity (213). Macrophages polarise into M1/M2 phenotype in response to the cytokine microenvironment they are exposed to. According to Martinez and Gordon (2014), M1/M2 polarisation represents the extreme ends of the spectrum; many macrophages are observed with characteristics in between M1 and M2 (214).

HBCs are stated to be of an M2 phenotype as demonstrated by their expression of the cell surface marker CD163 (172,213). In addition to their function in fetal defence, HBCs are thought to play an important role in angiogenesis, ECM remodelling, regulation of apoptosis, STB turnover and villous proliferation in response to hypoxia (170,212). Given these functions it is not surprising all HBCs appear to have a repair phenotype. However, our dual staining immunofluorescence has clearly shown CD163+ (M2) macrophages in VUE lesions co-localising with the pro-inflammatory cytokine IL-12 (Chapter 4). This is highly suggestive that HBCs are contributing to the cell-mediated inflammation in this condition. In organ transplant immunology it is known that during allograft rejection (host-versus-graft disease) competent antigen presenting cells (APCs) in the donor organ interact with recipient effector

cells (T cells). 'Passenger' APCs in the graft may process donor antigen (Ag) and present Ag + MHC to recipient T cells. Alternatively, recipient T cells may recognise donor MHC on APC as non-self (215). This acute rejection process is considered to be a T cell phenomenon. One must bear in mind that, unlike a grafted organ, the placenta does not make a complete vascular anastomosis with the maternal host. Another notable difference is that the graft (placenta) is part of a viable donor (fetus) (87). In VUE, elevated numbers of fetal (graft) HBCs and a prevalence of maternal (host) T cells in close association within the placental stromal core were observed. That HBCs appear to be producing IL-12 serves to indicate that these are activated macrophages and could be presenting antigen to maternal T cells. However, immunoperoxidase staining revealed low levels of MHCII in VUE lesions (unpublished data). What remains to be elucidated are the consequences of maternal and fetal immune cell interaction.

Though the preferred analogy for VUE is that of host-versus-graft disease there has been one study that suggested VUE represents graft-versus-host disease. Here, the maternal T cells are considered to be the graft as they are infiltrating the villous compartment, which makes the placenta the host (134). Whether classified as host-versus-graft or graft-versus-host immune rejection it is apparent that HBCs may have a key role in establishing and maintaining inflammation in VUE. It is perhaps a difference in macrophage and T cell interaction that determines the severity of the pathology and consequently the outcome.

7.1.5 An *In Vitro* Model of VUE

The development of an *in vitro* model of VUE permitted investigation into the functional consequences of an excess of maternal T cells in the placental environment. It was shown that the procedure of obtaining placental tissue and matched maternal blood was logistically possible.

Co-culture of placenta and isolated T cells over a period of 4 days resulted in a reduction in hCG secretion and an increase in pro-inflammatory cytokine production and secretion. The T cell lesions in the VUE model were relatively sparse thus it was assumed that the pro-inflammatory cytokines detected in the BioPlex assay were responsible for reduced hCG concentrations. It is not yet known if infiltrating CD8 T cells were acting in a cytotoxic manner. Immunoperoxidase staining for cytoplasmic perforin, an indicator of cytotoxic activation, would address this (216). hCG is pivotal in the maintenance of STB, regulation of trophoblast differentiation and stimulation of CTB incorporation into STB (70). Altered levels of hCG represent altered functional capacity of STB. Reduced maternal serum concentrations of hCG have been reported in FGR (192), intrauterine fetal demise (191) and pregnancies experiencing reduced fetal movements (135). A reduction in PlGF secreted from villous explants co-cultured with CD4 and CD8 T cells provides further evidence that the

presence of T cells compromised STB endocrine functional integrity. It has been suggested that PlGF can be used to identify pregnancies at risk of adverse outcome due to placental insufficiency (73). In the case of the *in vitro* model, more observations are required to confirm if PlGF is significantly reduced by the presence of T cells.

The activity of the system A transporter in villous explants was not affected by co-culture with T cells. System A was studied due to its critical role in fetal growth (184). However, transporters responsible for delivery of amino acids to the fetus which are not substrates of system A are also downregulated in FGR (217) and might be important in the pathogenesis of VUE.

These studies have shown that it is possible to co-culture maternal CD4 and CD8 T cells with placental villous tissue from the same pregnancy, maintain integrity of tissue and cells and achieve migration of T cells into the villous stroma over 4 days. The preparation is thus a model of VUE and CIUE. However, the lesions that were observed did not necessarily reflect the lesions seen in extensive high grade villitis. The tissue and blood utilised in these studies were from normal pregnancies so it is likely that maternal tolerance of the fetus was well established. That there was evidence of T cells migrating into the villous stroma in these healthy placentas is perhaps indicative that VUE has the potential to occur in every pregnancy (91).

It is worth noting that *in vitro* conditions over 4 days do not entirely mimic those *in vivo* so a model of VUE is unlikely to exactly replicate the condition itself. There are several potential modifications to the procedure, detailed in section 7.2 that may enhance the inflammatory process that this protocol attempts to reproduce. Furthermore, it should be tested whether it is specifically CD4 and CD8 T cells that reduce endocrine secretion from explants following co-culture or whether other populations of immune cells similarly influence placental function.

Quantification of T cell migration into villous stroma may be more informative if more specific immunostaining of the co-culture explant sections was carried out. There was an increase in leukocytes and a pro-inflammatory cytokine response in explants cultured with CD4 and CD8 T cells compared to controls. What has not been reported, thus far, was the type and proportion of cells present. Further staining for CD163, CD4 and CD8 may elucidate if a particular population of cells was prevalent, as in VUE.

7.1.6 Clinical Implications of VUE

One aim of a project attempting to address a clinical issue is to glean a better understanding of the pathophysiological mechanisms underlying the disorder of interest. Another aim is to identify signs or markers that enable detection of a problem and, ideally, be able to treat it

or prevent it. Biomarkers of inflammation that could be detected in maternal serum offer a promising option for antenatal screening and monitoring of high-risk pregnancies.

Recently published data from several groups have indicated that a pro-inflammatory cytokine/chemokine profile can be detected in maternal serum. A 2009 study described increased placental mRNA expression of pro-inflammatory chemokines CXCL9 and CXCL13 and significantly higher plasma concentrations of CXCL9, CXCL10 and CXCL11 in maternal and fetal plasma in cases of VUE (144). Girard et al. (2014) studied placentas and serum from pregnancies where there was maternal report of reduced fetal movements (RFM) (122). This study population was chosen as RFM pregnancies are at a higher risk of stillbirth that may be due to placental dysfunction (68). Girard et al. describe decreased circulating concentration of IL-10 and elevated concentration of IL-1Ra, an anti-inflammatory mediator used as a marker of prior activation of the IL-1 system. The serum biomarker changes were mirrored in placental tissue together with a pro-inflammatory bias represented by increased IL-1β and IL-18 and reduced IL-10 and IL-4 mRNA (122). In 2012, Mullins et al. systematically reviewed the literature relating to serum cytokine profiles in FGR. Whilst results were heterogeneous (presumed to be related to the difficulty in diagnosing FGR antenatally), in normotensive pregnancies delivering FGR or SGA infants the serum profile observed was largely pro-inflammatory (137).

There is scant evidence of successful treatment for VUE but as it is an inflammatory condition, treatment options may rely on administering corticosteroids. In 2008, Boog reported successful preventative treatment of 4 patients with a history of recurrent VUE. Patients were administered 20mg/day prednisone (which does not cross the placental barrier) from the second trimester, combined with 100mg/day aspirin between weeks 12-35 of gestation. This treatment regime produced 4 living infants. A fifth pregnancy in the study resulted in an intrauterine fetal death, though this was associated with severe intervillositis, another inflammatory condition that carries a much poorer prognosis (89).

There are other candidate medications that may be considered. Azathioprine is a cytotoxic antimetabolite often used following transplantation and to treat autoimmune disease. The use of azathioprine in pregnancy has been investigated in several prospective studies and whilst results show no increase in fetal defects, this drug is associated with lower birth weight, gestational age and prematurity (218,219). Ciclosporin is another immunosuppressive drug primarily used to prevent transplant rejection, but is also used in the treatment of autoimmune disease. Ciclosporin use during pregnancy appears to be associated with low birthweight and prematurity though the contribution of maternal co-morbidities to these is not entirely understood. Nonetheless, care should be exercised if these drugs are considered (220). Hydroxychloroquine (HCQ) is an anti-malarial drug used to treat antibody-mediated autoimmune conditions such as systemic lupus erythematosus

and anti-phospholipid syndrome. It is often continued throughout pregnancy to avoid an increase in disease activity. Drug usage in pregnancy and evidence that HCQ can cross the placenta raised concerns about fetal exposure to the drug. A systematic review and studies investigating HCQ use in pregnancy revealed no differences in live births, congenital defects, fetal deaths or prematurity in pregnancies treated with HCQ compared to those not treated with HCQ (221–224). There is little available evidence that HCQ could be used in the treatment of conditions related to sterile inflammation.

A burgeoning area of research is the use of homing peptides to specifically target and deliver drugs to a specific organ (225,226). Though in its infancy, early results of targeted drug delivery to the placenta in animal models are promising and could provide a valid method of treatment during pregnancy (227).

It is difficult to say that VUE is definitively linked to stillbirth based solely on the experimental data presented here. We can, however, state that VUE detrimentally affects placental function and that placental dysfunction underlies many cases of FGR and stillbirth. Our morphological studies were based on a single time point in a plastic process and the culture experiments may be more representative of acute inflammation rather than chronic. There is also a question as to whether infiltrating maternal T cells migrate beyond the placenta and into the fetus. There is no published evidence in humans to support this, though in rats Runting Syndrome is a lethal graft-versus-host disease that involves maternal T cell infiltration of the fetus (100). If it is the case that maternal cells can migrate into the fetus it may begin to explain why some cases of VUE result in FGR, some stillbirth and others a healthy live infant.

This project has produced solid foundations of novel experimental work on which to base further investigations.

7.2 Future Work

In order to enhance the findings detailed in this thesis the following suggestions for continuation and elucidation are proposed.

1. The systematic review of VUE studies highlighted that there remains uncertainty about the relationship of VUE with stillbirth (155).

- A study to address this uncertainty would confirm if there is an association of VUE with non-anomalous stillbirth. To power a case-control study in order to accurately estimate the incidence of VUE in stillbirths, 137 samples from non-anomalous stillbirths and 137 controls would be required.

2. Morphological characterisation of VUE lesions in stillbirth demonstrated high levels of macrophages, CD4 and CD8 T cells and evidence of pan-placental inflammation.

- A morphological study of placentas from live born infants with VUE using the same panel of antibodies would permit a comparison of both lesion and pan-placental immune cell composition. This may confirm or refute the theory that increasing severity of VUE inflammation leads to progressively poorer pregnancy outcome.

3. Studies of VUE in live born infants have described hyperplastic HBCs (87), trophoblast expression of intercellular adhesion molecule (ICAM)-1 (177,228) and C5b-9 deposition (163).

- Extending the immunohistochemical study of placentas from stillborn infants to include these markers may provide evidence of similarities or differences in the severity of the inflammation.

4. *In vitro* work showed that co-culture of explants with maternal T cells affected placental hormone and cytokine production.

- This work could be extended to include placentas and bloods from pregnancies at high risk of placental insufficiency. Women who experienced reduced fetal movements and are considered to be at a higher risk of stillbirth have been shown to exhibit a pro-inflammatory profile in serum (122). Utilising blood and placenta from a women with increased serum IL-1Ra may facilitate the migration of T cells into the villous stroma in the *in vitro* model. Obesity during pregnancy has been shown to stimulate inflammation in the placenta (139) and increasing maternal bodyweight and BMI linearly relate to increased severity of VUE (91). Therefore, using placentas from mothers with BMI >30 may increase the likelihood of mimicking high grade VUE *in vitro.* Placental injury and pro-inflammatory cytokine production, which can be experimentally induced by culture in hypoxic conditions (229), could be utilised to potentially stimulate T cell migration into the villous stroma.
- Modification of the co-culture process might improve the infiltration rates of T cells into villous stroma. If the syncytiotrophoblast could be stimulated to express ICAM-1 it may facilitate maternal leukocyte adhesion to and migration through STB. During the preliminary explant co-cultures explants were incubated with TNF-α in an attempt to stimulate ICAM-1 expression, however, rates of apoptosis appeared to be increased in the explants so this line of stimulation was not pursued (data not shown). In addition, if it were possible to lengthen the duration of the co-culture period the opportunity for cells to migrate and therefore the length of time for functional consequences to occur, might be increased.
- Incubation of co-culture explants with T cells on a rocker in the incubator could be a way to keep the isolated T cells in suspension throughout the culture period.

5. *In vitro* work showed that culture of placental explants with cytokines affected hormone production.

- An extended panel of pro-inflammatory cytokines and/or anti-inflammatory neutralising antibodies could be utilised to investigate the effects of a cytokine milieu on explant function.
- Employing a cytotrophoblast culture model could provide insights into the effects of cytokines on the function of the syncytium. Whilst it would not mimic the inflammatory process of VUE, it could prove useful in specifying which parts of the placenta undergo functional impairment.

This project has laid excellent foundations on which to understand the pathological processes involved in placental inflammation, and particularly VUE. It has also highlighted areas in which further research is required to unravel the complexities underlying conditions that have potentially devastating consequences.

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