Placental SLE like Autoimmunity and Fetal Loss

A Thesis Submitted to The University of Manchester for the Degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Abbreviations Used

2D	Two Dimensional
3D	Three dimensional
aCL	Anti cardiolipin antibody
Aix	Augmentation index
Anti-b2GPI	Anti-b2 glycoprotein I antibodies
Anti-RNP	Anti-Ribonuclear Protein antibody
Anti-Sm	Anti-Smith antibody
APCs	Antigen Presenting Cells
aPL	Anti-Phospholipid antibodies
APO	Adverse Pregnancy Outcome
APS	Antiphospholipid syndrome
BCR	B Cell Receptor
Beta2GP1	Beta2-glycoprotein I
BP	Blood pressure
C3	Complement factor 3
C3aR	Complement factor 3 Receptor
C4	Complement factor 4
C4d	Complement factor 4 split product d
CAD	Coronary Artery Disease
CD146	Constitutive Endothelial cell marker (microparticles)
CD235a	Erythrocyte Marker (microparticles)
CD4	Cluster of Differentiation 4
CD41a	Platelet Marker (microparticles)
CDr42	Tissue Factor Marker (microparticles)
CD45	Leucocyte marker (microparticles)
CD62e	Activated Endothelial marker (microparticles)
CH_{50}	Total haemolytic complement

CHI	Chronic Histiocytic Intervillositis
CHD	Coronary Heart Disease
CPRD	Clinical practice research Datalink
CRRY	Complement receptor-1–related gene/protein Y
CS	Caesarean section
CTD	Connective Tissue Disease
CVD	Cardiovascular disease
CV	Coefficient of variation
DAB	3, 3'-diaminobenzidine
DAF	Decay accelerating factor
DP	Diastolic Pressure
DNA	Deoxyribose nucleic acid
dNK	Decidual natural killer
ELISA	Enzyme Linked Immunosorbent Assay
EMPs	Endothelial Microparticles
F	Perivillous Fibrin Deposition (cohort for chapter 4)
FACS	Fluorescence-activated cell sorting
FARSITE	Feasibility Assessment and Recruitment System for Improving Trial Efficiency
FGR	Fetal Growth restriction
IBR	Individualised Birthweight Ratio
IHD	Ischaemic Heart Disease
IL	Interleukin
IL-8	Interleukin 8
IL-10	Interleukin 10
IFN-α	interferon-α
IFR5	Interferon Regulatory Factor 5
ISAC	Independent Scientific Advisory Committee
iTreg	Induced T regulocyte

JugSy	Jugular-symphysial height
LAC	Lupus anticoagulant
LGA	Large for Gestational Age
LLD	Lupus like disease
LMWH	Low molecular weight heparin
LPS	Lipopolyssaccharide
L:T	Length to thickness ratio
LR	Likelihood Ratio
MAC	Membrane attack complex
MAP	Mean arterial pressure
MCP	Membrane cofactor protein
MCTD	Mixed connective tissue disease
MDT	Multidisciplinary team
MHC	Major Histocompatibility Complex
MPs	Microparticles
mRNA	Messenger RNA
MVM	Maternal Vascular Malperfusion (cohort for chapter 4)
NSB	Normal Stillbirth (cohort for chapter 4)
NP	Normal Pregnant
nTreg	Naturally occurring T regulocyte
OR	Odds ratio
PAPP-A	Plasma protein-A
PAPS	Primary APS
PBMCs	Peripheral blood mononuclear cells (PBMCs)
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PET	Pre-eclampsia
Pg	Picograms

PI	Pulsatility index
PL	Previous Loss Pregnant
PlGF	Placental growth factor
PMA	Phorbol 12-Myristate 13-Acetate
PPROM	Preterm pre-labour rupture of membranes
PPT	Peak to peak time
PS	Phosphatidylserine
PWV	Pulse wave velocity
RCOG	Royal College of Obstetricians and Gynaecologists
RI	Reflection index (pulsewave) / Resistance Index (Doppler)
RM-ANOVA	Repeated measures ANOVA
RNA	Ribonucleic acid
RR	Relative Risk
SBP Ao	Central aortic systolic blood pressure
SGA	Small for Gestational Age
SLE	Systemic Lupus Erythematosus
SLEPDAI	SLE Pregnancy Disease Activity Index
sFlt-1	Soluble fms-like tyrosine kinase-1
SP	SLE pregnant
SP	Systolic Pressure
sVEGF R1	Soluble Vascular Endothelial Growth Factor Receptor 1
TCR	T Cell Receptor
TGF-ß	Transforming growth factor ß
Th cell	T helper cell
TLR	Toll Like Receptor
Treg cell	T regulatory cell
UAD	Umbilical artery Doppler
UCTD	Undifferentiated Connective Tissue Disease

UtAD	Uterine artery Doppler
V	Villitus of unknown aetiology (cohort for chapter 4)
VEGF	Vascular endothelial growth factor
VEGF R1	Vascular endothelial growth factor Receptor 1
VUE	Villitis of unknown aetiology
WHO	World Health Organisation

Abstract - Placental Systemic Lupus Erythematosus (SLE)-like Autoimmunity and Fetal Loss

The University of Manchester PhD 2018

Hannah Kither

Introduction: Systemic Lupus Erythematosus (SLE) is a multisystem autoimmune disease of significant female preponderance. It is associated with increased rates of adverse pregnancy outcomes (APOs), including stillbirth. The relationship between SLE and stillbirth is bidirectional, with women who have had a stillbirth at increased risk of developing SLE. The underlying cause of pregnancy pathologies with SLE has not been fully established, but aberrant placental formation and/or maternal maladaptation to pregnancy, at an immune or vascular level, are plausible. This thesis sought to explore the relationship between stillbirth and SLE in pregnancy, with the hypothesis that women who have a stillbirth display similar vascular and maternal immune issues.

Methods: A multi-method approach was taken to address this research question, involving both epidemiological and prospective studies. A large scale epidemiological study was undertaken utilising a UK national primary care database (Clinical Practice Research Datalink, CPRD) to ascertain the risk of developing SLE, following an APO. In the prospective studies, three pregnant cohorts were considered: (i) those with SLE, (SLE Pregnant (SP), n=51), (ii) prior stillbirth (Previous Loss (PL), n=29) or (iii) uncomplicated pregnancies (Normal Pregnant (NP, n=44). Maternal vascular changes were assessed by brachial blood pressure (BP), photoplethysmography (stiffness index (SI), and reflection index (RI)), arteriography (pulsewave velocity (PWV)), plasma placental growth factor (PlGF) and soluble FMS-like tyrosine-kinase 1 (sFLT-1), and uterine artery Doppler velocimetry (UtAD). Immune changes were monitored by peripheral blood microparticles (MPs), T helper 17 (Th17) and T regulatory (Treg) cell numbers. Placental assessments of *in utero* placental biometry were undertaken, along with placental histological examination and complement deposition (complement factor 4d (C4d) and complement factor 3a receptor (C3aR)). A retrospective study of stillbirth placentas was also conducted.

Results: The UK epidemiological study identified 20,123 cases of APO and 97,323 livebirths. The overall incidence of SLE was 0.05%. Women with a stillbirth had a relative risk (RR) of developing SLE of 4.10 (95% CI 3.14-5.36)) compared to livebirth outcomes. In the prospective study, the SP group had the lowest gestation at delivery, exaggerated by lupus nephritis. *In utero*, placental widths were smallest in the SP group and largest in NP. No difference in C4d staining was defined in livebirth placentas, although stillbirth cohorts had exaggerated deposition associated with villitis (V) and maternal vascular malperfusion (MVM). C3aR was also highest in NP and lowest in the SP group. In stillbirth cases, C3aR deposition was higher in those associated with MVM. Mean arterial BP was higher in PL and lower in NP across gestation. No drop in second trimester BP was seen in PL cases in their current pregnancy. PWV increased with gestation and was highest in SP at 17-28 weeks' gestation. UtAD measures fell with increasing gestation. In SP, the presence of abnormal UtAD was associated with lower gestation at delivery. PlGF fell with increasing gestation and was lowest in SP, whilst sFLT-1 was raised. 21% of PL patients displayed seropositive autoantibodies, with concomitant elevation in Th17 cells. No differences in Treg cells were defined, but total MPs were higher in PL, over SP and NP groups.

Conclusions: UK women with a prior stillbirth have increased lifelong risk of developing SLE. They demonstrate similarities (vascular and immunological) to women with confirmed SLE diagnosis. Their immunological changes in maternal autoantibodies, placental histology and complement deposition suggest pathological similarities to SLE, with possible targets for therapeutic intervention.

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.

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Journal Format

Journal format was chosen for the thesis as laboratory and clinical work and their corresponding chapters fell naturally into sections suitable for publication.

Chapter 3 is for submission to Paediatric and Perinatal Epidemiology

Chapter 4 is for submission to BMC Pregnancy and Childbirth

Chapter 5 is for submission to Clinical Science

Chapter 6 is for submission to Clinical Science

Statement of Contribution

The author of this thesis (Hannah Kither) was involved in all aspects of design of the studies involved in this thesis, including hypothesis development, experimental design and development of methodologies, ethics application, patient recruitment, data cleaning and data analysis. All blood sample analysis was also performed by myself.

The original idea for this thesis was provided by Dr Ian Crocker, this idea was then developed with myself and Dr Clare Tower.

Original ethics for recruitment of patients with SLE was provided by Dr Clare Tower.

Raw data for epidemiology was provided by the Feasibility Assessment and Recruitment System for Improving Trial Efficiency (FARSITE) team or pulled from Clinical practice research Datalink (CPRD) by Rosa Parisi in the Epidemiology department of The University of Manchester. All analysis of epidemiology data was performed by myself.

Ultrasound data was collated from clinics by clinicians including myself, Dr Clare Tower and Professor Alex Heazell.

Placental samples were processed and histological reports provided by the Royal Manchester Children's Hospital Histopathology department of Central Manchester University Hospitals NHS Foundation Trust. Anonymised placental samples were stained for complement deposition by the Adult Histopathology Department of Central Manchester University Hospitals NHS Foundation Trust. All analysis of this staining was performed by myself.

1. Introduction

It has long been acknowledged that autoimmune disorders have wide ranging effects, including an adverse impact on the long-term health of affected individuals. Predominantly affecting women, autoimmune diseases can have a significant impact on childbearing, with pregnancy adding an additional stress to the maternal system with potential detriment to mother and baby (1). Systemic Lupus Erythematosus (SLE) is a multisystem autoimmune disease shown to be associated with increased rates of adverse fetal outcomes, the most devastating being stillbirth, which at 3.6% prevalence is over 5 times that seen in healthy women (2,3).

The relationship between SLE and stillbirth is bidirectional, as research has shown that having a stillbirth increases a woman's lifetime risk of developing SLE (4,5). However, the underlying relationship between these is unclear, with uncertainty as to whether stillbirth occurs because of undiagnosed SLE, or if stillbirth itself is a trigger for subsequent SLE development.

This project investigated the relationship between stillbirth and autoimmune disease, studying women with established SLE and women in a subsequent pregnancy following a previous stillbirth. In considering areas of pathological overlap, this chapter firstly overviews SLE and its impact on pregnancy, including stillbirth, and secondly links stillbirth with the onset of maternal autoimmune disease.

1.1 Stillbirth

In the UK, stillbirth is defined as the birth of a baby without signs of life after 24 weeks gestation, irrespective of fetal birth weight (6). In 2017, there were 2,873 stillbirths in the UK, with an overall rate of 4.2 per 1000 births (1 in 238) (7); of these, approximately 15% were related to fetal abnormalities (8). On a global scale, there is significant variation in stillbirth definition, with some including birthweight, and some based on gestation alone; typically the gestational age range used for defining stillbirth ranges from 20-28 weeks of pregnancy (9–15). The World Health Organisation (WHO) defines stillbirth as the birth of a baby with no signs of life after 28 weeks gestation (16). In estimating the size of the problem worldwide, approximately 2.6 million stillbirths occur every year. Of those, approximately 84,000 occur in developed countries and, within these developed countries, 10% occur as intrapartum events (0.6 per 1000 births), meaning the majority (approximately 90%) occur in the antenatal period. Given these statistics, it could be argued that new methods are required to identify women at risk of antepartum stillbirth, and tailored care packages should be introduced to curb this lamentable rate of stillbirth (17).

Not only do the definitions of stillbirth vary, but so do the underlying causes of stillbirth, with multiple systems for their classification, the number of unexplained cases ranging from 14 - 47.4%, according to which system is considered (18). Broadly, the causes of stillbirth are grouped into congenital abnormalities, infectious causes, fetal growth restriction/placental insufficiency, umbilical cord accidents, maternal medical disease, intrapartum events and idiopathic, unexplained reasons (19). Irrespective of definition, there are consistent risk factors for stillbirth

that can be detected in the antenatal period. In identifying these, women can be recognised as high risk for stillbirth, with care modified to reflect this with the overall aim of reducing stillbirth rates. These include maternal factors such as ethnicity (increased stillbirth rate in non-Caucasian women), previous stillbirth, nulliparity, maternal age, raised body mass index (BMI) at conception and maternal disease (e.g. diabetes, hypertension) and fetal factors, such as growth restriction or multiple pregnancy, and duration of pregnancy (stillbirth rate increased after 41 weeks gestation) (12,20,21). Extremes of fetal size at either end of the spectrum of growth i.e. small for gestational age (SGA), fetal growth restriction (FGR) or large for gestational age (LGA), are also associated with increased stillbirth risk, equating to an odds ratio of 3 for SGA and 3.5 for LGA (22).

1.1.1 Pregnancy Outcomes Following Stillbirth

Previous stillbirth is an independent risk factor for subsequent stillbirth, with an overall increase in risk of 2-10 times that of women with a previous live birth (23). This risk is elevated in women with a second trimester loss (20-28 weeks), compared to that after 28 weeks gestation (hazard ratio of 10.3 vs 2.5) (24). Compared to women with a previous live birth, the adjusted odds ratio of a further loss in a subsequent pregnancy is 4.77 for women who have already suffered a stillbirth (25). This likelihood of further fetal death is increased if the original stillbirth was attributed to a placental cause (26). Maternal complications are also higher in a subsequent pregnancy following stillbirth, with increased rates of pre-eclampsia (PET) and gestational diabetes seen (26–28).

When compared to women with livebirths, women with a stillbirth in their first pregnancy have an increased risk of both ischaemic placental disease (OR 1.6, 95% CI 1.2-2.1) and preterm birth in a subsequent pregnancy (OR 2.25 – 4.2) (29,30), suggesting a potential, and therefore recognisable, recurrent aetiology. In addition, there are increased rates of composite adverse fetal outcomes, including placental abruption (OR 9.4), preterm delivery (OR 2.25 – 2.8) and fetal growth restriction (FGR) (OR 2.7) and SGA (6%) (28,31,32). In women with possible placental vascular disease (PET, abruption and FGR) associated with their previous stillbirth, there is an increased rate of poor neonatal outcomes in their subsequent pregnancies, as compared to women with other causes of prior stillbirth such as fetal anomalies (OR 2.1, CI 1.2-3.8) (30). The increased rate of poor neonatal outcomes in these women with pregnancy related vascular disease is likely to be as a result of increased preterm birth (OR 1.95, CI 1.06-3.61) and FGR (OR 3.65, CI 1.09-12.19) (30), both of which are associated with poor neonatal outcomes. Overall, women with a previous stillbirth are at increased risk of further adverse pregnancy outcomes compared to women with a live birth; this risk is particularly increased in women with placental disease associated with their previous loss.

1.1.2 Stillbirth and the Development of Maternal Cardiac Disease

In addition to the increased rates of pregnancy complications seen in subsequent pregnancies following a stillbirth, there is also increasing evidence to suggest that women who have a

stillbirth are at greater risk of cardiovascular (CV) disease in later life (33–35). As a risk factor for stillbirth, FGR is also associated with an increased risk of maternal development of ischaemic heart disease (IHD). For example, mothers to babies born in the lowest 20% birthweight for gestational age have a hazard ratio of 1.9 (95% CI 1.5-2.4) for developing IHD (as defined by IHD-related hospital admission or death) compared to mothers of babies in the upper 80% of birthweight. Similarly, preterm delivery and maternal PET have also been shown to increase maternal risk of developing IHD (HR 1.8 [95% CI 1·3–2·5] and HR 2·0 [95% CI 1·5–2·5], respectively). When these factors are considered in combination, the effect is additive, with mothers to babies with FGR pregnancies complicated by preterm delivery and PET having a risk of IHD related hospital admission or death of up to 7 times that for healthy pregnancies (95% CI 3·3–14·5) (36).

Stillbirth itself is a risk factor for maternal development of CV disease, with having a stillbirth giving an increased odds ratio (OR) of cardiac-related hospital admission of 1.5 (95% CI 1.2-1.8) compared to women who had a live birth (33). A similar increase in OR of CV disease is seen in recurrent early pregnancy loss (34). In a different study population (USA), Parker et al. (35) showed that a history of stillbirth is associated with coronary heart disease (CHD) (OR 1.27 (95% CI, 1.07-1.51)) compared to women with no history of stillbirth. Similarly, miscarriage was also associated with an increase in CHD, with a single miscarriage giving an OR of 1.19 (95% CI, 1.08-1.32), and ≥2 miscarriages an OR of 1.18 (95% CI, 1.04-1.34) (35). Overall, pregnancy loss, irrespective of gestation, is associated with increased risk of maternal development of CV disease.

Maternal hypertensive diseases of pregnancy are related to both FGR and stillbirth. Ray et al. performed a study comparing women with PET, gestational hypertension, placental abruption, and placental infarction (the composite of which they termed maternal placental syndrome) to women with uncomplicated pregnancies (37). In their Canadian cohort there was an increased hazard ratio (HR) of CV disease in women with maternal placental syndrome (HR 2.0, 95% CI 1.7-2.2), and when FGR was taken into account specifically, this rose to 3.1 (95% CI 2.2-4.5). Moreover, in cases of stillbirth, this risk increased further again, this time to HR of 4.4 (95% CI 2.4-7.9) (37). Overall, these studies suggest that adverse pregnancy outcome, in particular stillbirth, is associated with maternal CV disease, although it is unclear as to whether the adverse pregnancy outcome occurs as a result of underlying maternal CV disease or if CV disease develops as a consequence of adverse pregnancy outcome. It is likely, given the association with PET and maternal vascular disorders of pregnancy, that both of these components play a role, with women predisposed to CV disease experiencing increased hypertensive disorders of pregnancy as a result of the inability of their compromised cardiovascular system to cope with the additional health burden of pregnancy, and as a consequence increasing their overall lifetime risk of CV disease further.

1.2 Pregnancy, Stillbirth and the Placenta

In considering the link between maternal disease and adverse fetal outcomes, the human placenta holds considerable importance. The formation of a healthy placenta requires significant adaptation of both the maternal vascular and immune systems. In containing both maternal and paternal DNA, the placenta is a site of exposure to foreign DNA and, as such, is a potential trigger for the maternal immune system. Successful placental formation and implantation requires regulation of the maternal immune system at the interface between maternal and fetal surfaces.

Placental development begins with implantation of the blastocyst (embryo at days 5-7 of development) into the uterine endometrium, with consequent differentiation and decidualisation of the uterine endometrium. The blastocyst consists of trophoblast (the outer cell layer which forms the early placenta) and an inner cell mass (which develops into the embryo proper), both of which contain paternal DNA and pose a challenge for the maternal immune system. In both stillbirth and SLE, there is evidence of failure in the early stages of placental development, either through abnormal uterine vascular modifications or aberrant maternal immune adaptations. As a consequence, abnormal placentation develops, which eventually results in restrictions in fetal growth and, if this is undetected, stillbirth (see section 1.2.3).

1.2.1 The Placenta and the Maternal Vascular System

Uterine vascular remodelling is a key part of placentation. In healthy human pregnancy, uterine spiral arteries are responsible for blood flow through the decidua and proximal myometrium (38). Over the course of early pregnancy these spiral arteries are invaded by extravillous trophoblast, transforming them from their non-pregnant state of low flow, high pressure vessels into large high flow and low pressure conduits that are optimal for maternal blood delivery to the developing fetus. Abnormalities in this vascular remodelling are associated with maternal hypertensive diseases of pregnancy including PET (39–41). In linking placental pathology and circulating vascular factors, it has been demonstrated that women with massive perivillous fibrin deposition (excessive fibrinoid tissue deposition in the intervillous space), presumed to be as a result of maternal fetal rejection and failed maternal immune adaptation to pregnancy (42)) have lower levels of circulating placental-derived vascular factors, placental growth factor (PlGF) and higher levels of soluble Fms-like tyrosine kinase-1 (sFlt-1)/soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR-1) than controls (43). Screening for women at high risk of pregnancy complications now includes uterine artery Doppler flow velocimetry and measurement of circulating placental-derived vascular factors. These factors, including PlGF and sFLT-1 (44), have also been demonstrated to be useful in differentiating between active SLE (lupus nephritis) and PET, as further described in section 1.5 (page 36).

1.2.2 Placental Development and the Maternal Immune System

In addition to the vascular changes of pregnancy, the maternal immune system is faced with a unique challenge as pregnancy requires its adaptation and modulation to allow the development

of the placenta and fetus, both of which contain foreign paternal DNA, and therefore foreign antigens. The presence of paternal DNA within the developing fetus and placenta means that the fetus has the characteristics of a semi-allogeneic transplant. As a consequence, maintenance of a successful pregnancy requires the maternal immune system to undergo careful modulation in response to this non-self "invasion". In this regard, profound changes both locally within the uterus and systemically within the expectant mother are necessary.

In adapting to the presence of fetal tissue, the local immune response within the decidua is tightly controlled (45). In early pregnancy, the decidual cell population is predominantly formed of decidual natural killer (dNK) cells (large granular lymphocytes) and only a low percentage of T cells (10-15%) (46). At the blastocyst implantation site, the balance of Th1/Th2 cells within the decidua shifts in favour of anti-inflammatory Th2 production, whilst implantation is facilitated by dNK production of regulatory factors such as interleukin(IL)-8, IL-10, transforming growth factor ß (TGF-ß) and vascular endothelial growth factor (VEGF) (47–49), which lead to an anti-inflammatory decidual environment. Concurrently, Treg cells modulate the cytotoxic activities of the dNK population, promoting an anti-inflammatory environment (50,51).

Implantation of the blastocyst into the endometrium has characteristics of an inflammatory process, associated with invasion of foreign tissue. In order to counteract this inflammatory process in the first trimester of a healthy pregnancy, the levels of Treg cells increase, both in the maternal peripheral blood and the decidua, with Treg levels peaking in the second trimester before falling back to pre-pregnancy levels in the third trimester (52–54). The inflammatory reactions of the placenta are further controlled by the primary decidual antigen-presenting cells (APC), macrophages. These decidual macrophages produce a variety of factors such as IL-10, and stimulate indoleamine 2,3 dioxygenase ((IDO) which suppresses T cell proliferation) (55), VEGF and soluble Fms-like tyrosine kinase-1 (sFlt-1) (56), thus shifting the balance away from a pro-inflammatory response towards an anti-inflammatory one. Forming part of the cycle of immunosuppression within the decidua, cytotoxic T-Lymphocyte Antigen 4 (CTLA 4) on lysosomes in the Treg cells responds to antigen exposure by moving to the cell surface. Once at the cell surface, CTLA-4 acts on APCs to produce more IDO, thus further suppressing T cell proliferation (57,58), with a resultant effect of promoting the anti-inflammatory cycle.

Simultaneously to the cell-mediated pathways outlined above, the complement system also becomes activated by the presence of foreign antigens. Forming part of both the innate and adaptive immune systems (59), complement acts in various ways by mediating inflammation (C3a, C5a), opsonising pathogens, clearing immune complexes (C3b) and cell lysis (membrane attack complex, (MAC)). The complement system is activated via three different pathways, all of which relate to recognition of foreign antigen. This can be in the form of the classical pathway in which antigen-antibody complex is the stimulus, the lectin pathway in which lectin binds to glycan or glycoprotein components of pathogens or as a direct result of stimulation from the cell surface of the foreign antigen/pathogen (alternative pathway). These three pathways produce three difference routes to the formation of the two common pathways in which complement

factor 3 (C3) is converted into the active C3b, which is capable of forming complement factor 5 (C5) convertase, and the final common pathway of formation of the active component C5b9 membrane attack complex (Figure 1). In the context of pregnancy, altered complement activation has been described in the placenta, as it is here that exposure to paternal DNA occurs. In normal pregnancy, excessive complement activity and deposition is suppressed in the trophoblast membranes by sensitive control mechanisms (60), involving membrane cofactor protein (MCP), decay accelerating factor (DAF) and CD59. All three act synergistically to regulate complement activation at different points in the complement cascade (60–62). As a result of these tightly controlled mechanisms, complement is part of the process of vascular remodelling seen in the early stages of placental development (63), with complement deposition seen within the villi and decidua of normal placentas (64,65). Systemically during pregnancy, maternal serum levels of split complement components C3, C4 and total haemolytic complement (CH50) rise by up to 50% compared to non-pregnant levels, however in PET this increase is even more significant with an increase in the active C5b9 seen in PET pregnancies (66,67). The differences seen in the complement activity of pregnancy between healthy and pathological pregnancy suggest that the increase seen in healthy pregnancy is to clear the products of increased cellular breakdown and consequent immune complex formation seen in pregnancy, whereas in PET there is an imbalance between formation and clearance of these breakdown products, leading to a further increase in complement activation (68).

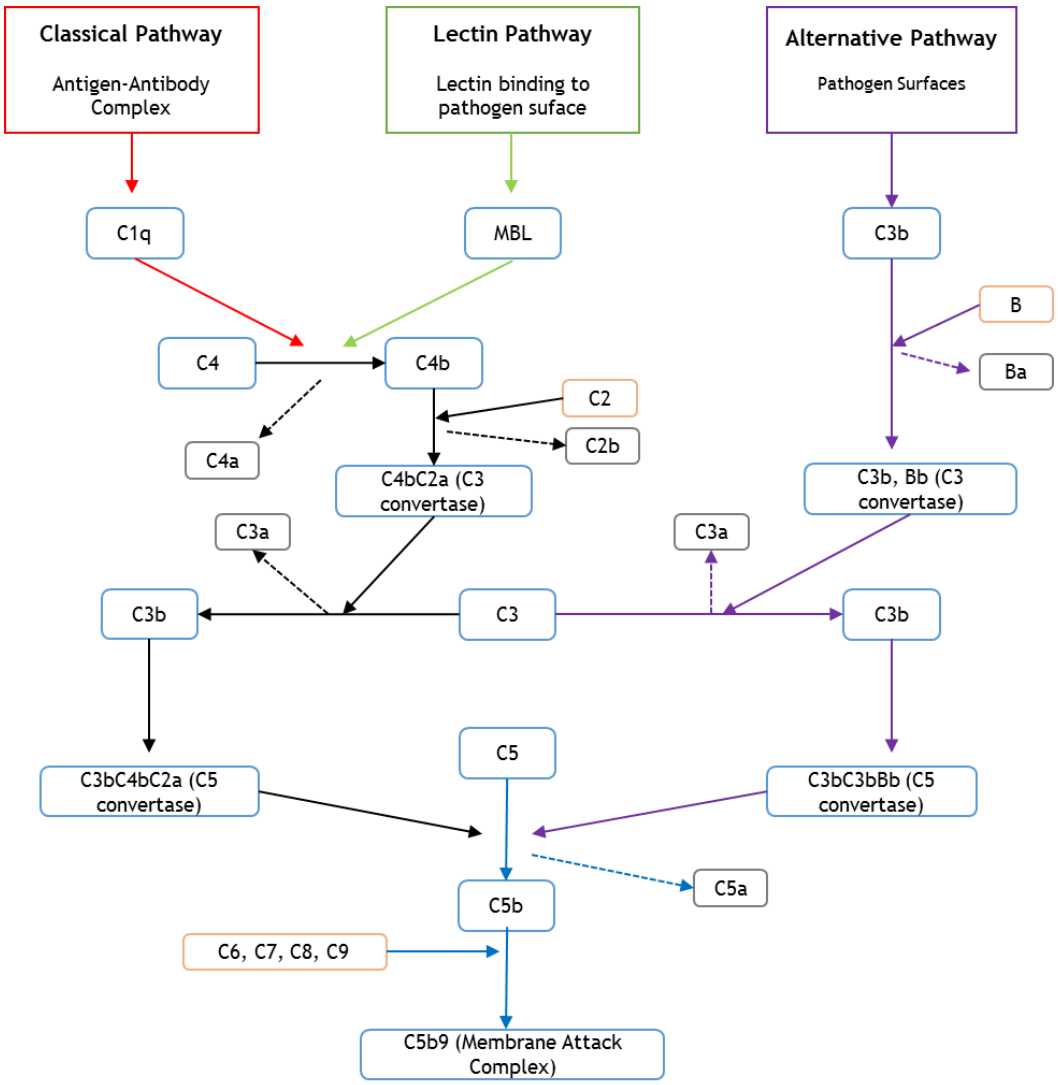


Figure 1- The Complement Cascade. C3 activation pathways lead to 2 common pathways both of which result in the formation of a C3 convertase to cleave C3 into the active component C3b to form C5 convertase, which in turn cleaves C5 to C5b. This then results in formation of the active C5b9 membrane attack complex.

1.2.3 The Placenta and Stillbirth

As previously described, the development of a healthy placenta requires significant changes to the maternal immune and vascular systems. Failure in these processes can result in abnormal placental development, which in turn is associated with poor pregnancy outcomes (69,70), arguably the most catastrophic being stillbirth.

As outlined above, the causes of stillbirth and its subsequent pregnancy implications vary depending on the underlying aetiology. Causes of stillbirth are multifactorial but up to 47% have their basis in placental disease/dysfunction (12,71). Higher rates of both structural (single umbilical artery, velamentous cord insertion) and abnormal placental histology are seen in stillbirth (compared to livebirths), including increased rates of villous immaturity, signs of inflammation, vascular irregularities and increased fibrin deposition (72–74). As part of the overall picture of abnormal placental function, placental size has also been associated with

adverse fetal outcomes. For example, a small placenta (as demonstrated by low weight at delivery compared to gestation-matched livebirths) is associated with stillbirth and neonatal death (75–77). Likewise, as previously suggested, women with a prior pregnancy loss also show an increased incidence of obstetric complications in their subsequent pregnancies, more frequently when their lost pregnancy was previously associated with placental disease (26); this suggests a predisposition to abnormal placentation which can recur. However, the underlying causes of abnormal placentation and consequent adverse outcomes are unclear, with both vascular and immune dysfunction implicated.

1.3 Systemic Lupus Erythematosus

A significant risk for stillbirth and adverse fetal outcome is the presence of maternal disease. Of these, maternal SLE is a key example. SLE is a multi-system autoimmune disease, with an incidence of 4.9 per 100,000 person years. It has a 6:1 female preponderance and primarily affects women of reproductive age, the peak incidence of SLE occurring between 15-40 years old (78,79). SLE has multiple clinical manifestations and variable severity. Therefore, diagnosis is not always straightforward. To enable consistency in diagnosis in the context of research, the classification criteria, as defined by the American College of Rheumatology (ACR), Table 1, is used with ≥4 criteria required for a diagnosis of SLE (80). Under the same criteria, Lupus-like Disease (LLD) is also defined, when ≥3 recognised criteria are met.

Given the variable clinical manifestations and severity of symptomology, the diagnosis of SLE is often protracted, with a mean time from first symptoms to formal diagnosis of 26.4 months (81). As such, there is significant overlap between SLE, LLD and other autoimmune connective tissue diseases (CTDs), including undifferentiated connective tissue disease (UCTD). UCTD is a condition in which in which there are signs, symptoms, and serological evidence of an autoimmune CTD but these fail to meet the diagnostic criteria for a single specific CTD (82,83). Approximately 30% of patients with UCTD will eventually be diagnosed with a specific CTD, most commonly SLE (up to 95%) (82).

There are several key immunological autoantibodies seen in SLE although, as outlined in Table 1, none of these are diagnostic. The most characteristic autoantibodies seen in SLE are antinuclear antibodies (ANA). As a diagnostic test, this is non-specific and an isolated positive result has a low positive predictive value (PPV) in the general population (PPV of SLE in the absence of symptoms 2.1%) (84,85). However, ANA are found in up to 100% of women with SLE, and in this context their presence has a high level of sensitivity at 93% (84). The most specific autoantibodies in SLE are those against double stranded DNA (dsDNA), which are found in 60% of patients (86). Antibodies to nuclear antigens are also common, including anti-Smith (anti-Sm) and anti-ribonucleoprotein (anti-RNP) antibodies. Approximately 13-40% of patients with SLE express an antiphospholipid antibody (aPL), compared to only 1-5% of the healthy population (87–89). Together, these autoantibodies (ANA, dsDNA, anti-Sm, anti-RNP and aPL) may be present for a significant period of time prior to clinical manifestations of the disease, with a lag time of up

to 9 years (90). The typical pattern of development of these autoantibodies changes with time, with ANA rising first, followed by anti-Sm and then anti-RNP prior to diagnosis; the cause for this chronological emergence is unknown (90). Of these autoantibodies, particularly levels of dsDNA rise with increasing disease activity, and fall with effective treatment (91,92).

In considering the expression of specific aPL in SLE, the prevalence of a positive Lupus anticoagulant (LAC) is 25-38%, and anticardiolipin (aCL) in 13-31% (86). Of those SLE patients with aPL, only 10-15% will have concurrent Antiphospholipid Syndrome (APS) (93–96). APS is an autoimmune disease characterized by the presence of elevated levels of aPL. It is associated with thrombotic events, fetal loss (miscarriage or stillbirth) and pregnancy morbidity (Table 2) (88). The diagnostic criteria for a diagnosis of APS are outlined in Table 2, with a diagnosis of APS made if there is one clinical and one laboratory criterion present. Whilst SLE itself confers a risk for the development of APS, this relationship is bidirectional, with 13-18% of patients with APS subsequently developing SLE (97), potentially indicating underlying common pathological processes.

Table 1 - The 1997 American College of Rheumatology Revised Criteria for Classification of Systemic Lupus Erythematosus. (80)

Criteria	Definition
1.Malar Rash	Fixed erythema, flat or raised, over the malar eminences (tending to spare the nasolabial folds)
2.Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3.Photosensitivity	Skin rash as a result of unusual reaction to sunlight by patient history or physician observation
4.Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5.Nonerosive Arthritis	Involving ≥ 2 peripheral joints, characterized by tenderness, swelling, or effusion
6.Pleuritis or pericarditis	**a.** Pleuritis-convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion **b** .Pericarditis-documented by ECG or rub or evidence of pericardial effusion
7.Renal disorder	**a.** Persistent proteinuria ≥ 0.5 grams per day or ≥ 3+ if quantitation not performed **b.** Cellular casts-may be red cell, haemoglobin, granular, tubular, or mixed
8.Neurologic disorder	**a.** Seizures-in the absence of offending drugs or known metabolic derangements; e.g., uraemia, ketoacidosis, or electrolyte imbalance **b.** Psychosis-in the absence of offending drugs or known metabolic derangements, e.g., uraemia, ketoacidosis, or electrolyte imbalance
9.Haematologic disorder	**a.** Haemolytic anemia-with reticulocytosis **b.** Leukopaenia-less than 4,000/mm^3 total ≥ 2 or more occasions **c.** Lymphopaenia-less than 1 ,500/mm^3 ≥ 2 or more occasions **d.** Thrombocytopaenia-less than 100,000/mm^3 in the absence of offending drugs
10.Immunologic disorder	**a.** Anti-DNA: antibody to native DNA in abnormal titre **b.** Anti-Sm: presence of antibody to Sm nuclear antigen **c.** Positive finding of antiphospholipid antibodies based on **1)** an abnormal serum level of IgG or IgM anticardiolipin antibodies **2)** A positive test result for lupus anticoagulant using a standard method **3)** A false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.
11. Antinuclear antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with “drug-induced lupus” syndrome

Table 2 - Revised Sapporo Criteria for the Classification of Antiphospholipid Syndrome (APS) (98)**.**

	Criteria	Definition
Clinical Criteria	**1.Vascular thrombosis**	≥1 episode of arterial, venous or small vessel thrombosis in any tissue or organ. Must be confirmed by objective validated criteria, if histopathological thrombosis should be present without significant vessel wall inflammation.
	2.Pregnancy related morbidity	≥1 unexplained deaths of morphologically normal fetus ≥10th week gestation
		≥1 premature birth ≤34 weeks gestation due to Pre-Eclampsia or severe Pre-Eclampsia Recognised features of placental insufficiency
		≥3 consecutive spontaneous miscarriages ≤10 weeks gestation in the absence of maternal structural, hormonal or chromosomal abnormalities or paternal chromosomal abnormalities.
Laboratory Criteria (2 occasions, ≥12 weeks apart)	**Lupus Anticoagulant (LAC)**	Presence of Lupus Anticoagulant in serum
	Anticardiolipin Antibody (aCL)	IgG +/- IgM in serum or plasma, present in medium or high titre (> 40 GPL or MPL, or > the 99th percentile)
	Anti Β2 Glycoprotein-1	IgG +/- IgM in serum or plasma (in titre >the 99th percentile)

1.3.1 Immunology of SLE

In a healthy immune system, the presence of foreign antigens triggers an immune response in which these antigens are recognised, taken up and processed by APCs. Consequently, APCs express the antigen on their cell surface in association with major histocompatibility (MHC) class II molecules or toll like receptors (TLRs) (Figure 2). Presentation of antigen via APCs to B cell receptors (BCR) or B cell TLR stimulates polyclonal activation of B cells and subsequent antibody production. Simultaneously, TLR activation causes stimulation of interferon regulatory factor 5 (IRF5) which mediates type one interferon-α (IFN-α) production, which subsequently causes differentiation of monocytes into dendritic cells (the main APC for presentation to T cells) which are then capable of further antigen presentation via MHC II (Figure 2). T cell receptor (TCR) binding to cluster of differentiation 4 (CD4) causes cytokine release initiating further T cell differentiation and upregulation. T cell subtypes Th1, Th2, Th17 and regulatory T cells (Tregs) are differentiated as a result of locally secreted cytokines (99). In broader terms, Th1 and Th17 cells are pro-inflammatory, Th2 cells are anti-inflammatory, and Treg cells play a dominant role in preventing the development of autoantibodies and suppressing the responses of other immune

cells (51). Concurrently to the T and B cell pathways outlined above, the presence of foreign antigens on APCs also activates the complement system (see section 1.2.2, page 24).

In SLE, the development of autoantibodies to self-DNA or RNA occurs as a result of abnormal recognition of self-DNA or RNA as foreign. This recognition of self-DNA or RNA is presumed to occur as a result of defective clearance of apoptotic cells, leading to an accumulation of these self-DNA/RNA breakdown products in the circulation (100,101). These become modified and are subsequently recognised as foreign. As a consequence, these self-DNA/RNA fragments are processed by APC, leading to the creation of antibodies to self-DNA or RNA, and the development of pathological DNA and/or RNA autoantibodies due to polyclonal activation of B cells (101). Similarly, TLRs recognise the nucleic acids in these immune complexes and consequently promote pathogenic autoantibody production, further propagating the cycle of immune system activation (102) (Figure 2). Individuals with SLE demonstrate an exaggerated B and T cell response culminating in an imbalance of Th cell production, most notably a decrease in Th2 and elevation of Th1 and Th17 cells (103). In SLE, this shift in the Th1:Th2 balance, combined with low levels of Treg cells, results in the promotion of a systemic pro-inflammatory state (104). During periods of active disease, there are high levels of IFN-α, which correspond to elevated anti-dsDNA titres (91). This is likely to be the resultant effect of IFN-α acting as a mediator of both the innate and adaptive immune responses, causing increased differentiation of monocytes into dendritic cells, which can then act as APC, and propagation of further T cell responses (Figure 2) (91,105). When considering the role of the complement system in SLE, the immune complexes observed initiate the complement cascade, consuming circulating C3 and C4, levels of which are subsequently reduced (106). As a result of this hypocomplementaemia, a reduction of immune complex clearance is forthcoming, with consequent elevation in immune complex deposition in tissues (107), further acting as stimuli for the complement cascade.

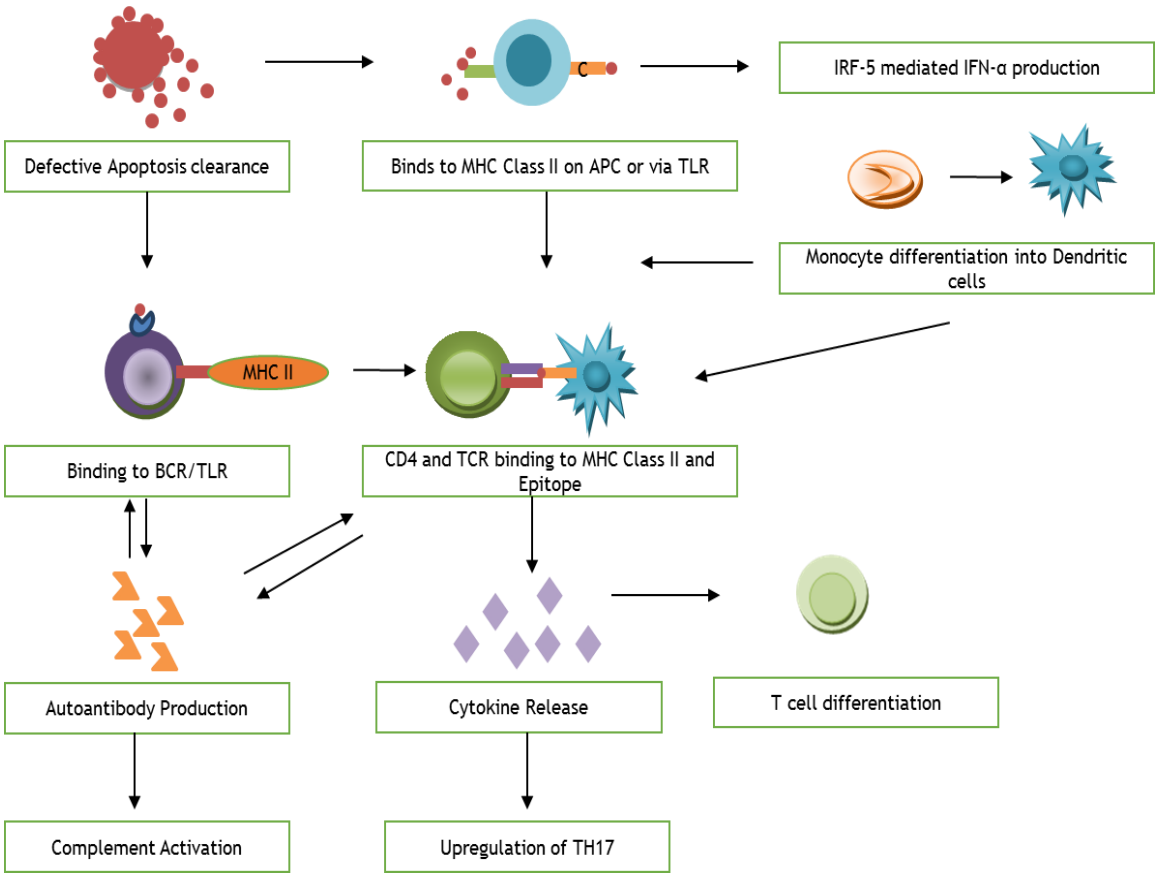


Figure 2 - Pathogenesis of SLE. Defective clearance of apoptotic cell debris results in i) presentation of self-DNA or RNA to antigen presenting cells (APC) via MHC II or toll like receptors (TLRs). ii) B cell receptor (BCR) or B cell TLR binding stimulates autoantibody production, formation of antibody-antigen complexes which then cause activation of the complement cascade. TLR activation also stimulates Interferon Regulatory Factor 5 (IRF5) mediated IFN-α production resulting in differentiation of monocytes into dendritic cells capable of further antigen presentation via MHC II. CD4/TCR binding causes cytokine release initiating further T cell differentiation and upregulation of Th17 cells and further presentation of antigen to B cells, stimulating excessive autoantibody production as defined in SLE.

1.3.2 Pathology of SLE

The underlying aetiology of SLE, consequent on aberrant recognition of self-DNA or RNA, has been described as a multiple hit model, based on genetic, environmental, hormonal and immune factors (105,108–113). In individuals genetically predisposed to SLE, a series of genetic variants has been described, the majority based on MHC haplotypes (114,115). In combination with environmental factors, such as smoking or viral infection, these immune transformations (116,117) are considered a backdrop to delayed apoptotic cell clearance and systemic circulating excess of cellular nuclear constituents. Elevations in cell-derived microparticles (MPs), seen in SLE, certainly concur with this suggestion (118–120). Containing both RNA and DNA, MPs are formed as a result of cell surface shedding, forming vesicles of 200-1000nm in size, and resembling the parent cells from which they were derived (121). These phenomena are hypothesised to be linked to the failed apoptotic clearance of these MPs, coupled with recognition of their RNA/DNA content acting as the primary stimulus for autoantibody production

(122,123) in SLE. The systemic nature of this inflammatory stimulus accounts for the varied clinical phenotype.

1.3.3 SLE and Cardiovascular Disease

As outlined above, SLE is a multisystem, multifactorial disease with varied clinical manifestations. The nature of SLE, and its end-organ effects, means that affected people have increased mortality compared to healthy individuals, with an elevated standardised mortality ratio of 2.2-2.4, predominantly related to infection or CV disease (124). In women with SLE, CV risk is particularly exaggerated, with the condition considered an independent risk factor for endothelial dysfunction and development of atherosclerosis (125). To emphasise this relationship, studies have shown an up to 50 times risk of development of CV disease in SLE compared to that seen in in healthy women (126–128). A risk factor for CV disease is atherosclerosis, which has been shown to be twice as prevalent in patients with SLE than the healthy population (129). A combination of low Treg and elevated Th17 levels has been demonstrated in SLE patients with atherosclerosis, suggesting that an abnormal immune response may likely contribute to the inflammatory process, required as part of the atherosclerotic plaque formation, as a cornerstone of CV disease (104).

As a precursor of atherosclerosis, research has looked at the impact of SLE on the vascular endothelium and has defined both increased levels of vascular stiffness and higher rates of hypertension, compared with age-matched healthy controls (130,131). More specifically, these endothelial changes have been linked to disease flare, with worsening activity concurrent with raised vascular stiffness (132,133). Endothelial cells cover the lumen of the maternal vasculature and secrete cytokines in response to stimuli. They are capable of promoting vascular inflammatory responses and are the site of atherosclerosis formation, alongside playing a role in angiogenesis and haemostasis (134,135). Endothelial cell dysfunction is associated with inflammation, hyperlipidaemia, hypertension and prolonged glucocorticoid use, all of which are prevalent in SLE (136).

As outlined in section 1.3.2, MPs are related to their parent cell. As such, endothelial MPs (EMPs), derived from the vascular endothelium, are reported to be markers of endothelial dysfunction (137). EMPs are elevated in both hypertension and acute coronary syndromes (138), although whether this is as part of a pathological process or a result of one, remains unclear. In SLE, studies have demonstrated a reduction in circulating EMPs with treatment regimens and reduced disease activity (139), supporting again their role as a marker of underlying disease activity (139) and a potential indicator of ongoing vascular dysfunction within this patient group.

1.4 SLE and Pregnancy

As an autoimmune disease, SLE is associated with abnormal systemic immune processes. Within this setting, pregnancy affords a unique challenge for maternal systems when superimposed with

SLE. Ultimately, maternal and fetal outcomes are negatively impacted in SLE-affected pregnancies, as detailed below.

1.4.1 Maternal Outcomes

1.4.1.1 Impact of Pregnancy on SLE

The impact of pregnancy on maternal health in SLE is hard to quantify, with conflicting reports on the flare rate of disease activity with gestation. Typically, disease flare is defined as worsening or new clinical symptoms with concurrent serological abnormalities (140). Meta-analysis of the published literature performed by Smyth et al. (2) in 2010 looked at 37 studies of pregnancy outcomes in patients with SLE, with numbers of study participants ranging from 11 to 203. Within these studies, the documentation of aPL was variable, with only 32 reports including status. Variations in the definition of disease flare (either by use of ACR criteria or investigator defined criteria) and differing patient cohorts across the studies, means that reported SLE flare rates in pregnancy range from 13-68% (2), dependent on criteria used and patient group studied.

In assessing the impact of pregnancy on SLE, there is evidence linking disease activity at conception with maternal and fetal outcomes, with the presence of active disease at time of conception associated with increased rates of adverse fetal and maternal outcomes, and conversely quiescent disease with positive outcomes (141,142). Considered one of the most severe complications of SLE, lupus nephritis is further associated with poor pregnancy outcomes, with active lupus nephritis, within 6 months preceding conception, strongly associated with increased rates of pre-eclampsia (30% vs 8% in non-nephritis patients) (2,143,144). Pre-pregnancy lupus nephritis is also associated with a 15% recurrence rate during pregnancy and within the first postnatal year (145). Similarly, non-renal flares are increased in this cohort, with reported rates of 25.6% (2). As a marker of end organ damage in SLE, the presence of lupus nephritis is associated with a more severe disease phenotype, and as a consequence with worse pregnancy outcomes for both mother and fetus.

1.4.1.2 Impact of SLE on Pregnancy Outcome

As described previously, SLE is an independent risk factor for hypertension with 25% prevalence at the time of conception, rising to 44% in women with a history of lupus nephritis (146,147). This increased background hypertension manifests in pregnancy as a 25% prevalence of PET (148), a 3 to 5-fold increased rate compared to healthy pregnant women (149). Given variability in flare rates in SLE in pregnancy, it is difficult to quantify the impact of pregnancy on SLE activity *per se*. Moreover, there are multiple other factors, such as medication changes and disease activity at time of conception, that could play a role on the impact of pregnancy on maternal disease (2,3).

1.4.2 Fetal Outcomes

SLE is associated with an increased risk of poor fetal outcomes, including a fetal loss rate of up to 5 times that of healthy women (2,3), and premature birth (below 37 completed weeks gestation) of up to 39.4% (2,150). Within this loss rate is an overall spontaneous miscarriage rate of 16% and incidence of stillbirth of 3.6% (2). The reasons for this high incidence of fetal demise is poorly understood, but it is postulated that placental dysfunction plays a pivotal role (2,151–153). SLE-affected pregnancies also show increased rates of FGR (12.7%), with babies born smaller for any matched gestational age. Broadly considered as placental in origin, reported rates of FGR in women with SLE are variable according to the classification used (12.7% in meta-analysis, with range 8.8-16.7%), either customised growth charts or population based charts (with a lower rates of FGR detection) (154–156). In considering poor fetal outcomes associated with SLE, the increased rates of FGR and rates of preterm birth mean that babies born to affected mothers also face an additional burden of neonatal morbidity and mortality due to their prematurity and small size (2,143,157–159).

Similarly to the increased rates of adverse maternal outcomes defined in women with lupus nephritis, there are also increased rates of adverse fetal outcomes associated with the condition, especially in those with active maternal lupus nephritis in the 6 months preceding conception. Here, the impact may manifest as a preterm labour rate of 60% and FGR rate of 46.7%, compared to rates of 24% and 20% respectively in equivalent women with stable disease (160). However, a more recent study failed to replicate these differences, despite persistence of maternal complications (161), perhaps explained by the high incidence of fetal complications in the "control" cohort (women with stable SLE) and small study numbers (39 women and 55 pregnancies included).

In the absence of lupus nephritis, women with SLE and dual positivity for aCL and LAC are at greatest risk of adverse pregnancy outcomes (162). In these cases, placental infarction, secondary to decidual vasculopathy and thrombosis, is the most frequently described pathology (163,164).

1.5 The Pathology of Adverse Outcomes in SLE Pregnancies

As described above, the increased rates of adverse pregnancy outcome associated with SLE are thought to have their origins in either aberrant placentation or as a result of maternal systemic influences proffered by SLE, or possibly a combination of the two.

1.5.1 Placental Causes of Poor Outcomes in SLE Pregnancies

Although routinely implicated in the adverse outcomes of SLE-affected pregnancies, poor placental function is seldom defined and explained. Nevertheless, several key differences are identified in placentas of women with SLE, which may offer part explanation of placental involvement.

1.5.1.1 Placental Structure

Studies of pathological findings in placentas from pregnancies complicated by SLE have shown evidence of an excess of thrombotic events (such as decidual vasculopathy) impacting on placental reserve (164), increased perivillous fibrin deposition (164–166) showing evidence of placenta lesions/repair and excessive syncytiotrophoblast basement membrane thickening and syncytial knots (167), providing evidence of abnormal placental cell turnover and stress (168,169). These findings are evident but more rarely seen in placentas from live births (166) which supports the theory that placental anomalies underpin at least some of the pregnancy complications associated with SLE (170–172).

Given the rarity of SLE as a disease, the majority of the work identifying placental abnormalities has been performed on small cohort studies with less than 50 participants (164,172). Differing disease activity scales have also been used, making associations with disease activity infeasible. This task is further hampered by APS. Like SLE, APS is also associated with higher rates of placental anomalies, with aPL positivity similarly marked by increased placental fibrin deposition (164). Therefore, inclusion of APS in any patient set makes conclusions on the origins of placental pathologies in SLE more challenging and less conclusive.

1.5.1.2 Placental Derived Hormones

The placenta produces both pro- and anti-angiogenic factors throughout its life-time. These include anti-angiogenic protein sFlt-1 and pro-angiogenic VEGF and PlGF and the balance between these factors plays a significant role in placental development. In maternal pregnancy-related vascular disease, such as PET, the relationship between these pro-and anti-angiogenic factors is disrupted although it is uncertain whether this is causal or reflective of underlying vascular disturbance (173,174).

In a healthy pregnancy, levels of PlGF rise until approximately 30 weeks gestation, at which point they gradually fall, returning to values at term equivalent to those previously seen at 20 weeks of pregnancy (175,176). Levels of sFlt-1 increase longitudinally throughout pregnancy to maximal levels at term, with the ratio of sFlt-1 to PlGF increasing to reflect the fall in PlGF and rise in sFlt-1 (177). VEGF levels undergo minimal longitudinal changes in pregnancy, with lower levels reported in women with hypertension and PET throughout pregnancy as compared to healthy uncompromised controls. (173,175,176).

Acting by binding to pro-angiogenic VGEF and PlGF, sFlt-1 is higher in SLE than in normal pregnancies, supporting the theory that abnormal placentation underlies both pathogenesis and adverse pregnancy outcomes in this group (178). In non-SLE pregnancies, an imbalance between these factors has also been demonstrated clinically, using a ratio of sFlt-1/PlGF, with an increase negatively associated with birthweight and placental dysfunction (179,180) and predictive of the development of PET. The relationship between these vascular factors and stillbirth has yet to be investigated fully.

1.5.1.3 Placental Vasculature

Another presumed cause of adverse fetal and maternal outcomes is a failure of the maternal vasculature to adapt to the demands of pregnancy. This is considered to have a negative impact on utero-placental perfusion (181). Certainly, defective remodelling of the maternal spiral arteries is noted in pregnancies complicated by early-onset PET and FGR (182,183). In SLE, it is thought that vascular irregularities associated with active disease and immune system imbalances at the time of spiral artery transformation could act synergistically to cause abnormal placentation and subsequent pregnancy complications (152).

1.5.2 Systemic Causes of Adverse Outcome in SLE Pregnancies

From a systemic immune perspective, the hypocomplementaemia seen in active SLE is associated with both early and late pregnancy failure, as well as higher rates of neonatal death (184). Preterm birth in women with SLE has also been shown to link to low circulating maternal levels of C3 and C4 in the first and second trimester respectively, and spontaneous premature rupture of membranes (before 34 weeks) (185,186). As a breakdown product of C4, C4d binds to cell surfaces and causes a localised inflammatory response. Deposition of C4d at the syncytiotrophoblast membrane is found in 50-62% of placentas of women with SLE, where it is associated with growth restriction and preterm labour. In the same study, C4d deposition was rarely seen in placentas of healthy uncomplicated pregnancies (187). In women without SLE, similar deposition of C4d in the placenta is associated with placental abnormalities. It has been found in 88% of cases of villitis of unknown aetiology (VUE), pre-eclampsia (50% expression), and FGR (21%) (187–190), proposing a more generalised pathophysiological phenomenon which is most notable in inflammatory disorders of the placenta.

Evidence from women with aPL further implicates complement as a pathological feature of poor placental development. Studies from women with APL have shown increased levels of C4d and C3b deposition in the placenta compared to healthy uncomplicated pregnancies, suggesting an abnormal immune interaction at the maternal fetal interface (189). In an extension of the theory that successful placental development relies on control of placental complement mechanisms, murine studies have shown complete fetal loss in mice deficient in complement inhibitory protein CRRY, which can be rescued by mating with C3 deficient males (191). The CRRY protein is absent in humans, in whom C3 regulation is carried out by DAF and MCP (192). Both DAF and MCP control C3 regulation by inhibition of the C3 and C5 convertase, reducing the formation of MAC, the final stage of the complement cascade (Figure 1) (192). In humans, levels of DAF and MCP are elevated in organs of patients with SLE and complement related damage (such as lupus nephritis), providing further evidence of the importance of complement and tissue damage (193–195).

Overall, excess complement activation at the maternal fetal interface in SLE causes excess deposition of complement in the placental tissues, resulting in consumption of systemic complement and hypocomplementaemia (196). This excess complement deposition in the placenta is linked with adverse fetal outcomes and therefore potentially plays a pathological

role. This has not been looked at in SLE in the absence of APS or in cases of pregnancy following stillbirth.

1.5.3 Screening for Placental Complications in SLE

As women with SLE are recognised as high risk for pregnancy complications, a method for screening these women for placenta-related anomalies or vascular irregularities in pregnancy may be beneficial in adapting care to optimise pregnancy outcomes in this high-risk group. Current approaches to screening pregnant women with SLE are broadly aimed at assessing uterine vascular adaptation (by assessment of uterine artery blood flow by Doppler ultrasound) and placental function by measurement of placental-derived hormones. The benefits and limitations of both approaches are discussed below.

1.5.3.1 Uterine Artery Doppler

Utilising ultrasound based flow Doppler the pattern of flow within the uterine artery can be assessed giving quantitative values. The flow of blood within the uterine arteries, as measured by uterine artery Doppler (UtAD) is believed to represent downstream resistance of the spiral arteries (197). Although this is an oversimplification of the pathophysiology (198,199) uterine artery blood flow patterns have been used to screen for underlying placental disease (200). Within a high risk population, abnormal UtAD (high resistance or associated with a diastolic notch, see Chapter 2 - Methods, page 45) is associated with increased rates of stillbirth, FGR and pre-eclampsia (201). However there is also variation in UtAD with ethnicity, BMI and maternal disease state (including SLE). In a review of published data by Papageorghiou et al, in 2004, including 15 studies and over 22,000 pregnancies, the likelihood ratio (LR) of developing PET in the presence of a normal UtAD was 0.5, compared to a LR of 6 in the presence of abnormal UtAD (200). Similarly, both UtAD resistance index (RI) and pulsatility index (PI) (measures of uterine artery blood flow) have been shown to be higher in SLE pregnancies with adverse outcomes compared to those unaffected (202,203). As a confounder for the use of UtAD in isolation as a predictor of pregnancy outcome in SLE, Gheita et al. demonstrated SLE activity to be higher in pregnancies with adverse outcomes, meaning UtAD may also reflect maternal disease state as a measure of poor vascular compliance (202).

Supporting the theory that maternal disease impacts on UtAD, a study looking at autoantibody levels in pregnant women with symptoms of connective tissue disease (CTD) reported higher rates of abnormal uterine artery waveform and elevated UtAD resistance in women with autoantibodies (ANA, dsDNA, anti-Sm, ani-RNP, aCL) present compared to those with only CTD symptoms alone (204). This suggests a potential link between antibody status and UtAD, implying a possible role for autoantibodies in aberrant placental development, maternal vascular adaptation and defective spiral artery transformation.

1.5.3.2 Placental-Derived Hormones

In an attempt to improve the diagnosis and prediction of the development of PET, measurements of maternal circulating PlGF and sFlt-1 have been utilised. Low levels of PlGF (<12pg/ml, between 19 and 35 weeks' gestation) are associated with the development of PET (205). Moreover, acting reciprocally, PlGF and sFlt-1 have been considered in combination, with the ratio between PlGF and sFLT-1 used to predict development of PET: a ratio >38 was found to be predictive of development of PET (in the absence of maternal SLE) (44,206). However, SLE complicates the diagnosis of PET as the renal disease seen in SLE manifests similarly, and has a clinically similar picture, with proteinuria and maternal hypertension as its hallmarks. Consequently, in the presence of SLE with worsening hypertension and proteinuria, a PlGF/sFLT-1 ratio of >85 has been suggested to indicate PET over the alternative diagnosis, lupus nephritis, although this has yet to demonstrated in larger scale studies (207).

1.6 SLE and Stillbirth

Of all complications seen in pregnancies affected by SLE, stillbirth is arguably the most catastrophic, with a prevalence of 3.6% (2); this is significantly higher compared to that of the UK population rate (0.4%) (208). Within the population of pregnant women with SLE, higher disease activity at conception is associated with highest risk, although the extent of this has yet to be quantified (159,160).

1.6.1 Stillbirth, SLE and Abnormal Immunology

Underlying immune dysfunction is thought to underpin some of the pregnancy pathology observed with SLE. As previously described in section 1.2.2, in a normal uncomplicated pregnancy, levels of Treg cells increase in both maternal blood and decidua, with a peak in the second trimester (54). This pattern is considered, but certainly not proven, to aid maternal immune regulation and tolerance of the antigenically foreign fetus. In recurrent miscarriage, an inverse relationship has been found between Treg cells and Th17 cells in the blood and decidua of affected women (209), with elevated Th17 cells seen at time of pregnancy loss, compared to women with either spontaneous miscarriage or undertaking elective termination of pregnancy (210,211). It is this shift from immunosuppression to pro-inflammatory state that is thought to underpin the abnormal placentation and adverse pregnancy outcomes anticipated or associated with SLE. Arguably still poorly explained in SLE-affected pregnancies, the putative relationship between inflammation and stillbirth or late miscarriage in the absence of SLE or overt autoimmune disease, has never been explored.

In such an undertaking, APS and aPL may be undoubted confounders, given their relationship with SLE. In APS, there are increased levels of complement split products in the circulation, suggesting activation (212). Administration of aPL results in more frequent fetal loss and decreased birthweights in murine studies which suggests that it may be aPL that is critical in determining adverse pregnancy outcome (213). The conventional treatment for APS in human pregnancy is low molecular weight heparin (LMWH), which has been shown to inhibit

complement in murine models of APS (214), thus again emphasising the importance of complement. To date, the activation and deposition of complement in the placenta has not been characterised in stillbirths without APS.

Another marker of disease activity in SLE are MPs, and as previously described they are implicated in its pathogenesis (118–120). In pregnancy, endothelial and platelet MPs have been shown to increase with gestation in the maternal circulation (215). In the context of healthy, low risk pregnancies, research into MPs and pregnancy outcomes has not demonstrated a link between altered MP levels and prediction of adverse pregnancy outcomes (216). MPs have, however, been implicated in PET with elevated levels of EMPS seen in women with PET or recurrent miscarriage (in the first trimester) when compared to healthy women (217–221). When considering SLE and MPs, elevations in MPs outside of pregnancy are well described in women with SLE (222). However, there is little literature on the pattern of MPs in pregnancies complicated by SLE, and none specifically on stillbirth or late pregnancy loss.

1.7 Stillbirth and subsequent development of Autoimmune Disease

In view of the established relationship between stillbirth and SLE, and the known time lag between antibody positivity and formal diagnosis (82,90), studies have linked stillbirth and subsequent SLE development. Of the two largest retrospective studies (both of Danish origin), the largest (of 51,732 women) demonstrated an increased prevalence of SLE in women following a stillbirth, with relative risk of onset in the subsequent 4 years of 6.3 (95% CI 3.38-10.68) (4,5). The timescale for development of SLE in these Danish studies fits with the time lag displayed between antibody positivity and disease diagnosis in other cohorts (4,5,90,223). In the study of Jørgensen et al., further pregnancy complications were included, such as hyperemesis, miscarriage (spontaneous and missed) and hypertensive diseases. Whilst the presence of any of these pregnancy pathologies resulted in an increased relative risk of SLE, this was most striking again with stillbirth (overall RR 3.03, CI 1.88-4.58, RR at 0-4 years following stillbirth 6.33, CI 3.38-10.68), again suggesting a link between pregnancy loss and SLE. These studies were based on Danish hospital records and looked at pregnancy outcomes and hospital admissions related to SLE, comparing women with livebirth, miscarriage and stillbirth and their subsequent risk of SLE (as defined by hospital admission). This, therefore, only addresses those women with disease serious enough to require inpatient treatment, and as such is a severe disease phenotype. This link between stillbirth and subsequent development of SLE nevertheless remains contentious, with some studies showing increased rates prior to diagnosis and others increased losses after disease confirmation (224–227). It should be noted that these studies were predominantly retrospective in design, with small numbers of participants, varying definitions of adverse outcomes and differing control groups.

1.8 Summary

SLE is associated with elevated rates of pregnancy loss, including miscarriage and stillbirth. This is presumed to be due to abnormal placental pathology and poor maternal vascular adaptation to

pregnancy, although decisive evidence linking these observations is weak. The underlying pathological processes of these placental and vascular complications are thought to be related to inflammation, with more severe maternal disease (and therefore the greatest degree of inflammation) associated with worsening maternal and fetal outcomes, linking maternal immune dysfunction with adverse pregnancies. However, in the case of SLE and stillbirth, this relationship may be bidirectional, with SLE being a risk factor for stillbirth and stillbirth being a risk factor for the development of subsequent autoimmune disease, including SLE and future cardiovascular disease, inside or outside of a subsequent pregnancy.

1.9 Hypothesis

The overarching hypothesis being investigated here is that otherwise unexplained antepartum stillbirth is related to an autoimmune-mediated process similar to that underlying adverse pregnancy outcomes in SLE. Specifically, it is hypothesised that (i) women with a previous fetal loss have an increased risk of developing SLE and (ii) these women display similarities in vascular and immune dysfunction (in a subsequent pregnancy) to that identifiable in SLE-affected patients and pregnancies.

1.10 Objectives

1. Examine the relationship between pregnancy outcomes and subsequent development of autoimmune disease in the UK population using a national primary care database focussing on stillbirth and adverse pregnancy outcomes compared to uncomplicated livebirth pregnancies (Chapter 3).
2. Look at the impact of maternal disease phenotype and autoantibody status on maternal and fetal outcomes in pregnancies affected by SLE or prior stillbirth, comparing both to healthy uncomplicated control pregnancies (Chapter 4).
3. Assess *in-utero* differences in placental size in pregnancies affected by SLE or prior stillbirth, compared to healthy pregnancies. Furthermore, to assess complement deposition in the placenta of stillbirth with differing histological findings, to define previously unrecognised maternal autoimmunity (Chapter 4)
4. Characterise the longitudinal vascular changes in pregnancies affected by SLE or prior stillbirth, compared to healthy pregnancies and relating these defined changes to circulating maternal anti- and pro-angiogenic factors in the second and third trimesters of their pregnancies (Chapters 5).
5. Assess differences in maternal immune system response in pregnancy, as defined by Th17, Treg and MP levels, in pregnancies affected by SLE or prior stillbirth, as compared to healthy controls (Chapter 6)

2. Methods

The following methods were used in 3 pregnant cohorts of women: women with SLE (SLE Pregnant, SP), healthy pregnant women (Normal Pregnant, NP) and women with a previous stillbirth (Previous Loss, PL). SLE was defined by the American College of Rheumatologists (ACR) criteria (Appendix 3 - American College of Rheumatologists Criteria for diagnosis of SLE, page 170) (228). All pregnancies were singleton pregnancies, with no congenital abnormalities. Unless otherwise specified, measurements were performed longitudinally throughout pregnancy at 17, 22, 28 and 36 weeks' gestation (+/- 2 weeks) and postnatally (6-20 weeks) in all 3 cohorts. An additional first trimester measurement was taken in both SP and NP cohorts at 10 (+/- 2 weeks) gestation.

2.1 Recruitment

The following flow diagram shows how many women were recruited, including numbers of those lost to follow up or excluded; summarising the final cohorts (Figure 3).

2.2 Ethical Approval

Ethics for the epidemiological aspect of this study was granted on 01/07/2014 (protocol 14_106R). Ethical approval for the placental part of the study was granted on 08/09/2014 (14/LO/1352). The ethics for performing vascular and immunological measurements on previous stillbirth women was approved on 15/09/2014 (14/NW/1149). The full patient information and consent forms can be found in Appendix 1 – Patient Information (Undiagnosed Lupus-Like Autoimmunity and unexplained stillbirth), page 165 and Appendix 2 – Consent Form (Undiagnosed Lupus-Like Autoimmunity and unexplained stillbirth), page number 168. Pre-existing ethics covered this testing on the SLE cohorts (13/NW/0158).

Figure 3- Recruitment to the studies in this thesis; showing n numbers included in each group

2.3 Ultrasound-Based Parameters

Ultrasound measurements were performed by a Royal College of Obstetricians and Gynaecologists (RCOG) accredited sonographer using a Voluson E6 (GE Healthcare, Hatfield, UK) ultrasound machine, using a 3D, 4-8Hz curvilinear probe (all measurements were taken in 2D). *In utero* placental biometry was measured at 17 and 23 weeks gestation (+/- 2 weeks). Fetal and maternal Dopplers were performed at every visit from 17 weeks gestation (+/- 2 weeks).

2.3.1 *In utero* Placental Biometry Assessment

Maximal placental thickness (t) and maximal placental length (l) were recorded using 2D ultrasound (Figure 4). Length to thickness ratio (l:t) was calculated with an abnormal value considered to be a l:t under 2 in line with published research (229).

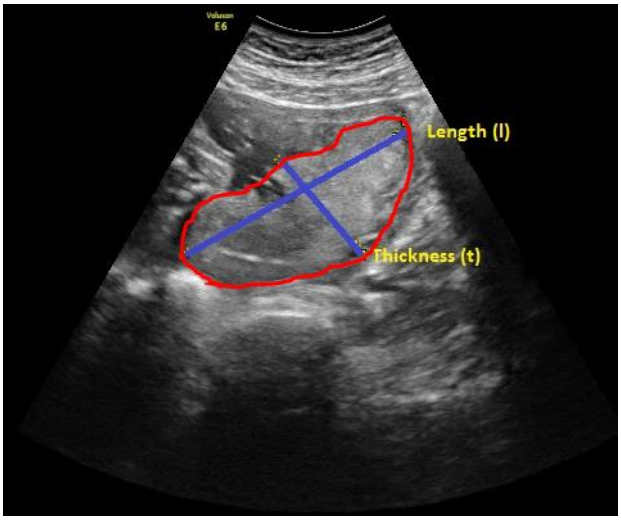


Figure 4- Measurement of In-Utero Placental Biometry using 2D ultrasound. Maximal placental thickness (t) and maximal placental length (l) indicated. Placenta delineated in red.

2.3.2 Uterine Artery Doppler Assessment

The uterine artery was identified as it crossed the external iliac artery using colour Doppler, with measurements taken on the medial aspect of the intersection (Figure 5). Resistance index (RI) and pulsatility index (PI) were calculated automatically by the Voluson E6 (Figure 6A). The presence of a diastolic notch, was considered abnormal (197,230), as shown in Figure 6B and C.

2.3.3 Umbilical Artery Doppler (UAD) Assessment

A free loop of umbilical artery was used to measure UAD. Both resistance index and pulsatility index (RI and PI respectively) were recorded alongside the presence of end diastolic flow. An RI or PI over the 95th percentile was considered abnormal, as was the absence or reversal of end diastolic flow (231). Where abnormalities were detected, both umbilical arteries were measured and the mean value determined and used.

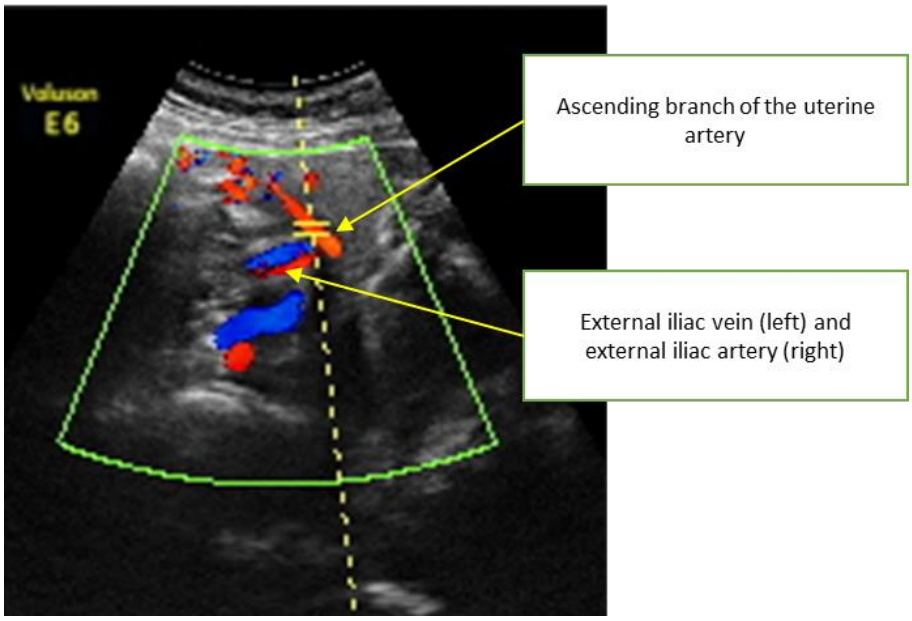


Figure 5 - Uterine artery identification. Demonstration of the identification of the uterine artery at the point of crossing the external iliac artery and vein. The gate shows the point at which the measurement was taken (medial aspect).

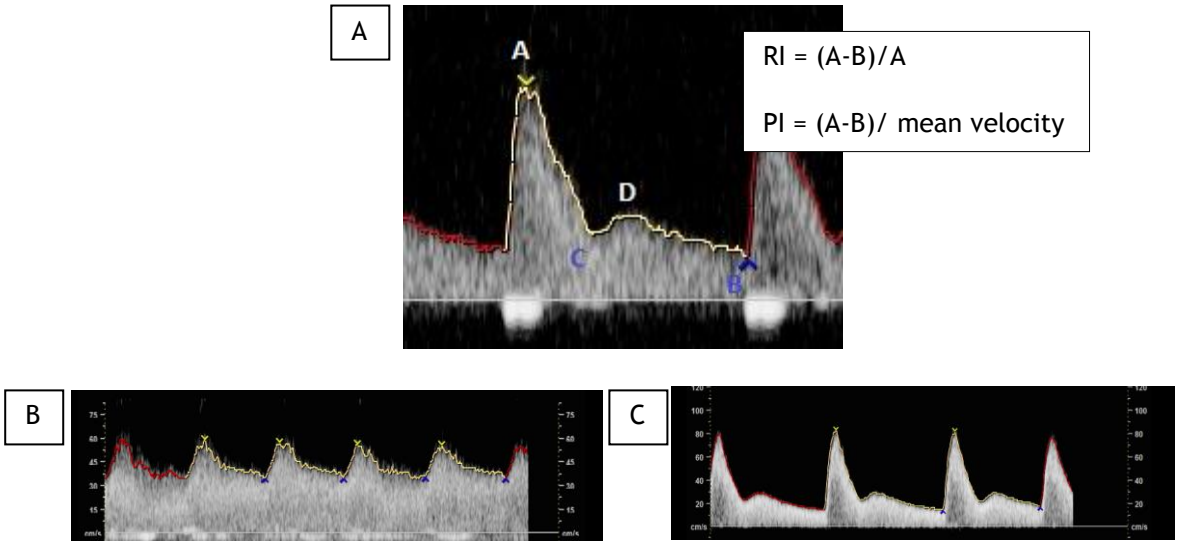


Figure 6 - Uterine Artery waveform. A) Demonstration of Pulsatility Index (PI) and Resistance Index (RI) calculation, with A=systolic peak, B=end of diastole, C=start of diastole and D=maximum diastole. B) Normal uterine artery waveform, and C) High resistance uterine artery with the presence of a diastolic notch.

2.4 Maternal Systemic Vascular Assessment

2.4.1 Brachial blood pressure

Brachial blood pressure (BP) was recorded at each visit, in line with national recommendations for antenatal care. Until 01/09/2014, this was performed using a manual sphygmomanometer over the brachial artery. Thereafter, an automated blood pressure monitor was used to reduce observer bias (Alere Microlife, Alere Ltd, Massachusetts, USA). This automated method has

previously been validated for use in pregnancy (232,233). Measurement of brachial BP was performed with the patient seated after 5 minutes rest.

2.4.2 Oscillometric Assessment

An Arteriograph (TensioMed, Budapest, Hungary) was used to measure pulse wave velocity (PWV), central aortic systolic blood pressure (SBPAo), central pulse pressure (PPAo) and both brachial and aortic augmentation index (Aix B and Aix A, respectively). This was performed in left lateral position with the cuff placed on the right arm over the brachial artery. The measured distance from suprasternal notch to symphysis pubis (Jugular-Symphysial height, JugSy) was used as a proxy for aortic length (234). The Arteriograph cuff senses early and late systolic waves (P1 and P2) and the diastolic wave (P3) (Figure 7). Changing levels of pressure within the brachial artery were detected and recorded by the software (TensioMed Arteriograph, version 1.10.1.11): Calculations as shown in Table 3 (235,236).

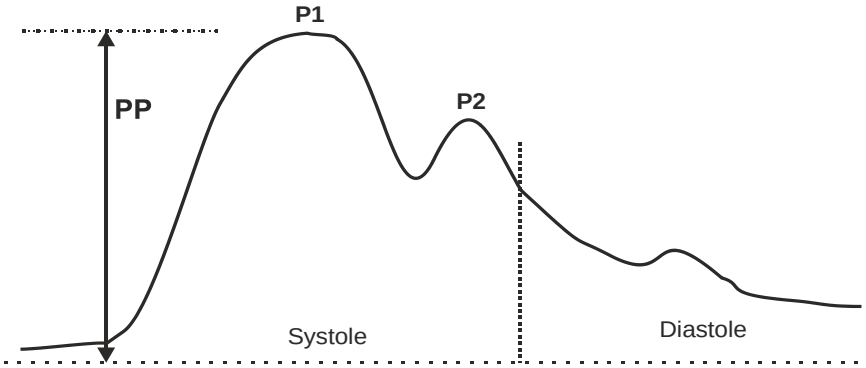


Figure 7- Arteriograph Waveform. Waveform produced by the Arteriograph demonstrating the double waveform of systole as a result of the forward early systolic wave (P1) and late systolic reflected wave (P2). P1 = early systolic wave. PP = pulse pressure.

Table 3 - Calculation of Arteriograph-based parameters.

Parameter	Measurement	Calculation
Augmentation Index (Aix)	Small arterial tone / vasodilatation	(P2-P1)/PP*100
Pulse Wave Velocity (PWV)	Aortic Stiffness	JugSy/P2-P1

Aix = Augmentation index, PWV= pulse wave velocity, P1= early systolic wave, P2 = late systolic reflected wave, PP = pulse pressure, JugSy = length from suprasternal notch to symphysis pubis.

2.4.3 Photoplethysmography Assessment

Photoplethysmography measures light absorption by blood and tissue; changing blood volumes with the cardiac cycle gives the pulsatile pattern of flow (Figure 8) on which measurements are calculated. The two peaks identified represent systole and diastole, respectively. Stiffness index (SI) represents large artery stiffness, whilst reflection index, RI, represents small arterial tone.

For patient assessments, a Pulse Trace PCA2 (Care Fusion, Chatham, UK) was used in the left lateral position, with probe attached to the patients left index finger. Photoplethysmography

analysis was performed 3 times at 2-5 minute intervals, with a mean value recorded. Measurements were customised to maternal parameters (i.e. age, height, weight and smoking status).

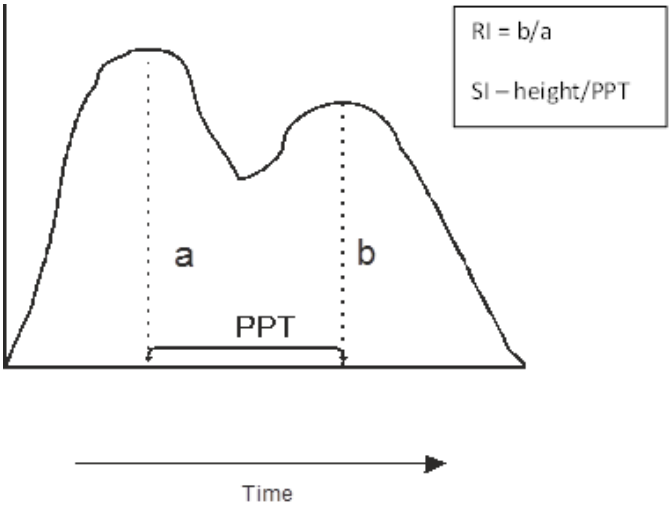


Figure 8 - Photoplethysmography (Digital Volume Pulse) Waveform. a = Systole, b= diastole, RI=reflection index, SI=stiffness index, and PPT=peak to peak time.

2.5 Blood collection

Blood samples were taken peripherally with a vacutainer system (Fisher scientific, Loughborough, UK). Each vacutainer was centrifuged at 4^{o}c for 10 minutes at 3000rpm. Serum and citrated samples then had plasma removed and further spun (3500rpm, 10 minutes, 4^{o}c). Aliquot samples were stored at -80^{o}c before further use. All samples were processed within 2 hours of blood being drawn.

2.6 Serum Vascular Markers

To further examine the systemic vascular changes seen in pregnancy, maternal circulating vascular markers were measured at 28 and 36 weeks (+/- 2 weeks) using the methods outlined below.

2.6.1 Placental Growth Factor

Maternal plasma levels of Placental Growth factor (PlGF) were measured in 250µl EDTA plasma samples using Triage PlGF test with Triage MeterPro point of care analyser (Alere, Massachusetts, USA). The Triage test is a single use flourescence immunoassay containing mouse monoclonal antibodies against PlGF. The maternal sample reacts with the antibody conjugates at the sample port and then is pulled through the device, using capillary action, into the measurement zone in which the antibody conjugate complexes are captured. This is then analysed in the MeterPro analyser which detects flourescence with a measureable range of 12-3000picograms/millilitre (pg/ml), with a coefficient of variation (CV) of 12.8-13.2%. Table 4 shows the classification of PlGF results used when considering PlGF as a categorical variable; this was based on published

research and manufacturers recommendations (237,238). PlFG was analysed both as a categorical variable and a continuous variable.

Table 4 - Classification of Placental Growth Factor (PlGF) results (10,11)

Result (pg/ml)	Classification	Interpretation
<12	Highly Abnormal	Suggestive of severe placental dysfunction
≥12, <100	Abnormal	Suggestive of placental dysfunction
≥100	Normal	Normal, unlikely to have placental dysfunction

2.6.2 Vascular Endothelial Growth Factor

Maternal circulating soluble Vascular Endothelial Growth Factor Receptor 1 (VEGF R1)/ Soluble Fms-like tyrosine kinase-1 (sFlt-1) was measured in maternal EDTA plasma samples using R&D Systems Quantikine ELISA (Bio-Techne, Minnesota, USA). Assay range was 31.2-2000pg/ml, with a sensitivity of 13.3 pg/ml, intra-assay CV of 2.6-3.8%, and an inter-assay CV 5.5-9.8% (239).

The ELISA was performed in-line with manufacturer's instructions. Maternal EDTA plasma samples were diluted 4:1 with Calibrator Diluent RD6-10. Assay diluent (100µl) was added to each well. Wells were then covered and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (500rpm). Four wash cycles were performed using 400µl Wash Buffer. 200µl of Human VEGF R1 Conjugate was added to each well. Wells were then covered and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (500rpm), followed by a further 4 washes. 200µl of Substrate Solution was added and wells then covered and incubated for 30 minutes at room temperature in the dark. 50µl of Stop Solution was subsequently added and optical density of each well measured with microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) at 450nm with the wavelength correction set at 570nm. The 450nm reading was subtracted from the 570nm reading for final analysis.

2.7 Maternal Circulating Immunology Markers

In order to assess maternal immune response to pregnancy, two T cell subsets and microparticles were measured. Circulating maternal T regulatory (Treg) cells and T helper 17 cells (Th17) were measured at 17 week's gestation (+/- 2 weeks). Microparticles were measured longitudinally in citrated plasma samples at 22, 28 and 36 week's gestation (+/- 2 weeks).

2.7.1 Peripheral Blood Mononuclear Cell isolation

Blood was collected as previously outlined in section 2.5. Unless otherwise stated all centrifuge cycles were performed at 23^{o}c at 100g. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood (EDTA sample) using a Ficoll gradient preparation with Histopaque 1077 (Sigma-Aldrich, Dorset, UK). 6ml of whole blood was pipetted onto 6ml of Histopaque and then centrifuged at 23^{o}c for 30minutes (400g). The PBMC layer was removed and added to 12ml Phosphate Buffered Saline (PBS); followed by a further centrifugation cycle of 10 minutes. On

resuspension a cell count was performed and sample diluted with PBS to a final concentration of $1x10^6$ cells/ml. These cells were then used in the T cell measurements outlined below.

2.7.2 Treg Cell Measurement

A commercially available kit (BioLegend, San Diego, California) was used for the identification of CD4CD25FoxP^3 triple positive Treg cells, following the protocol outlined below. Unless otherwise stated, all centrifuge cycles were performed for 5 minutes at 23^{o}c at 250g.

Isolated PBMCs ($1x10^6$ cells as per section 2.7.1) were added to 0.5ml Staining Buffer (Biolegend, Catalogue 420201) and 20µl of CD4PE-Cy5/CD25PE to the test sample only. Samples were incubated at room temperature in the dark for 20 minutes followed by centrifugation. The supernatant was then removed and cell pellet resuspended in 1ml Fixation/Permeabilization Buffer. Following incubation at room temperature in the dark for 20 minutes, a further centrifugation was then performed, prior to supernatant removal. 1ml of staining buffer was then added to the cell pellet followed by a 16 hour incubation at 2-8^{0}c (in the dark). Cells were then centrifuged before removal of the supernatant and addition of 1ml Permeabilization buffer. This was followed by a second permeabilization wash after cells were incubated at room temperature in the dark for 15 minutes. Cells then underwent a further centrifuge cycle prior to supernatant removal and addition of 100µl Permeabilization Buffer. 5µl Fox P3 was subsequently added to the test samples and 5µl isotype control to the control samples. Cells were further incubated for 30 minutes in the dark (room temperature) before addition of 1ml staining buffer. Following centrifugation, the supernatant was removed and 1ml staining buffer added. A final centrifuge cycle was undertaken and the cell pellet resuspended in 0.5ml of staining buffer. Cells were analysed by FACS (as outlined in section 2.7.4).

2.7.3 Th17

Th17 cell assessments in PBMCs were performed using a commercial kit (R+D Systems, Abingdon, UK), either directly on fresh isolates or after stimulation, following optimisation as below. Unless otherwise stated, all centrifuge cycles were performed for 5 minutes at 5^{0}c at 250g.

2.7.3.1 Optimisation of Th17 cell analysis

Several protocols for stimulation of Th17 cells were initially trialled. Initially, 5µl Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich, Dorset, UK) (50ng/ml), 5µl Ionomycin calcium salt (Sigma-Aldrich, Dorset, UK) (200ng/ml), 5µl interleukin 23 (IL-23) (R+D Systems, Abingdon, UK) and 5µl Lipopolyssaccharide (LPS) (Sigma-Aldrich, Dorset, UK) (1mg/ml) were used in an overnight stimulation. However, this failed to improve the detection rate of Th17 cells. Similarly, the stop phase of the Th17 stimulation protocol was optimised. Initial experiments utilised 5µl Monensin Sodium (5mmol), 5µl PMA (50ng/ml) and 5µl Ionomycin calcium salt (200ng/ml) for either 2 or 4 hours, but produced no difference in Th17 cell detection, compared with 2 hours of 5µl Monensin Sodium alone. The full details of this optimisation can be found in

Appendix 7, page 191. Therefore final protocols for Th17 cell stimulation and assessment were defined as below:

2.7.3.2 Stimulated Th17 Protocol

Isolated PBMCs ($1x10^6$ cells/well) were placed in a 12 well culture plate (Scientific laboratory Supplies Ltd, Nottingham, UK). 2 wells per patient - control and test. 490µl media (RPMI60, 10% heat inactivated FBS, 2mmol Glutamin, 200u/ml Penicillin, 100 µl/ml streptomycin (G1146 Sigma)) was then added alongside 5µl PMA (50ng/ml) and 5µl Ionomycin calcium salt. Samples were then incubated at 37^0c for 16 hours before addition of 5µl Monensin Sodium (5 mmol). A further 2 hour incubation was undertaken followed by two wash phases with 1ml PBS. Cells were then stained as per the unstimulated protocol (section 2.7.3.3), with the exception of the incubation phase after antibody addition, which was reduced to a 1 hour incubation.

2.7.3.3 Unstimulated Th17 protocol

Isolated PBMCs ($1x10^6$ cells) were added to 0.5ml Fixation/Permeabilization Buffer and incubated in the dark for 30 minutes at 2-8^0c before centrifuging. The supernatant was then removed and 100µl Permeabilization/Wash buffer added. 10µl of each antibody, or corresponding isotype control, were added prior to 16 hours of incubation at 2-8^0c (in the dark). An additional 1ml Permeabilization / Wash buffer was then added prior to centrifugation. Following this, the supernatant was removed and cell pellets resuspended in 500µl PBS prior to FACS analysis (section 2.7.4).

2.7.4 Fluorescence Activated Cell Sorting (FACS) analysis

All T cell measurements and analysis were performed using a BD Accuri C6 Flow Cytometer (BD Biosciences, San Diego, California). Colour correction was applied, in line with manufacturer's instructions as per Table 5, and confirmed using individual antibody conjugates.

Prior to analysis of isolated PBMCs, a lymphocyte cloud was identified as shown in Figure 9. A gate was applied and subsequent analysis performed on this subgroup. In the case of Treg cells, a positive gate for CD4 and CD25 was first established, as shown in Figure 10A. This population was subsequently investigated for FoxP3 positivity (Figure 10B), with triple positive cells considered as regulatory T cells. For recognition of Th17 cells, dual CD3 and IL-17 positivity was employed (Figure 11A-B).

Table 5 – Fluorescence Compensation Corrections used for all Fluorescence Activated Cell Sorting (FACS) analysis

Channel	Correction Factor			
	FL1	FL2	FL3	FL4
FL1	*	+6.9	*	*
FL2	+8.5	*	*	*
FL3	+1.6	+20.9	*	+0.8
FL4	*	+0.7	*	*

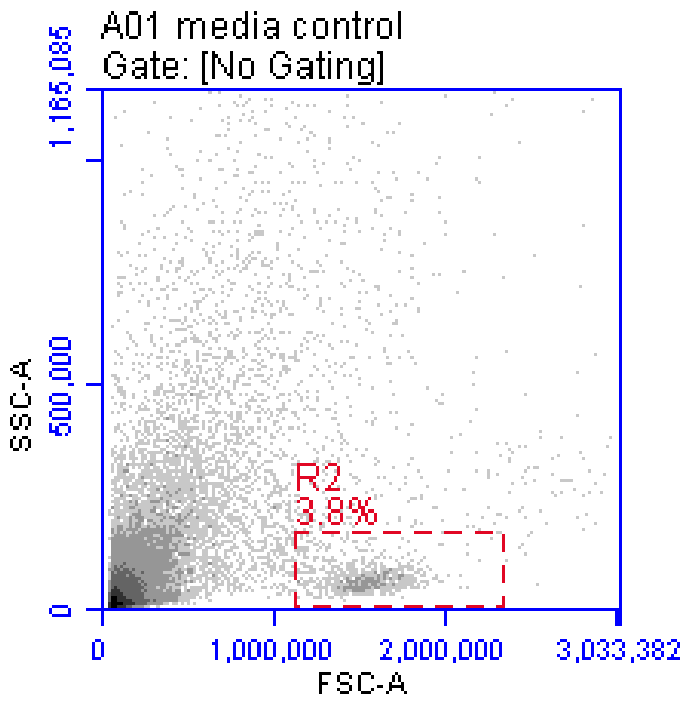


Figure 9 - Flow Cytometric Identification of lymphocytes and gate setting. R2 = T and B cell cloud of distinct FSC (forward side scatter) and SSC (side to side scatter) profile.

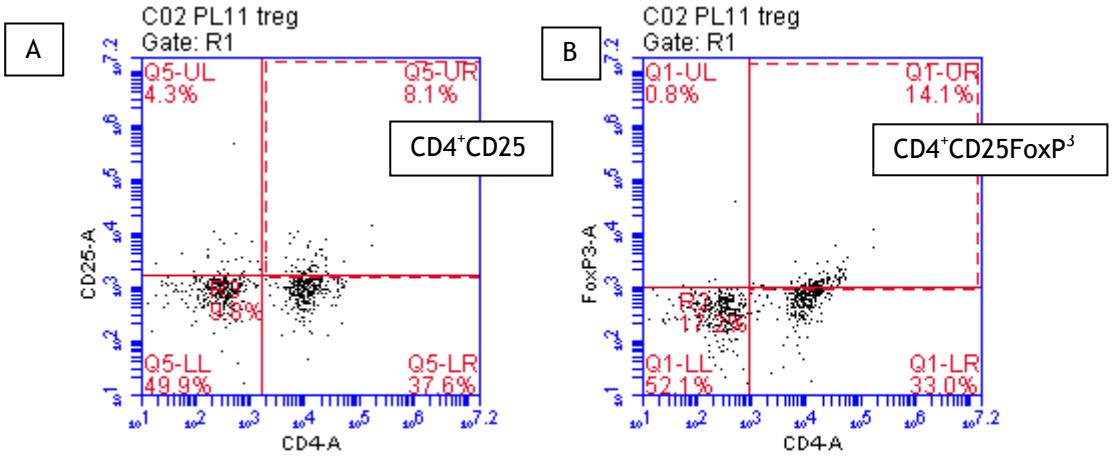


Figure 10 -Flow Cytometric Identification of CD4CD25FoxP3Treg cells. A) Highlighted quadrant defines CD4+CD25+ cells B) Subsequent gating on this CD4+CD25+ population allowed FoxP3 positivity to be superimposed, giving triple CD4CD25FoxP3 positive cell identification.

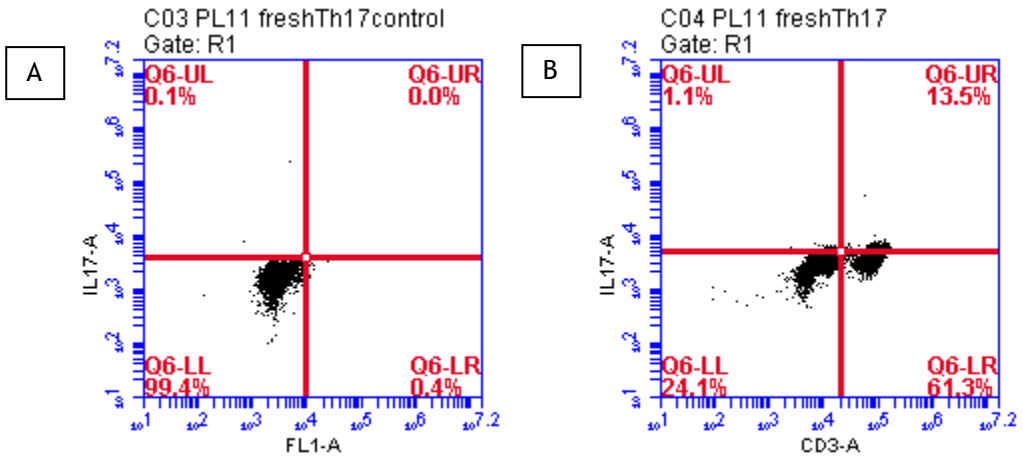


Figure 11 - Flow Cytometric Identification of Th17 cells. Isotype control for Th17 demonstrating gate setting using CD3 and IL17 as markers. A) Control and B) Sample.

2.7.5 Microparticles

Initial experiments used annexin V (Becton Dickinson), as a marker of cellular origin, encountered problems due to clumping; thus, this more traditional marker was abandoned for an alternative, Biodipy FL maleimide (FL N-(2-aminoethyl)) malemide (Life technologies). Biodipy FL maleimide binds to cell membranes via cysteine residues and thiol groups and has been shown to be comparable in identifying microparticles to methods utilising annexin V (240).

30µl of citrated plasma sample was used per test. 0.25µl FCR blocker (MiltenyiBioteck, BergischGladbach, Germany) was added to each sample, followed by 10µl - 20µl antibodies as detailed in Table 6 (241). Samples were incubated for 30 minutes at room temperature (in the dark) prior to addition of 30µl Flow Net Fluorospheres (Beckman Coulter Ltd, High Wycombe, UK). PBS was then added to give a total volume of 475µl. Finally 25µl Biodipy (final concentration 2.5µM) was added prior to FACS analysis.

Table 6 - Microparticles in maternal plasma, their identifying monoclonal antibodies and isotype controls for flow cytometric analysis.

Type of Microparticle	Monoclonal Antibody Used	Volume of Antibody Used per test (30µl plasma)	Supplier
Platelet	CD41a	15µl	Becton Dickinson
Constitutive Endothelial Cell	CD146	20µl	Becton Dickinson
Activated Endothelial Cell	CD62e	15µl	Becton Dickinson
Leucocyte	CD45	20µl	Becton Dickinson

Erythrocyte	CD235a	15µl	Becton Dickinson
Tissue Factor Expressing Cell	CD142	20µl	Becton Dickinson
Isotype Control		20µl	Becton Dickinson

Fluorospheres (Beckman-Coulter, High Wycombe, UK) were used to identify MPs at 10µm in diameter with a fluorescence range of 525nm to 700nm. Anything smaller than this size was considered an MP (Figure 12A). For each type of MP, a monoclonal antibody control (Table 6) was used to gate the sample (Figure 12B) enabling identification of antibody-positive microparticulates only (Figure 12C).

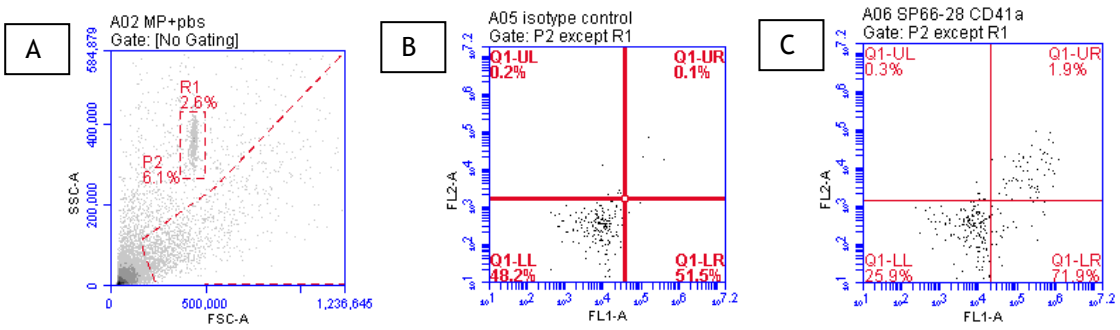


Figure 12 - Flow Cytometric Identification of microparticles. A) Microparticle cloud identification with count beads labelled in R1, and microparticle cloud. B) Microparticle populations using FL1 as marker for Biopdipy and C) FL2 as marker for antibody

2.8 Complement deposition within the placenta

Two cohorts of samples were used for analysis; a retrospective cohort of stored samples with known adverse pregnancy outcomes, and a prospective cohort of women with SLE, a prior stillbirth or an uncomplicated healthy pregnancy recruited as part of the current study.

2.8.1 Sample identification

Placental histological reports from Royal Manchester Children's Hospital Histopathology department were examined for the time period 01/01/2011 – 31/12/2013. 1195 reports were examined. 121 had no information regarding fetal outcome and so were excluded, leaving 1074 suitable for analysis (Figure 13). Each was classified according to underlying pathology findings. Prospective samples were identified through antenatal clinics at St Marys Hospital, Central Manchester University Hospitals Foundation NHS Trust. Women were asked to donate their placenta after delivery if they had SLE, had a suffered a prior stillbirth, or had an uncomplicated healthy pregnancy. Placentas were then treated in the same manner as the historical samples.

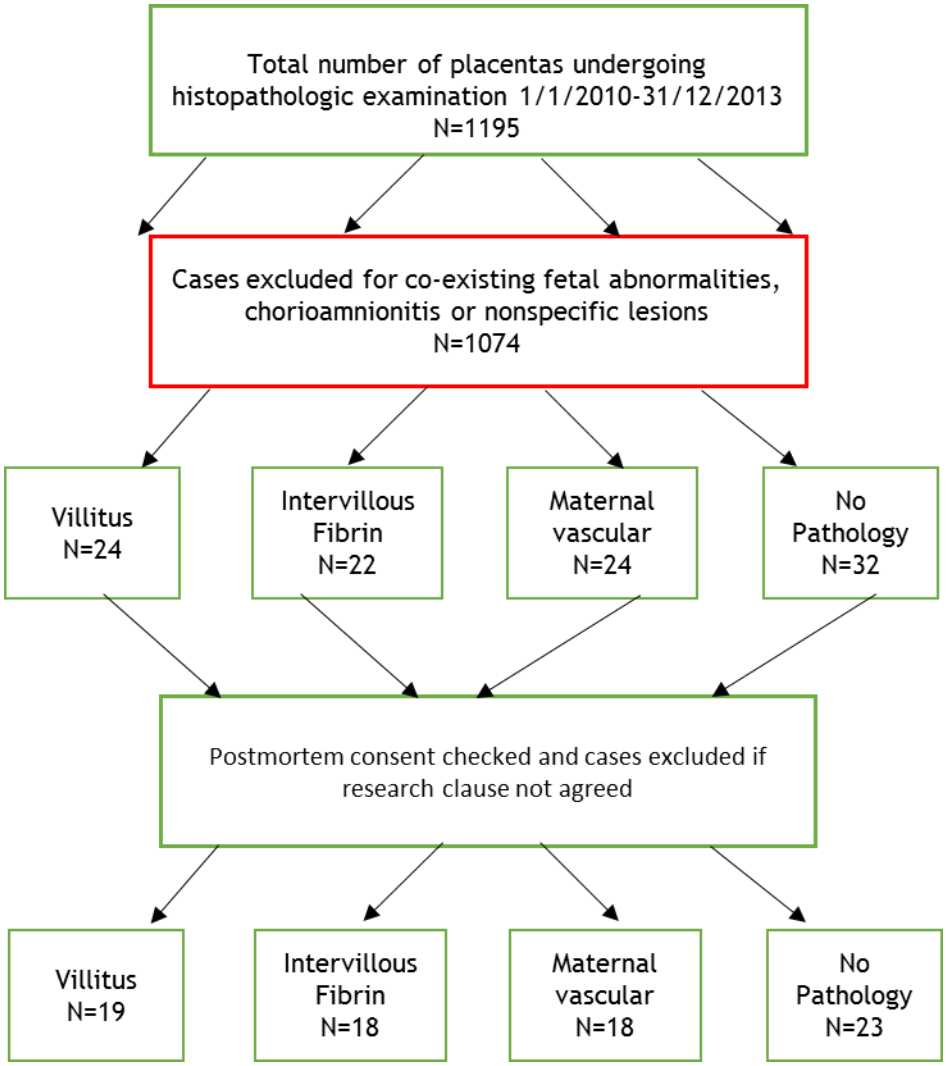


Figure 13 - Pathway of retrospective placental tissue identification. Pathway for identification of appropriate placental tissues for staining for complement family activity from the archives at Royal Manchester Children's Hospital histopathology Department.

2.8.2 Placental complement deposition staining

Placentas were formalin fixed (10% formalin) in the Royal Manchester Children's Hospital Histopathology Department and processed using a Leica Polaris (Leica Microsystems, Milton Keynes, UK). Placental blocks were paraffin embedded in a 16 hour process (dehydration with ethanol, clearing with xylene then wax infiltrated).

An automated Ventana BenchMark ULTRA IHC/ISH Staining Module (Ventana Co., Tucson, AZ, USA) was used for all staining, together with the Ultraview 3, 3' diaminobenzidine (DAB) version 3 detection system (Ventana Co.). This was performed in the Adult Histopathology Department of Central Manchester University Hospitals NHS Foundation Trust by the Adult Histopathology Team. Deparaffinized tissue sections (4 µm) incubated in EZPrep Volume Adjust (Ventana Co.) were subjected to the following protocols. At intervals between steps, slides were washed with a TRIS-based Reaction Buffer, pH 7.6.

2.8.2.1 Complement Factor 4d (C4d)

A heat-induced antigen retrieval protocol, set for 64 min, was carried out using a TRIS–ethylenediamine tetracetic acid (EDTA)–boric acid pH 8.4 buffer (Cell Conditioner 1). The sections were incubated with ultraviolet inhibitor blocking solution for 4 min, then with antibody to C4d (Cell Marque (Catalogue number CMC40411021), Anti- C4d Rabbit Polyclonal, at 1:75 concentration) for a set time of 32 min at room temperature. This was followed by incubation with horseradish peroxidase-linked secondary antibody (8 min), followed by DAB chromogen and substrate (8 min), and copper enhancer for 4 min. Counterstain (haematoxylin II) was applied for 12 min, before an incubation of 4 min with bluing reagent. A known C4d positive kidney was used as the positive control (Figure 14B).

2.8.2.2 Complement Factor 3a Receptor (C3aR)

A heat-induced antigen retrieval protocol set for 36 min was carried out using a TRIS– EDTA–boric acid pH 8.4 buffer (Cell Conditioner 1). The sections were incubated with ultraviolet inhibitor blocking solution for 4 min, then with antibody to C3aR (Santa Cruz Biotechnology, Anti- C3aR (H-300) Rabbit Polyclonal, at 1:75 concentration) for a set time of 36 min at room temperature. This was followed by incubation with horseradish peroxidase-linked secondary antibody (8 min), followed by DAB chromogen and substrate (8 min), and copper enhancer for 4 min. Counterstain (haematoxylin II) was applied for 12 min before an incubation of 4 min with bluing reagent. Figure 14C shows an example of a negative control and Figure 14D C3aR positive placental staining seen within the placenta.

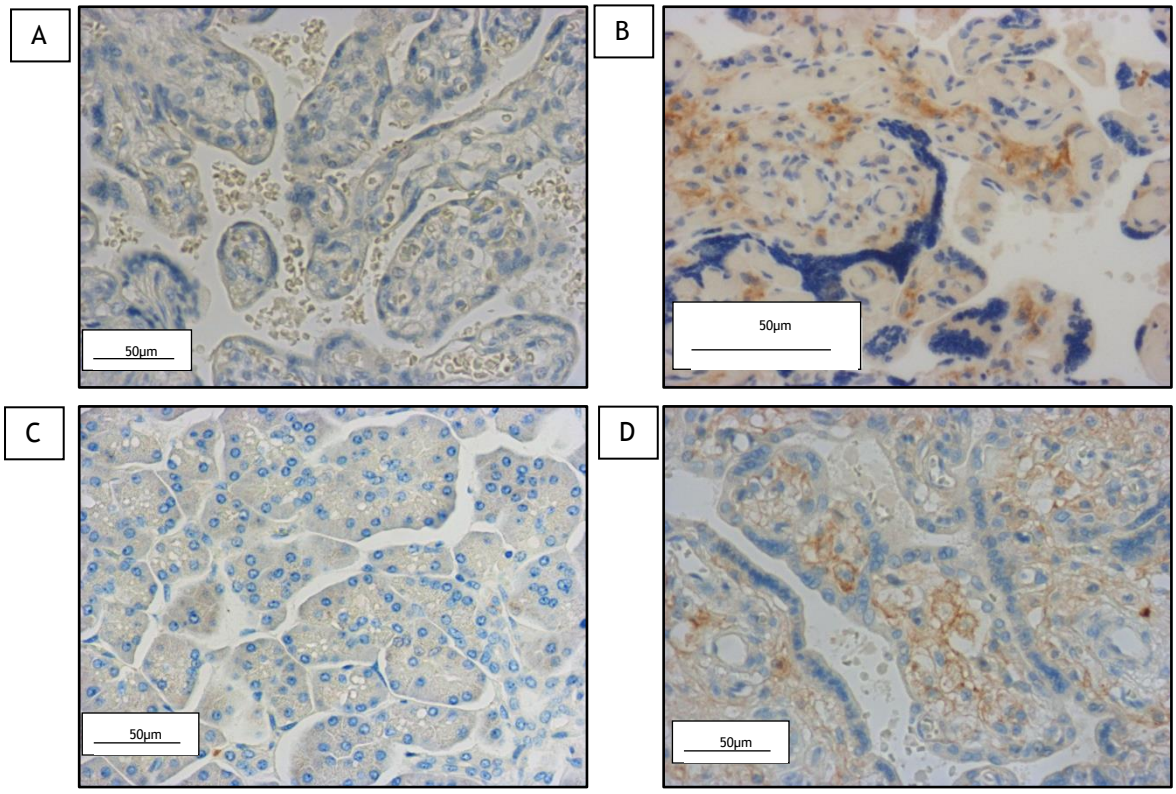


Figure 14 - Complement deposition in the placenta. A) Negative control omplement factor d (C4d), B) Positive C4d staining of the placenta, C) Negative control Complement factor 3a receptor (C3aR), D) Positive C3aR staining of the placenta. Scale bar = 50µm in all images, original magnification x10-20.

2.8.3 Image analysis for placental complement deposition

Images were captured using an Olympus BX41 light microscope (Essex, UK) and attached QIcam Fast 1394 camera (QImaging, British Columbia, Canda). Image Pro-Plus 7 (media Cybernetics, Maryland, USA) software was used to view and capture images. Brightness and contrast were adjusted to reduced grey effect. 10 random images were captured per slide at x10 magnification. Images were pooled to account for variations across the placenta and to give a single representative value per placenta (Figure 15). C4d and C3aR were assessed as total area, intensity of stain and percentage of DAB to haemotoxylin, i.e. total placenta tissue using HistoQuest image analysis software (TissueGnostics, Vienna, Austria). This software converts the image into greyscale, and quantifies the intensity of staining (scale 0-256). The software then converts this to allow differentiation of DAB positive and haemotoxylin positive cell/areas.

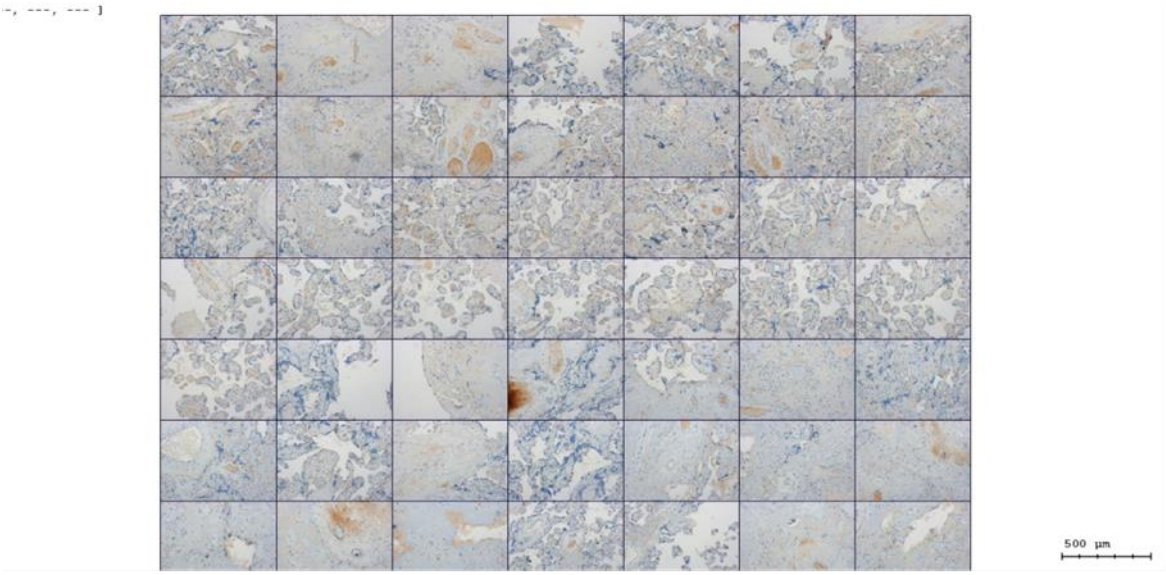


Figure 15 – Example of HistoQuest image analysis. Total images captured for one placenta, each frame represents one image. Haemotoxylin positive areas shown in blue, diaminobenzidine (DAB) positive shown in brown.

2.9 Epidemiology Data

Given the rarity of SLE as a disease, a population based approach was taken, in addition to the methods outlined above, to address issues relating to small study cohort size. In the UK population based databases are predominantly based in primary care. This study aimed to utilise the UK wide Clinical Practice Research Datalink (CPRD) which represents approximately 5% of the population.

Primary care databases use alphanumeric READ codes for medical diagnoses and pregnancy outcomes, these codes are transcribed into medical records as a result of "real time" coding at point of contact with a GP, or retrospectively following communication from secondary/tertiary care. This transcription may be performed by non-medical staff and therefore we sought to assess the feasibility of using these databases to look at pregnancy information. This was done using the NorthWest primary care database, the Feasibility Assessment and Recruitment System for Improving Trial Efficiency (FARSITE) system. This preliminary study demonstrated sufficient pregnancy outcome data within primary care records to enable application to the Independent Scientific Advisory Committee (ISAC) to use CPRD. This enabled more detailed analysis including linear data which allows for time-event analysis. This was important as the hypothesis of this thesis is that some stillbirths/pregnancy losses occur as a result of an autoimmune reaction in the maternal systems akin to that seen in SLE, or as a result of undiagnosed SLE/ LLD. The ability to look at the time from index event (adverse pregnancy outcome) to development of autoimmune CTD was therefore key to the research question, hence the need to access linear data.

In order to increase statistical power, five healthy livebirths were identified per APO. Given a background risk of developing SLE of 0.05%, this gave the study a statistical power of 80% to detect a doubling of this rate.

2.9.1 Feasibility Assessment and Recruitment System for Improving Trial Efficiency

As described above, a preliminary search of the NorthWest GP database FARSITE (Feasibility Assessment and Recruitment System for Improving Trial Efficiency, NorthWest eHealth) was performed as a pilot study. This search covered 609,691 people, using basic search terms for stillbirth and SLE (Appendix 3). FARSITE identified 26,929 pregnancies, including 556 cases of stillbirth (Table 7).

Table 7 – Pregnancy outcomes captured from the 26929 total pregnancies identified in the Feasibility Assessment and Recruitment System for Improving Trial Efficiency (FARSITE) dataset.

	Stillbirth	Livebirth	Miscarriage	Ectopic Pregnancy
Number	556	23,047	1,580	1,746

2.9.2 Clinical Practice Research Datalink (CPRD)

Analysis of the FARSITE data showed that testing the hypothesis using a bigger dataset was feasible. CPRD includes data on approximately 5% of the UK, and is thought to be representative of the population as a whole (242). It contains longitudinal patient data in an anonymised form; based on the READ code system (a list of alphanumeric codes that represent a diagnosis and its sub classifications e.g. L264.00 Intrauterine death, L264100 Intrauterine death - delivered) it provides diagnoses in a searchable, categorical form, in addition to medication and medical test information.

2.9.2.1 Search term and READ code generation in CPRD

The READ code dictionary was searched for key terms for adverse pregnancy outcome, livebirth, and autoimmune connective tissue disease as shown in Table 8. Composite adverse pregnancy outcomes were considered, with stillbirth considered the potential endpoint of multiple pregnancy pathologies. These adverse pregnancy outcomes specifically included: fetal loss (miscarriage and stillbirth), fetal growth restriction, premature delivery, placental insufficiency and placental abruption. Maternal complications, were likewise considered, included hypertensive disease, cardiovascular complications, and renal complications. In considering the diagnosis of an autoimmune disease, codes for SLE, LLD, connective tissue diseases (CTDs) and mixed connective tissue disease (MCTD) were used. Positive test results for autoimmune markers were also included, as were codes for neonatal lupus syndromes as this implies the presence of maternal antibodies.

Table 8 – Search terms used in the READ code dictionary

Adverse Pregnancy Terms	Livebirth terms	SLE codes	Autoimmune Test Result codes
Stillbirth Fetal death Hypertension (pre-existing, gestational, pre-eclampsia, HELLP, eclampsia) Fetal growth restriction Placental abruption Preterm labour and delivery	Livebirth Pregnant Normal delivery Caesarean delivery Instrumental delivery	SLE Lupus nephritis Systemic Sclerosis CREST syndrome Polymyositis Dermatomyositis Rheumatoid Arthritis	Anticardiolipin antibody Antiphospholipid antibody dsDNA anti-Ro anti-La ANA

Codes were then streamlined, removing those that were not commonly used, to produce a final list that captured as many cases as possible, but still enabled efficient data handling (Figure 16). The final list of READ codes used is shown in Appendix 4.

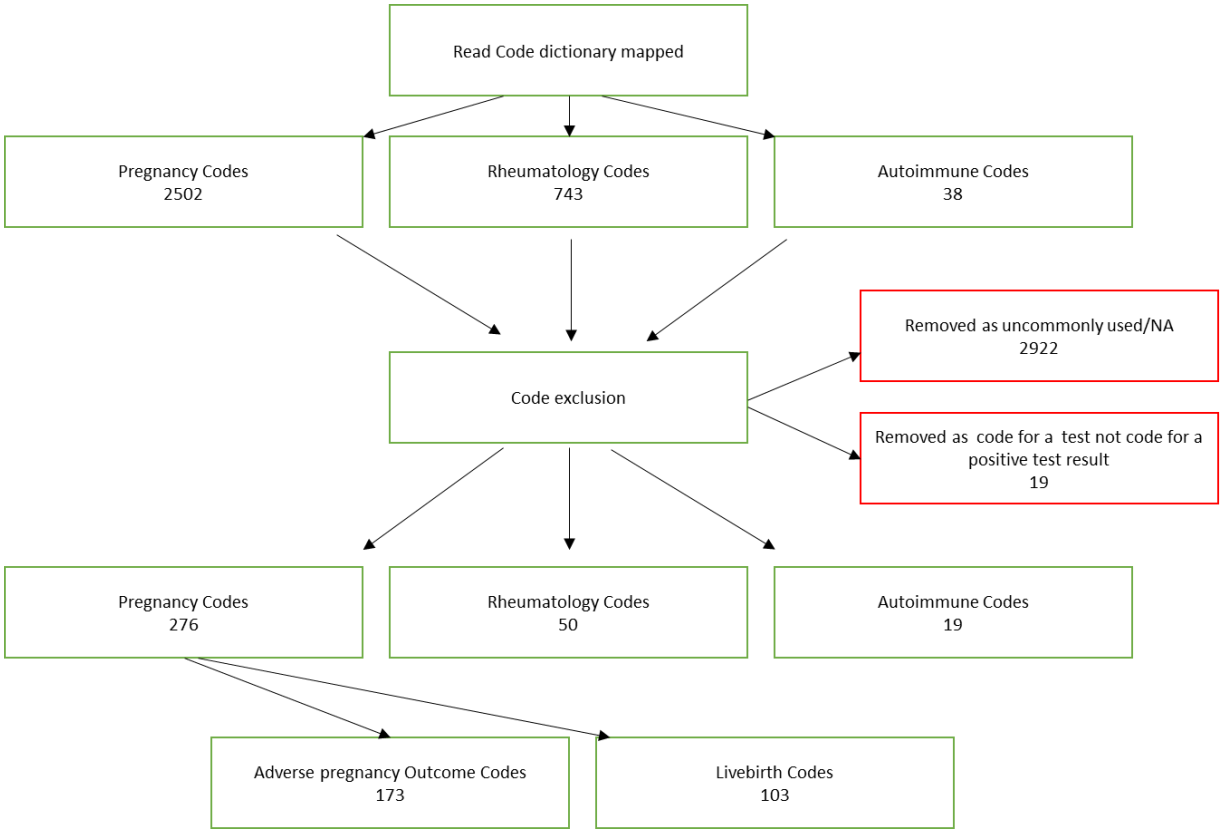


Figure 16 - READ code generation. Pathway of READ code list generation showing numbers of codes included and excluded to give final code list.

2.9.2.2 Data Extraction

Entry criteria were the presence of an adverse pregnancy code, at any time-point between 01/01/2000 and 31/12/2010, and age of over 18 at time of the event. Follow up was to 01/12/2014 (date of data extraction).

Prior code for autoimmune disease and age under 18 at entry were exclusion criteria. As outlined in section 2.9, page 59, 5 live birth “controls” were matched to each “case” of APO. These were matched to GP practice and maternal age at entry. The full protocol is available in appendix 5.

Data was extracted from CPRD into 14 subset files based on the information contained within (e.g. referral data or immunisation record). All files were collated based on unique patient ID. A check process was carried out using the Readcodes outlined in Appendix 4 to ensure cases had an adverse pregnancy outcome. Entry time and exit time were defined as shown in Figure 17. The same process was repeated to identify matched control. With an end result of 4.84 matched control cases per adverse outcome case.

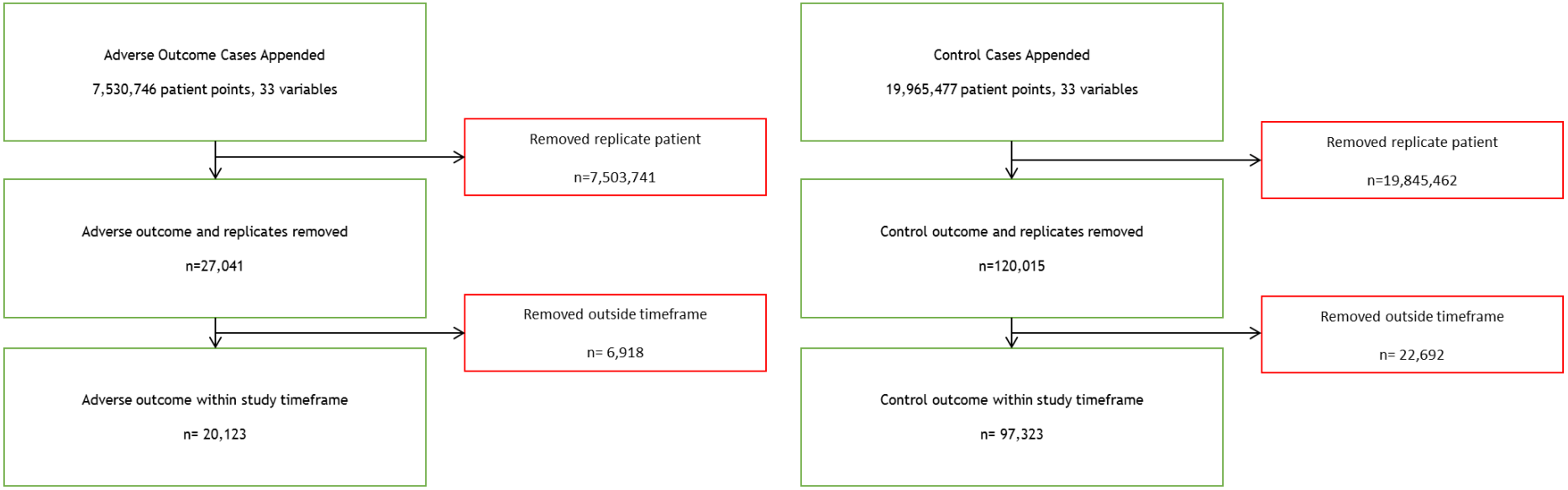


Figure 17 - Identification of cases and matched controls in Clinical Practice Research Datalink (CPRD)

2.10 Statistical Analysis

With the exception of epidemiological data all results were analysed with GraphPad Prism 7 (GraphPad Software Inc, USA). Repeated measures analysis of variance (ANOVA) or Kruskall Wallis was used to compare changes across pregnancy. T tests or Mann Whitney tests were used for matched gestational comparisons. Chi squared test was used to compare cohort percentages. Data is displayed as median and interquartile range (IQR), unless otherwise specified. Significance was set to $p<0.05$. All data was tested for normality.

Epidemiological data was analysed using STATAIC 13 (64-bit) (StataCorp LP, Texas, USA). Cox regression was performed on data using date of study entry as time 0 (either date of adverse pregnancy outcome or adverse outcome), and date of development of autoimmune disease as study exit. This was graphically represented using hazards ratio survival curves. Poisson regression was performed to look at count data and give exact risk ratios. Outcomes were considered both as a composite adverse pregnancy outcome, and as single risk factors.

3. - Is Stillbirth a Risk Factor for Development of SLE?

3.1 Abstract

Introduction – Autoimmune Connective Tissue Diseases (CTDs), such as Systemic Lupus Erythematosus (SLE), have a female preponderance and higher prevalence in women who experience an adverse pregnancy outcome (APO), including stillbirth. This study assessed the prevalence of CTDs, antiphospholipid syndrome (APS) and autoimmune antibodies, including antiphospholipid antibodies (aPL), in women with an APO, compared to those with an uncomplicated livebirth.

Methods - An initial feasibility study was performed using a primary care database, FARSITE (Feasibility Assessment and Recruitment System for Improving Trial Efficiency, NorthWest eHealth) to assess the utility of a UK database for identifying APOs. A national study was then performed, using the primary care database Clinical Practice Research Datalink (CPRD) for the timeframe 01/01/2000-31/12/2013. CPRD was searched for key pregnancy terms for APOs. Each case of APO was matched to 5 livebirths by GP practice and maternal age. Women were then followed serially for development of codes consistent with SLE diagnosis, CTD, APS or autoimmune antibodies. Poisson regression was performed to calculate relative risk ratios (RR) and 95% confidence intervals (CI) for the development of these conditions for APO vs. livebirth.

Results – The FARSITE search identified 26,929 pregnancies, including 556 stillbirths (stillbirth rate 2.1%). The prevalence of SLE diagnosis with stillbirth was 7.3% vs 3.0% with livebirth (RR of 2.41 (95% CI 1.77-3.26)); the timeline of diagnosis was not available within the FARSITE dataset. CPRD identified 20,123 APOs, which were matched to 97,323 matched livebirths, with a total of 875,590 person-years follow-up available. Mean follow-up time from study entry was 7.45 years (SD 4.39). 1,850 new autoimmune codes were recorded, giving an SLE incidence of 0.05%. Compared to women with an uncomplicated livebirth, women with an APO were at increased risk of developing CTD or autoimmune antibodies (RR 3.20, CI 2.90-3.51). Stillbirth was associated with an increased risk of developing specific autoimmune conditions (RR 5.82 (95% CI 4.97-6.81)), as was miscarriage (RR 3.41 (95% CI 3.03-3.85)), hypertensive disorders of pregnancy (RR 2.05 (95% CI 1.7-2.48)), premature delivery (RR 2.35 (95% CI 1.89-2.92)), fetal growth restriction (RR 2.69 (95% CI 1.50-4.83)) and placental abruption (RR 3.39 (95% CI 1.96-5.89)). For SLE specifically, the risk was greatest in those with stillbirth (RR 4.10 (95% CI 3.14-5.36)). For CTD, SLE and development of non-aPL antibodies, the risk was greatest within 4 years of their APO. Both aPL antibody prevalence and APS diagnosis were highest ≥ 5 years from APO.

Conclusions – APO is associated with an increased risk of subsequent development of maternal CTD, including SLE. This risk appears to be independent of that seen with aPL and APS. This observation may result from an underlying immunological factor which potentially predisposes women to both APO and subsequently CTD development, with risk highest within 4 years of their affected pregnancy. Alternatively, APO initiates an autoimmune process which culminates in the development of clinically evident CTD.

3.2 Introduction

In pregnancies complicated by maternal SLE, the incidence of stillbirth is increased by 3.6% compared to 0.5% in the general population (2). This increased risk of stillbirth is further exaggerated prior to diagnosis and treatment (224). It has been demonstrated previously that antibodies, typical of SLE, can develop up to 9 years prior to formal diagnosis (82,90) and that these, or other elements of its linked pathophysiology, may predispose to pregnancy complications, including stillbirth, even in the absence of clinically-evident disease. Two large epidemiology studies in Denmark have shown an association between stillbirth and an increased risk of subsequent maternal autoimmune disease (including SLE), with a peak relative risk of 6.3 in the first 4 years following pregnancy loss (4,5). This suggests that undiagnosed or subclinical SLE, could be in part responsible for unexplained stillbirths, or that stillbirth itself is a risk factor for the development of SLE or its associated conditions i.e. lupus-like disease (LLD) and connective tissue disease (CTD).

Previous epidemiology studies on the relationship between APO and CTD are based in Denmark; as such, they are not directly applicable to the UK. This is predominantly due to the homogeneity of the population in Denmark as women from ethnic minority groups are at a higher risk of both SLE and APO (243–248), therefore we sought to address this in a UK cohort. To examine this relationship in the UK population, a similar epidemiological approach was adopted. Firstly, by defining the feasibility of using a local primary care database, and then by full-scale national investigation using a primary care database, the Clinical Practice Research Datalink (CPRD). The initial feasibility study was conducted with FARSITE (Feasibility Assessment and Recruitment System for Improving Trial Efficiency), NorthWest eHealth GP database. This primary care database covers over 600,000 people in the North West of England. However, these data are not available in a longitudinal format. The CPRD collates anonymised information from GP practices across the UK, representing approximately 5% of the total UK population. As such, it reportedly reflects its epidemiologic features (242). The CPRD dataset has been validated and used for both pregnancy and SLE cohorts separately (7–10), but this is the first time the dataset will be interrogated using the combination of both disorders in a longitudinal context (242). Capable of time-event analysis, CPRD was thought to be the most appropriate data source to answer our research question of whether or not stillbirth is a risk factor for the development of SLE in the UK population.

3.3 Methods

3.3.1 Feasibility Assessment and Recruitment System for Improving Trial Efficiency (FARSITE)

The North West GP database, FARSITE, was used to assess study feasibility, defined as the ability to identify livebirths, APOs and cases of SLE in a UK primary care dataset. This search covered the records of 609,691 people in total, using basic search terms for stillbirth, miscarriage and SLE. The system automatically matches codes and so no further search term mapping was

required. The full list of terms and search parameters are given in Appendix 4 – FARSITE Search terms, page 171. The FARSITE search identified 26,929 pregnancies, including 556 cases of stillbirth and 3,326 early pregnancy losses. However, the absence of longitudinal data within FARSITE meant it was not possible to determine if these stillbirths happened in women with pre-existing SLE or if SLE was diagnosed after the stillbirth.

3.3.2 Clinical Practice Research Datalink (CPRD)

CPRD contains anonymised longitudinal data with diagnoses based on the READ code system, which is a list of alphanumeric codes that represent a diagnosis and sub classification e.g. L264.00 = Intrauterine death, L264.100 = Intrauterine death – delivered. Ultimately, it provides diagnoses in a searchable, categorical format, in addition to prescribed medication and medical test information.

3.3.2.1 Study Protocol

Ethical approval was granted by the Independent Scientific Advisory Committee (ISAC) (protocol 14_106R). Records from 01/01/2000 – 31/12/2013 were screened for the presence of APO (as identified using the READ code dictionary, outlined below). Five cases of livebirth controls with no adverse outcomes were matched per "case" adverse outcome (matched to maternal age, year of pregnancy outcome and GP practice); this was based on the power calculation also outlined below. All included women were over 18 at the stage of study entry. Pre-existing cases of SLE were excluded. If there was a code consistent with a diagnosis of SLE prior to the pregnancy code, it was assumed the women had SLE or were under investigation and so were also excluded (see Appendix 6 – Clinical Practice Research Datalink (CPRD) Research Protocol, page 181 for details).

3.3.2.2 Search term and READ code generation in CPRD

The READ code dictionary was searched for a list of APO codes and also for a list of autoimmune diagnoses, to generate a full code list for analysis, APOs were considered both as individual factors (e.g. stillbirth) and also as a whole group encompassing all the APOs considered (composite APO).

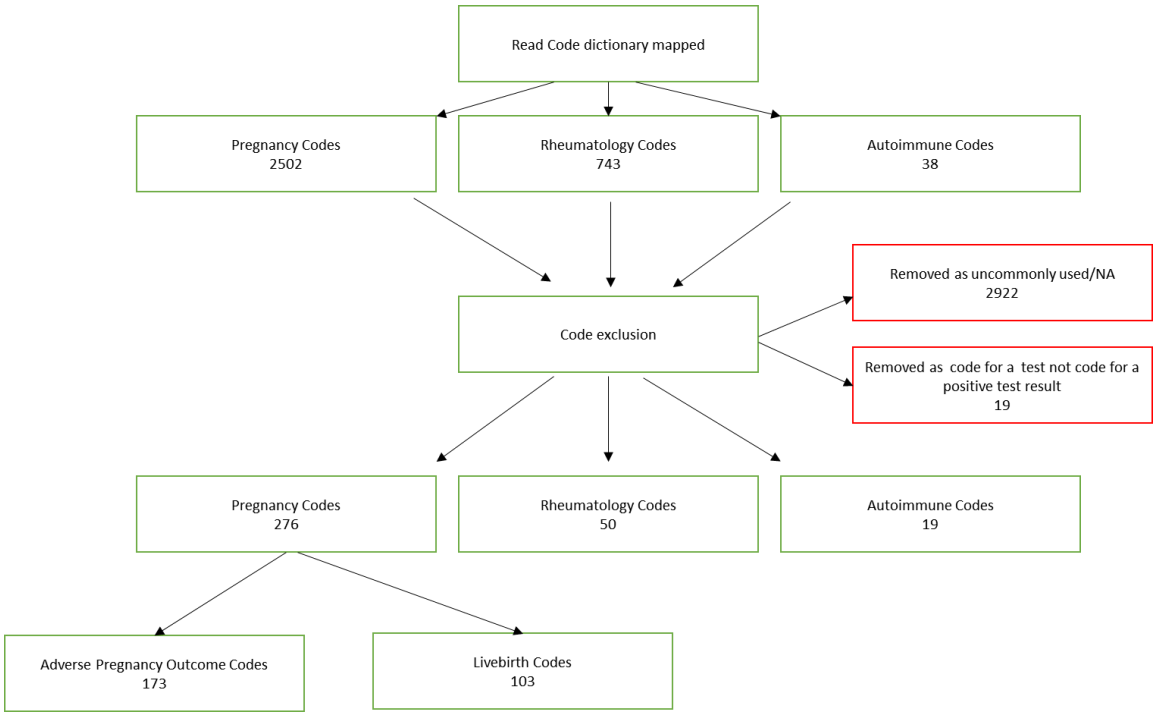
Table 9). Positive test results for autoimmune markers were also included, as were codes for neonatal lupus syndromes, as these imply the presence of maternal autoantibodies during pregnancy. The autoimmune and rheumatology codes were further examined to exclude those merely indicating a test, rather than a positive result. In view of the large crossover between autoimmune CTDs, a decision was made to keep all major CTDs within the search criteria. However, non-autoimmune rheumatic diseases e.g. osteoarthritis, were removed. The READ code dictionary was mapped against these key search terms, producing a comprehensive code list which was subsequently compared to published identifiers of pregnancy and SLE, to crosscheck that chosen codes were utilised in practice, and that key terms had not been missed (249). Codes were removed if not in general use and a final list produced to capture as many cases as

possible, but with retained efficiency in data handling (Figure 18). The final list of search terms and READ codes are shown in Appendix 5 – READ codes and Medcodes used within the Clinical Practice Research Datalink (CPRD) search criteria, page 173. APOs were considered both as individual factors (e.g. stillbirth) and also as a whole group encompassing all the APOs considered (composite APO).

Table 9 – Search terms for the Clinical Practice Research Datalink (CPRD).

Adverse Pregnancy Terms	Livebirth Terms	Connective Tissue Disease/Immunology Codes
Stillbirth Fetal death Hypertension (pre-existing, gestational, pre-eclampsia, HELLP, eclampsia) Fetal growth restriction Placental abruption Preterm labour and delivery	Livebirth Pregnant Normal delivery Caesarean delivery Instrumental delivery	SLE Lupus nephritis Systemic Sclerosis CREST syndrome Polymyositis Dermatomyositis Rheumatoid Arthritis Anticardiolipin antibody Antiphospholipid antibody dsDNA anti-Ro anti-La Anti-nuclear antibodies (ANA)

Figure 18 - Pathway of READ code list generation showing numbers of codes included. For full list of codes see Appendix 5



3.3.2.3 Data Extraction

As previously indicated, the entry criterion was the presence of an adverse pregnancy code at any time-point between 01/01/2000 and 31/12/2010, and maternal age of over 18 at time of

event. Follow-up was to 01/12/2014 (date of data extraction). Cases of APO were matched using GP practice, year of pregnancy outcome and maternal age at study entry. As shown in Figure 19, 20,123 APOs were identified within the study timeframe. 97,323 livebirths were matched, giving 4.8 controls per "case" of adverse outcome. Each patient in the database contributed towards the "years at risk" from time of entry (either APO or livebirth) to time of study exit (either through development of autoimmune disease, death, transfer out of GP practice, or end of follow-up 31/12/2010).

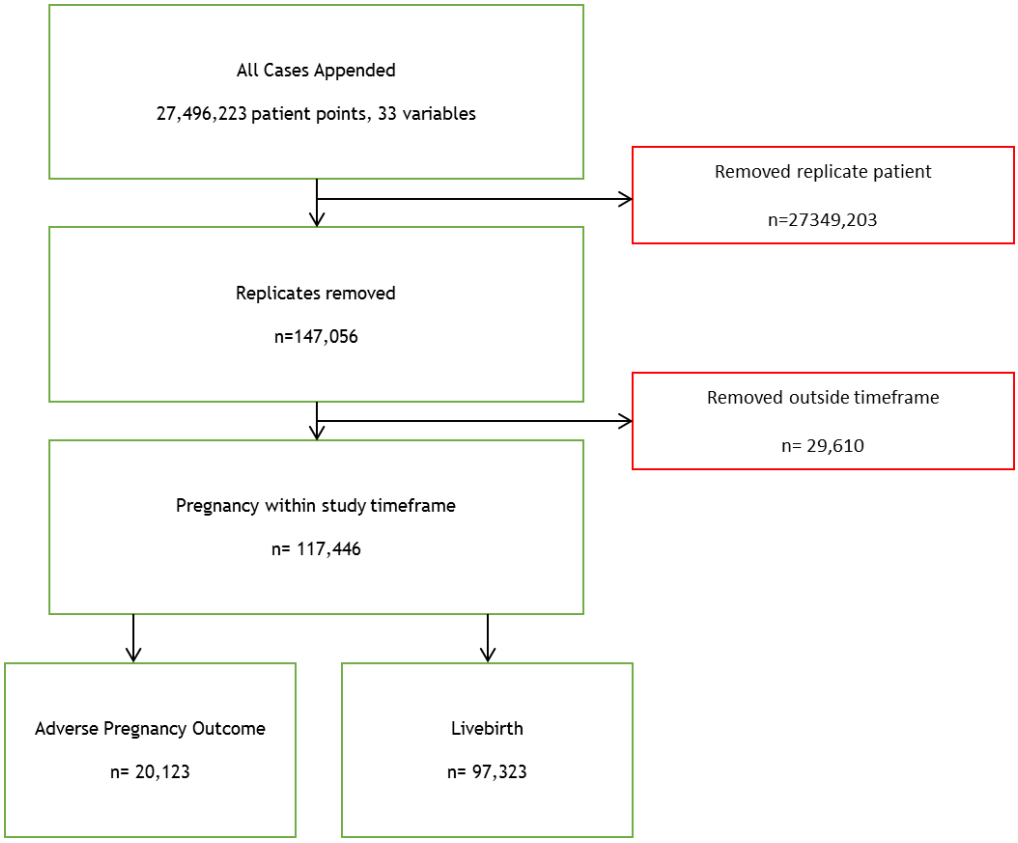


Figure 19 - The identification of adverse outcome pregnancies and matched livebirth controls within the study timeframe using Clinical Practice Research Datalink (CPRD).

3.4 Statistical Analysis

Data analysis was preformed using STATAIC 14 (64-bit) (StataCorp LP, Texas, USA). Data were extracted from CPRD into 14 subset files based on the information contained within (e.g. referral data or immunisation record). All files were collated based on a unique patient ID. Poisson regression was performed to generate relative risk ratios (RR) using date of study entry as time 0 (either date of APO), and date of development of autoimmune disease at study exit. RR were determined for both total time within the study and subdivided into risk within 4 years of pregnancy outcome vs that ≥5 years. Statistical significance was set at $p \leq 0.05$.

3.4.1.1 Power calculation

Preliminary data for CPRD suggested over 24,000 cases of composite APO cases (as defined in Table 9), with an assumed background risk of developing SLE of less than 0.05% in the general

population (250,251). A power calculation was performed and showed that matching 5 controls per case would provide a statistical power of 80% to detect a doubling of prevalence of SLE (relative risk of 2) in the adverse pregnancy outcome-related group.

3.5 Results

3.5.1 Feasibility Assessment and Recruitment System for Improving Trial Efficiency (FARSITE)

The primary feasibility outcome was to assess the ability to capture pregnancy outcome information from a primary care database. As shown in Figure 20, this aim was met with the identification of 26,929 pregnancies in the FARSITE search, of which 23,047 were livebirths and 556 were stillbirths (out of 3,882 pregnancy losses).

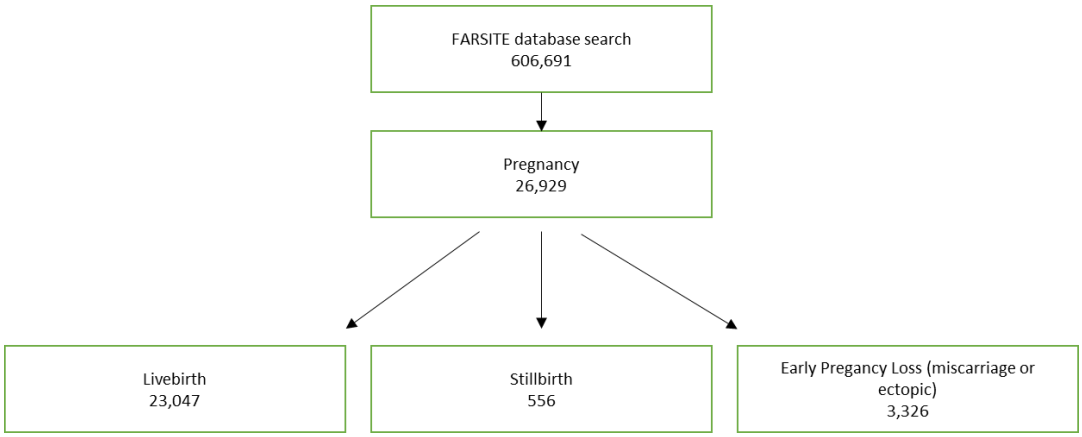


Figure 20 – The identification of pregnancy outcomes in Feasibility Assessment and Recruitment System for Improving Trial Efficiency (FARSITE).

The overall livebirth rate from FARSITE was 85.6%, with combined miscarriage and ectopic rate of 12% and stillbirth rate of 2.1%. The FARSITE data does not allow for time-event analysis, so no causality can be examined, meaning that some cases of stillbirth may have occurred in women with pre-existing SLE. As shown in Table 10, there was a significantly higher prevalence of SLE diagnosis in the stillbirth cohort compared to that of livebirth (7.3% vs 3.0%, n=41/556 stillbirths and 706/23,047 livebirths respectively, $p<0.001$), giving an overall relative risk (RR) of SLE in women with a stillbirth of 2.41 (95% CI 1.77-3.26), compared to those with livebirth. When miscarriage and ectopic pregnancy were considered, the RR of having SLE was 2.24 (95% CI 1.9-2.6). When considered as a composite outcome of all pregnancy losses, the RR was 2.3 (95% CI 1.99-2.6) for diagnosis of SLE, compared to women whose pregnancies resulted in a livebirth.

Table 10- Prevalence of SLE with a livebirth, stillbirth, miscarriage or ectopic pregnancy in the Feasibility Assessment and Recruitment System for Improving Trial Efficiency (FARSITE) database.

	Livebirth	Stillbirth	Miscarriage /Ectopic Pregnancy
Total number	23047	556	3326
SLE diagnosis	706 (3%)	41 (7.3%)	229 (6.9%)

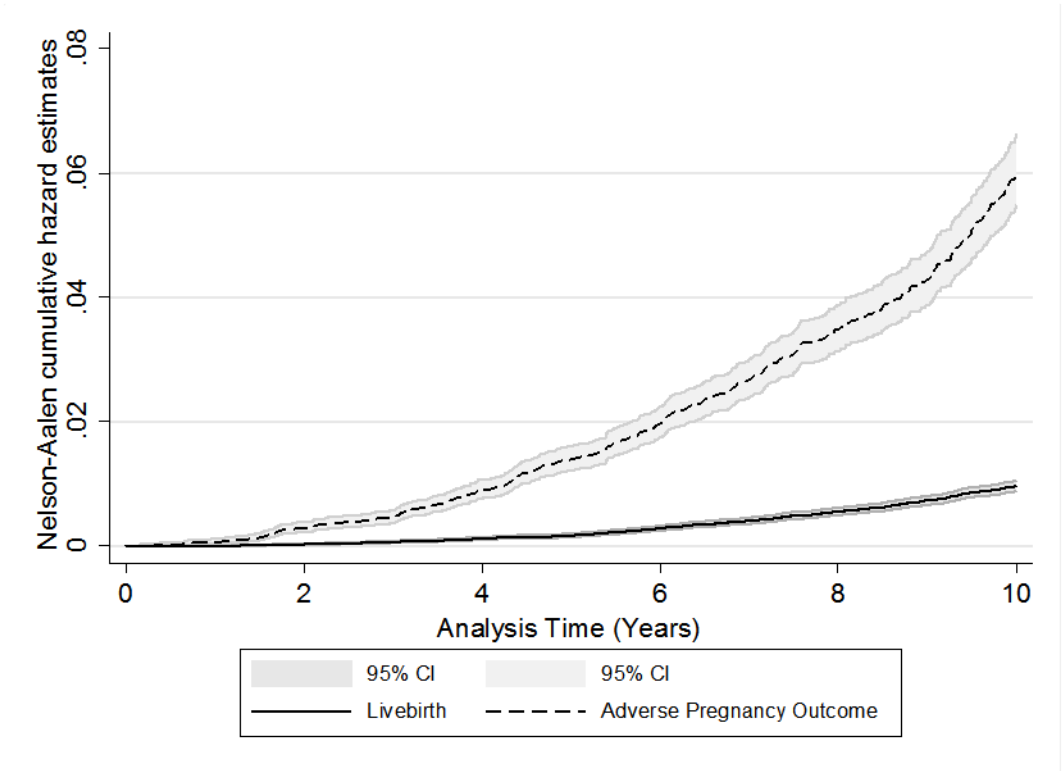
3.5.2 CPRD Analysis

As stated above, 20,123 cases of APO were identified within the study timeframe, along with 97,323 matched livebirth controls (Figure 19). A total of 875,590 person-years of follow-up were available for analysis. 1,850 women had an autoimmune disease code within the study timeframe with an overall prevalence of 1.75%. Of these, 61 new cases of SLE were identified, giving an incidence of 0.05% within the study population, which, as expected was higher than the background incidence of SLE in the UK population as a whole (6.5/100,000) (252). The mean age at the time of pregnancy outcome was 30.90 years (SD 6.47 years). Mean time of follow up was 7.45 years (SD 4.39 years), following either livebirth or adverse pregnancy outcome within the study window. Unfortunately, ethnicity was not available within this dataset.

3.5.2.1 Adverse Pregnancy Outcome

When considered as a composite risk factor, APO was associated with a RR of 3.20 (95% CI 2.90-3.51) for subsequent development of any autoimmune condition; this risk increased with duration of time since pregnancy outcome (Figure 21). As shown in Table 11, a composite APO was not associated with an increased risk of developing SLE [RR 1.05 (95% CI 0.62-1.79)] or CTD [RR 1.05 (95% CI 0.62-1.79)], although there was a significant association with positive immunology testing [RR 3.16 (95% CI 2.98-3.36)] and being diagnosed with APS [RR 4.84 (95% CI 4.22-5.57]. The risk of positive aPL and subsequent APS diagnosis was greatest at >5years following composite APO (aPL RR 3.61 <5 years vs 3.73 ≥5years, APS RR 3.61 <5 years vs 5.81 ≥5 years ,Table 12), although due to overlapping 95% CI these failed to demonstrate a statistically significant difference. Non aPL-antibody positivity was unaffected by time since the APO, with rates unchanged.

Figure 21 – Cumulative prevalence of composite autoimmune Connective Tissue Disease (CTD) within Clinical Practice Research Datalink (CPRD) for years 2000-2013, following either a livebirth or adverse pregnancy outcome.



3.5.2.2 Stillbirth

2,520 stillbirths were identified within the study timeframe, representing 12.5% of all APOs identified. A history of stillbirth was associated with a significantly increased risk of developing composite autoimmune disease, with RR of 5.82 (95% CI 4.97-6.81, p<0.0001). This included a significant increased risk of subsequent SLE diagnosis (RR 4.10 (95% CI 3.14-5.36), p<0.0001)). As shown in Table 12, such a risk was greatest within the first 4 years following stillbirth (RR 9.2 vs 5.3). There was no increased risk of subsequent connective tissue disease (RR 1.41, 95% CI 0.79-2.51). Stillbirth was also associated with a significantly increased risk of positive non-APL immunology (RR 3.30, 95% CI 2.60-4.10, p<0.00001), with greatest risk again within the first 4 years (RR 5.41 vs 3.24, although this was not statistically significant. When considering antiphospholipid (aPL) antibodies and diagnosis of APS, there was a significant increase in both prevalence of aPL positivity (RR 16.8, 95% CI 12.9-21.8) and APS diagnosis (RR 3.6, 95% CI 3.33-3.86, p<0.0001). The RR of APS diagnosis was highest at ≥5 years following stillbirth (5.1 vs 2.66), although the greatest RR for aPL was again within the first 4 years following stillbirth (15.44 vs 12.11).

3.5.2.3 Miscarriage

8,631 cases of miscarriage were identified in the study timeframe (42.9% of all APOs). This was also associated with an increased incidence of developing autoimmune disease, as shown by RR of 3.41 (95% CI 3.03-3.85), p<0.0001. Specifically, this was due to an increased rate of both non-aPL and aPL positive immunology testing, along with APS diagnosis in subsequent years. The risk of diagnosis of APS increased with time, with the risk highest ≥5 years following miscarriage (Table 12), although as shown it was statistically significant. Conversely, the risk of positive immunology was highest within the first 4 years following miscarriage. Neither SLE nor CTD was found to be significantly associated with miscarriage [RR 1.51 (95% CI 0.75-3.02), p=0.28 and RR 1.13 (95% CI 0.79-1.62), p=0.48, respectively].

3.5.2.4 Hypertensive disease

Hypertensive disease complicated 4,927 pregnancies (pre-eclampsia (PET) 4,719 and eclampsia 208). As shown in Table 11, this was associated with increased composite autoimmune disease, due to both non-aPL and aPL positive immunology, with overall increased RR for APS development (RR 5.64, 95% CI 1.17-27.16, P0.02). The RR of developing CTD or positive immunology (both aPL and non-aPL) was greatest within 4 years, but APS diagnosis was greatest at ≥5 years following a pregnancy complicated by hypertensive disease (Table 12).

3.5.2.5 Fetal Complications

3,164 preterm deliveries (≤36^{+6} weeks) occurred, associated with an increased risk of non-SLE connective tissue disease diagnosis, alongside an increased rate of APS diagnosis and non-aPL and aPL positive immunology. As shown in Table 12, the risk of development of APS was highest ≥5 years following birth, but the risk of developing a CTD or having positive immunology was highest within the first 4 years after pregnancy.

357 cases of fetal growth restriction (FGR) were identified, as were 404 cases of placental complications (abruption, placental insufficiency or abnormal placentation), both of which were associated with an increased RR of positive immunology. However, given the low numbers defined, the study was underpowered to detect any additional aspects of development of autoimmune CTD. The nature of the data meant it was not possible to adjust for multiple pregnancy complications.

Table 11 – Relative Risk of Connective Tissue Disease /autoimmune disease following an adverse pregnancy outcome or livebirth within Clinical Practice Research Datalink (CPRD) for years 2000-2013 (Risk Ratio and 95% Confidence Intervals shown).

	Composite Autoimmune Disease (n=1,852)	SLE (n=61)	Connective Tissue Disease (n=411)	Positive non-APL Immunology test (n=1,281)	Positive APL Antibody (n=469)	APS (n=59)
Livebirth (n=97,323)	**0.31 (0.28-0.34) P<0.001**	0.93 (0.49-1.81) P=0.83	0.82 (0.64-1.04) P=0.24	**0.31 (0.24-0.39) P<0.001**	**0.06 (0.05-0.08) P<0.0001**	**0.04 (0.02-0.10) P<0.0001**
Composite Adverse Pregnancy Outcome (n=20,123)	**3.20 (2.9-3.51) P<0.0001**	1.05 (0.62-1.79) p=0.81	1.41 (0.79-2.51) P=0.23	**2.3 (2.01-2.54) P<0.0001**	**7.49 (6.2-9.0) P<0.0001**	**4.84 (4.22-5.57) P<0.0001**
Stillbirth (n=2,520)	**5.82 (4.97-6.81) P<0.0001**	**4.10 (3.14-5.36) P<0.0001**	1.40 (0.79-2.47) P=0.25	**3.3 (2.6-4.1) P<0.0001**	**16.8 (12.9-21.8) P<0.0001**	**3.6 (3.33-3.86) P<0.0001**
Miscarriage (n=8,631)	**3.41 (3.03-3.85) P<0.0001**	1.51 (0.75-3.02) P=0.28	1.13 (0.79-1.62) P=0.48	**2.4 (2-2.8) P<0.0001**	**7.8 (6.3-9.8) P<0.0001**	**8.71 (6.73-11.27) P<0.0001**
Hypertensive Disease of Pregnancy (n=4,927)	**2.05 (1.7-2.48) P<0.0001**	1.18 (0.37-3.80) P=0.78	1.02 (0.63-1.66) P=0.92	**1.65 (1.3-2.1) P<0.001**	**4.1 (2.9-5.8) P<0.0001**	**5.64 (1.17-27.16) P=0.02**
Premature Delivery (n=3,164)	**2.35 (1.89-2.92) P=0.004**	0.62 (0.09-4.45) P=0.6	**1.78 (1.12-2.82) P=0.02**	**2.18 (1.7-2.8) P=0.012**	**3.51 (2.2-5.5) P<0.0001**	4.39 (0.54-35.7) P=0.12
Placental Abruption (n=341)	**3.39 (1.96-5.89) P<0.0001**	-	-	-	-	-
Fetal Growth Restriction (n=357)	**2.69 (1.50-4.83) P<0.005**	-	0.83 (0.12-5.90) P=0.84	1.56 (0.6-3.8) P=0.35	**8.89 (3.9-20.0) P=0.001**	-

Table 12 - Relative Risk of development of autoimmune connective tissue disease following an adverse pregnancy outcome by time from adverse pregnancy index event using Clinical Practice Research Datalink (CPRD) for years 2000-2013 (Risk Ratio and 95% Confidence Intervals shown).

	Composite Autoimmune Disease (n=1,852)		SLE (n=61)		Connective Tissue Disease (n=411)		Positive non APL Immunology test (n=1,281)		Positive APL Antibody (n=469)		APS (n=59)	
Time from index event (years)	**0-4**	**≥5**	**0-4**	**≥5**	**0-4**	**≥5**	**0-4**	**≥5**	**0-4**	**≥5**	**0-4**	**≥5**
Livebirth (n=97,323)	0.5 (0.42-0.6)	0.75 (0.71-0.8)	0.91 (0.58-2.2)	0.97 (0.72-1.3)	0.77 (0.71-0.82)	0.9 (0.87-0.92)	0.68 (0.56-0.83)	0.82 (0.77-0.88)	0.26 (0.18-0.38)	0.54 (0.47-0.64)	0.26 (0.06-1.02)	0.19 (0.08-0.45)
Composite Adverse Pregnancy Outcome (n=20,123)	2.77 (2.41-3.19)	2.06 (1.85-2.30)	1.29 (0.32-5.15)	1.15 (0.6-2.22)	1.45 (1.37-1.54)	1.39 (1.35-1.46)	2.12 (1.72-2.63)	2.06 (1.85-2.30)	3.61 (2.99-4.38)	3.73 (3.22-4.33)	3.61 (1.81-7.22)	5.81 (3.96-8.54)
Stillbirth (n=2,520)	9.2 (7.16-11.84)	5.33 (4.37-6.53)	2.98 (1.2-7.09)	2.22 (1.56-3.14)	1.39 (1.34-1.46)	1.37 (1.34-1.41)	5.41 (3.61-8.1)	3.24 (2.44-4.3)	15.44 (11.03-21.6)	12.01 (8.91-16.17)	2.66 (1.48-4.81)	5.1 (4.67-6.88)
Miscarriage (n=8,631)	4.06 (3.28-5.03)	2.90 (2.56-3.29)	3.63 (0.70-18.70)	1.05 (0.24-4.54)	1.64 (1.52-1.79)	1.47 (1.39-1.54)	2.74 (1.98-3.82)	2.26 (1.92-2.66)	6.33 (4.72-8.49)	4.84 (3.89-6.02)	6.01 (1.94-18.66)	10.21 (6.04-17.25)
Hypertensive Disease of Pregnancy (n=4,927)	3.07 (2.77-3..39)	2.59 (2.43-2.765)	-	1.11 (0.11-6.32)	1.51 (1.45-1.57)	1.41 (1.38-1.45)	2.34 (2.0-2.73)	2.1 (1.93-2.77)	4.01 (3.49-4.6)	3.88 (3.48-4.33)	4.13 (2.53-6.75)	6.35 (4.81-8.38)
Premature Delivery (n=3,164)	2.86 (2.59-3.17)	2.56 (2.4-2.72)	1.41 (0.5-3.8)	1.17 (0.7-1.9)	1.47 (1.41-1.53)	1.40 (1.36-1.43)	2.14 (1.82-2.50)	2.06 (1.89-2.23)	3.8 (3.3-4.36)	3.89 (3.5-4.32)	3.96 (2.42-6.46)	6.14 (4.66-8.08)

3.6 Discussion

For the first time in a large UK population cohort, this study has shown that having a history of APO, in particular stillbirth, is associated with an increased risk of development of autoimmune connective tissue disease, including SLE, in subsequent years. This risk was temporally related to the index pregnancy, as it was greatest within the first 4 years of experiencing an APO. An increased risk of positive immunology testing and APS was also observed. Conversely, having a livebirth was protective against a diagnosis of CTD or APS. Miscarriage was also associated with an increased risk of non-SLE connective tissue disease, alongside increased prevalence of non-aPL autoimmune antibody positivity and elevated rates of aPL antibody positivity and subsequent APS diagnosis.

SLE and CTD diagnosis rates were highest within the first 4 years of an adverse pregnancy outcome. However, the prevalence of an APS diagnosis increased with increasing time from the adverse pregnant event. The high rates of aPL antibodies and diagnosed APS may be partially explained by increased screening within this high risk population, as adverse pregnancy outcomes are already known to be associated with APS (253,254). However, the increased rates of non aPL antibodies also seen, suggests that these results are not solely related to patient selection bias, but could instead be due to symptomatology. If the primary cause of pregnancy loss were due to APS, it would be expected that the highest rates of diagnosis would be closest to the time of APO, however the converse was seen in this study. This supports the hypothesis that APO is associated with the subsequent development of autoimmune disease.

This study has demonstrated the utility of primary care data, held in CPRD, as a resource for observational studies in pregnancy, both for live birth and adverse pregnancy outcomes. However, caution must be exercised. The preliminary findings from the more local NorthWest database, FARSITE, showed a lower than expected level of miscarriage, perhaps demonstrating a lack of coding of early pregnancy loss within this dataset. Similarly, when examining the CPRD data, there were lower numbers than expected for fetal growth restriction (1.8%), considerably lower than the population definition [i.e. birthweight less than tenth centile (10%)]. This discrepancy, and under-representation of FGR, is likely due to poor reporting of diagnosis or mis-transcription of data between place of delivery (usually secondary or tertiary level care) and primary care. It is well established that fetal growth restriction is poorly documented within cases of stillbirth (255) and therefore potentially poorly documented with livebirths also. An additional confounding factor is the difference in diagnostic criteria for FGR between maternity and neonatal services [with maternity services using below the 10^{th} centile, and neonatal services using a typically lower non-customised standard (256,257)]. Underreporting of FGR in this study would wrongly classify FGR cases as “normal” outcome, and as such reduce the association seen between FGR and SLE/autoimmune CTD. As such it is unlikely to have introduced bias into the study.

The numbers of women coded as having SLE within the CPRD dataset was relatively low, which may reflect the younger age of the population studied (mean age 30.9 years) compared to the mean age of diagnosis of SLE, which is typically in the 4th or 5th decade (258). However, the increased rates of positive immunology seen could ultimately translate into more formal diagnoses of SLE in later years. The average follow-up time was limited to 7.45 years, due to the tight inclusion criteria of the study (minimum entry 01/01/2000, maximum exit 31/12/13). This was conducted to optimise the quality of data available within CPRD, and therefore again may under-represent the true prevalence of SLE following an APO. Nevertheless, the study was adequately powered to detect a difference in the frequency of SLE between women experiencing an APO and those who had an uncomplicated livebirth.

The rates of aPL positivity were highest in the first 4 years following adverse pregnancy outcome, although not statistically significantly, and diagnosis of APS was greatest ≥5 years afterwards (again, not statistically significantly due to overlapping 95% confidence intervals). This delay may represent time between testing and referral to specialist services for formal diagnosis. Overall, the rate of APS was higher than expected in this study cohort, which may be the result of positive selection bias (with women undergoing routine testing following stillbirth). The elevated rate of non aPL antibodies seen in the study, which suggest an underlying immune pathology that cannot be explained by APS alone, suggests that the results seen in this study are not a consequence of positive selection bias, as non aPL antibodies are not routinely screened for at time of stillbirth.

Similar to the studies by Ulff Moller et al. and Jorgensen et al., performed in a Danish cohort, this UK-based study has shown an increased risk of developing SLE following a stillbirth within 4 years of the index event (RR 2.98 (95% CI 1.25-7.09)). This level was comparable to that seen in the Danish cohort, in which RR was 3.03 overall (95% 1.88-4.58) (4,5), but lower than the risk defined in the Danish cohort in the first 4 years (RR 6.33 (3.38-10.68). Levels of CTD in this study were also similar to that seen in the Danish cohorts. The lower SLE risk seen within 4 years of the index adverse pregnancy in this UK cohort may be due to smaller study numbers, and shorter follow-up period (875,590 person-years of follow up vs 43.6 million). As such, the lower rates of SLE/CTD seen within this study may represent the time lag from antibody positivity to formal diagnosis, which may be protracted (up to 9 years) and therefore missed by the small study inclusion window observed here (259). Such a proposition is supported by the concomitant increase in rates of non aPL antibodies, also observed within this UK cohort.

The longitudinal nature of this study is a strength when considering the links between pregnancy outcome and CTD, as it allows assessment of relationships between pregnancy outcome and subsequent maternal health. The lag time to diagnosis of CTD, in particular SLE, from detection of the first positive autoantibody to formal diagnosis is significant, at up to 9 years (259); as such it requires longitudinal data in order to assess the relationship to obstetric risk factors. In this regard, this is the first longitudinal study to look in the UK population. A potential weakness of the study is that fetal prematurity was considered an APO. The data could not distinguish

between iatrogenic preterm delivery and spontaneous preterm delivery within the CPRD READ codes. The inability to differentiate between iatrogenic and spontaneous preterm birth means there is potential crossover between maternal disease, such as hypertension, or fetal disease, such as FGR and preterm delivery. All of these can be indications for preterm delivery, for either fetal or maternal wellbeing, and not a reflection of spontaneous preterm birth as a primary risk factor. This then becomes a potential confounding factor and means data on the risk of CTD following a preterm birth need to interpreted with caution and require further study to distinguish whether this risk is mediated by placental disease necessitating preterm birth or spontaneous preterm birth, which is in itself associated with placental dysfunction.

Studies using similar methodology to that described here, have demonstrated that stillbirth, recurrent miscarriage and preterm delivery are independent risk factors for the long term development of cardiovascular disease in women (33–35). Interestingly, CTD is also a risk factor for cardiovascular disease. As this study has demonstrated that women with an APO, in particular those who experienced stillbirth, are at risk of developing CTD, this may contribute to the increased cardiac risk profile in these mothers. Thus, these data provide further support for a link between APO and adverse long term maternal health. Thus, consideration should be given to additional screening in later life for those affected by pregnancy complications, or a lowering of threshold of suspicion for diagnosis of CTD and early referral for specialist care. Early diagnosis would optimise treatment and potentially reduce lifelong cardiovascular risk.

This study has clearly shown that stillbirth and other APOs are associated with increased future prevalence of autoimmune CTD. Whilst this link has been established, the pathological processes underlying this observation are unclear. It has yet to be established as to whether stillbirth triggers autoimmune disease development or if stillbirth occurs as a result of an undiagnosed, and therefore untreated, autoimmune disease. As such this relationship needs delineating further. In order to do this a large scale study prospective study would be required, with women undergoing screening for autoimmune disease in pregnancy. Long-term follow up for both pregnancy outcomes and development of autoimmune disease would help to further evaluate this relationship.

4. An Observational Study of Pregnancy Outcomes in Systemic Lupus Erythematosus and in a Subsequent Pregnancy Following a Stillbirth, including *In Utero* Placental Biometry and Histological Placental Examination

4.1 Abstract

Background – Systemic Lupus Erythematosus (SLE) is a multisystem autoimmune disease associated with increased rates of adverse pregnancy outcomes, including stillbirth. In pregnancies affected by either SLE or stillbirth, there is increased prevalence of histologically abnormal features, including abnormal levels of complement deposition in the placenta. SLE is associated with smaller placentas at delivery compared with those seen in healthy women, and small *in-utero* placentas have been shown to be associated with an increased rate of adverse pregnancy outcomes. We sought to examine pregnancy outcomes, *in-utero* placental size and placental histology at delivery, including complement deposition in women with either: (i) SLE, (ii) prior stillbirth or (iii) uncomplicated pregnancies.

Methods – Pregnant women with singleton pregnancies were recruited at St Mary's Hospital, Manchester, UK. Women with SLE (SLE Pregnant (SP), n=51), prior stillbirth (Previous Loss (PL), n=29) and healthy low risk women (Normal Pregnant (NP, n=44) from 1/12/12-1/1/16. Outcome data for pregnancies were collated, *in-utero* placental dimensions were measured using 2D ultrasound at 17 and 22 (+/- 2) weeks' gestation and placentas were prospectively collected at delivery (SP=13, PL=19, NP=9) for formalin fixation and histological examination. Retrospective identification of placentas from stillbirths was undertaken from the archives at the Department of Histopathology, Royal Manchester Children's Hospitals for the timescale 01/01/2011-31/12/2013. From these, four distinct histopathological groups, commonly seen in SLE, were identified: no histological abnormality seen (normal stillbirth, NSB, n=25), excess perivillous fibrin deposition (F, n=16), villitis of unknown aetiology (V, n=16) and maternal vascular malperfusion (MVM, n=31). Paraffin embedded samples of both these stillbirth and livebirth placentas were stained for anti-complement factor 4d (C4d) or anti-complement factor 3a receptor (C3aR) with a haematoxylin counterstain. The area of positive staining was then calculated using image analysis software.

Results – As expected, the lowest gestational age at delivery was seen in SP ($p<0.01$), and highest percentage of preterm (<37 weeks gestation) births ($p<0.01$) compared to NP and PL. Maternal lupus nephritis was associated with a lower gestational age at delivery than uncomplicated SLE ($p<0.01$, median 249 days vs 273). Customising for gestation using Individualised Birthweight Ratio (IBR) centile showed no difference across the groups. There was no difference in the mode of delivery seen despite a high rate of induction in PL (65%) and SP (47%). Second trimester *in-utero* placental dimensions (width) were smallest in SP and largest in NP ($p<0.05$), differences in depth were not significant but showed a trend towards being highest in SP and lowest in NP; this was reflected in a trend towards lower placental weight at delivery in SP. Complement deposition differed with livebirth and stillbirth placentas. Overall levels of C3aR were highest in livebirth placentas compared to stillbirths ($p <0.0001$), with greatest deposition seen in MVM and NP and lowest in SP. C4d deposition was highest in V and MVM placentas when compared to NSB ($p<0.01$); no difference was seen in C4d deposition between livebirth and stillbirth placentas.

Conclusions – This study shows that pregnancy outcomes for both women with SLE or prior stillbirth can be comparable to those seen in the general population. There was no increased rate of caesarean section in women with SLE, nor a change in IBR, suggesting pregnancy outcomes in this well controlled cohort are better than those previously reported. Complement deposition varied according to the underlying pathology of stillbirth, offering a potential pathological mechanism and therapeutic target for high risk women.

4.2 Introduction

Systemic Lupus Erythematosus (SLE) has a 6:1 female preponderance (78) and is largely a disease of women of reproductive age, with an incidence of 4.9 per 100,000 person years. Maternal SLE is associated with increased rates of early and late fetal loss (3.6% vs 0.05% stillbirth rate in the general population), increased preterm delivery and a higher risk of complications, including fetal growth restriction, hypertension and pre-eclampsia (PET) (2). There is evidence from large cohort studies linking SLE disease activity with adverse obstetric outcomes, with active disease associated with worsening outcomes, but this relationship has yet to be quantified (159,160).

A relationship has, however, been established between the prevalence of histopathological placental abnormalities and maternal SLE, with increased rates of placental pathologies such as exaggerated perivillous fibrin deposition, maternal vasculopathy, villitis of unknown aetiology (VUE) and observed placental infarcts (164–167,172). The increased prevalence of these aberrant features in pregnancies affected by SLE suggests these placentas have reduced reserve, abnormal cell turnover and have undergone excessive lesion repair (164,168,169). The relative rarity of these findings in uncomplicated pregnancies supports the idea that pregnancy complications are increased in SLE patients by exacerbations in placental abnormalities (166,170–172).

The importance of placental abnormalities in stillbirth has already been emphasised, with placental dysfunction playing a reported role in approximately 50% of all cases (44.5-64.3%) (260), with histopathological abnormalities including elevated rates of fibrin deposition, avascular villi, villous immaturity and inflammation compared to those found in equivalent livebirths (72). Although such microscopic changes cannot be detected *in utero* during pregnancy, studies employing ultrasound have demonstrated abnormal placental size in healthy women associated with adverse fetal outcomes. Specifically, pregnancies with abnormally small or thick placentas, identified antenatally, have higher rates of fetal growth restriction, preterm birth and stillbirth (261,262). However, here again the underlying histology in these placentas has not been fully described.

Although SLE is associated with the presence of inflammatory lesions in the placenta, such as VUE, information about the origins and consequences of these lesions is sparse. Studies have demonstrated increased Complement factor 4d (C4d) deposition in the placentas of women with SLE, hypertensive diseases of pregnancy and fetal growth restriction (FGR) compared to healthy uncomplicated pregnancies (187–190). C4d is formed as part of the breakdown of Complement factor 4 (C4) and is typically associated with antibody-mediated rejection, as such it is used as marker of graft rejection in solid organ transplants (263). Excess C4d deposition in the placenta has also been shown to be associated with specific placental lesions, with its expression seen in up to 88% of placentas in which VUE is evident (264). A further association between complement deposition and fetal loss has also been seen with increased levels of C4d deposition in tissue

samples from placentas from first trimester miscarriages (irrespective of chromosome status), and in placentas from recurrent miscarriage (265–267).

Although there is excess presence of C4d in placentas from women with SLE, this may also result from antiphospholipid antibodies (aPL), which are reported in 13-40% of patients with SLE (25-38% Lupus anticoagulant positive, 13-31% anticardiolipin positive, with 10-15% of SLE cases having concurrent antiphospholipid syndrome) (87–89,93–96). There are increased levels of complement split products in the maternal circulation in the presence of aPL, as further evidence of consumption of complement products (212). In addition to the changes seen in C4d levels, the receptor for complement factor C3a (C3aR) has been shown to play an important role in pregnancy pathology. C3aR has been implicated in the development of PET, with studies demonstrating its activation causing increased levels of soluble fms-like tyrosine kinase-1 (sFLT), with a resultant anti-angiogenic effect *in vitro* (268). Similarly increased levels of C3aR have been shown in the serum of pregnancies affected by PET compared to normotensive preterm pregnancies (269). C3aR messenger RNA (mRNA) levels are reduced in placentas of pregnancies complicated by FGR and PET compared to normotensive gestational age-matched controls (270). In murine models of SLE, C3a appears to be pathogenic for the neurological damage seen, with blockade of C3aR in murine models associated with a reduction in neuronal apoptosis (271). In the context of lupus nephritis, increased C3aR deposition is seen in the glomeruli and the intensity of staining is proportional to disease activity (272). Elevated levels of C3aR in the first trimester of pregnancy have been shown to be associated with increased adverse pregnancy outcomes, up to three times that of women with low levels of C3a in serum in the first trimester (185). Thus, both C4d and C3a/C3aR may have a role in mediating the placental lesions observed in pregnancies in women with SLE.

As described above, both SLE and stillbirth are associated with abnormal placental histology. The links between SLE and stillbirth are not, however, limited to the placenta. Firstly, there is an elevated risk of stillbirth in women with SLE. Secondly, stillbirth is also a risk factor for the future maternal development of autoimmune disease, including SLE (RR 3.9, CI 1.96-6.96) (4); this is further discussed in chapter 1 Introduction and chapter 3: - Is Stillbirth a Risk Factor for Development of SLE?. The risk of development of autoimmune disease is highest in the first 4 years following a stillbirth and it has been demonstrated that there can be a significant lag from the development of pathological autoantibodies to the diagnosis of SLE (up to 9 years) (90); given this time lag between antibody development and diagnosis, there may be crossover between the time of pregnancy loss and the presence of autoantibodies. Critically, stillbirth is an important risk factor for poor future outcome; having suffered one stillbirth increases the likelihood of further future stillbirth, with an OR of 4.83 (3.77-6.18) (25). The reasons for this association are not understood, but it is plausible that persistence of SLE or undiagnosed autoimmune disorder could lead to recurrent placental problems. Evidence from large studies into SLE pregnancies have shown the presence of specific autoantibodies to be linked with adverse outcome, with lupus anticoagulant (LAC) being most highly associated with adverse

pregnancy outcomes (162). In the context of stillbirth, 5% of previously healthy women will be shown to have antiphospholipid (aPL) antibodies present at the time of their pregnancy loss (273); this is further described in chapter 6: Pregnant Women with Prior Stillbirth Display a Systemic Immune Response Similar to those with Systemic Lupus Erythematosus, alongside a description of changes at the maternal systemic immune level with pregnancy.

As a result of the data on placental histological changes in SLE and stillbirth, and differences in *in-utero* placental size and outcome, we hypothesised that SLE affected pregnancies and women in a subsequent pregnancy after stillbirth would have worse pregnancy outcomes than their healthy counterparts and that *in-utero* placental biometry and autoantibody profile in these high-risk groups would differ from those seen in healthy uncomplicated pregnancies. In considering the underlying pathological process, we hypothesised that placental complement deposition would be different according to placental histopathology at the time of stillbirth, and in the presence of maternal SLE, or in women with a prior stillbirth. To address these hypotheses, we conducted a cohort study to examine pregnancy outcomes in women with prior stillbirth and women with SLE, and to compare this to health controls. Placentas were examined both for biometry *in utero*, and after delivery. Placental histological examination was performed and compared to stillbirth placentas with specific histological abnormalities as well as quantification of deposition of C4d and C3aR.

4.3 Methods

4.3.1 Recruitment

Pregnant women with singleton pregnancies were recruited at St Mary's Hospital, Manchester, UK, via either a specialist Rheumatology antenatal clinic for women with SLE (SLE Pregnant (SP), n=51), a specialist antenatal clinic for women who previously experienced a stillbirth (Previous Loss (PL), n=29) or routine antenatal clinics for healthy low risk women (Normal Pregnant, (NP), n=44) between December 2012 and January 2016. The study received a favourable opinion from a Research Ethics Committee (13/NW/0158 and 14/NW/1149). All women gave informed written consent before recruitment.

Placentas were prospectively collected at delivery from SLE-affected pregnancies (SP, n=13), women in a subsequent pregnancy following a stillbirth (PL, n=19) and healthy pregnant women with no previous adverse outcomes (NP, n=9). All placental samples underwent full histopathological examination within the Royal Manchester Children's Hospital Pathology Department, and full histology reports were generated. In addition, retrospective identification of antepartum stillbirth placentas was also performed from historic placental histology reports from archive records within the Department of Histopathology, Royal Manchester Children's Hospitals, for the timescale 01/01/2011-31/12/2013 (under ethical approval (14/LO/1352)). For these samples, placentas were excluded if chorioamnionitis (infection of the placenta) was present, or if an associated structural or chromosomal fetal abnormality was indicated in the post-mortem report. Placentas were also excluded if the research clause within any associated

post-mortem had not been agreed. Placentas were grouped according to their histology into 4 categories: no specific histological abnormality (NSB, n=25), excess perivillous fibrin deposition (F, n=16), villitus of unknown aetiology (V, n=16) and maternal vascular malperfusion (MVM, n=31). These categories were as defined by the 2014 Amsterdam Placental Workgroup Criteria (274,275).

4.3.2 Placental complement deposition

Placentas were fixed in neutral buffered formalin and wax embedded for histological analysis at Royal Manchester Children's Hospital Histopathology Department. Tissue sections (4 μm) were counterstained using haematoxylin and blueing reagent and stained with either an Anti-C4d Rabbit Polyclonal (Cell Marque, 1:75 dilution) or an Anti-C3aR Rabbit Polyclonal (Santa Cruz Biotechnology, 1:75 dilution) antibody. Human kidney was used as a positive control. Negative control was achieved by omission of the primary antibody (Figure 22). Staining was kindly performed by the Adult Histopathology Department of the Central Manchester Foundation Hospital.

Images were captured using an Olympus BX41 light microscope at x10 magnification. Each placenta had between 4 and 10 sections available for analysis. A single slide was taken per section and 10 random images captured per slide. Image analysis was performed using HistoQuest image analysis software, DAB (3, 3'-diaminobenzidine) positive and haematoxylin positive cell areas were calculated.

4.3.3 In utero placental biometry

In utero measurements (2D maximal width and maximum depth) of placentas were undertaken at 17 and 22 weeks' gestation (+/- 2 weeks) (276). Measurements were made using a Voluson E6 (GE Healthcare, Hatfield, UK) ultrasound machine with 3D (4-8Hz) curvilinear probe. All measurements were taken and recorded by a Royal College of Obstetricians and Gynaecologists (RCOG) accredited sonographer. Length to thickness (l:t) ratio was subsequently calculated, with an abnormal value at either gestation considered when l:t <2, width <10cm or depth >4cm. (76,229,277).

4.3.4 Disease activity

SLE activity was assessed longitudinally through pregnancy using the SLE Disease Activity Index (SLEDAI)(278). The SLEDAI system uses a numerical value (0-20) to represent disease activity, based on both clinical and laboratory features, with higher scores equating to higher disease activity.

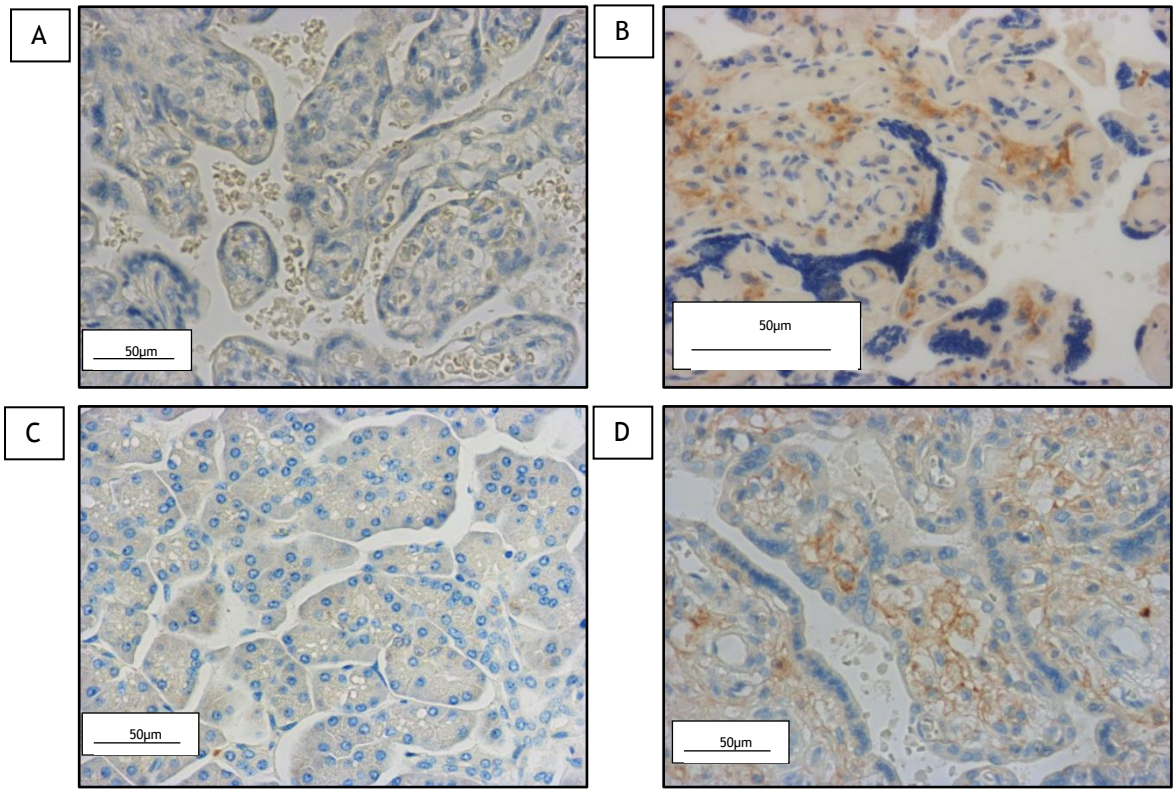


Figure 22 – Examples of complement deposition within the human placenta. A) Negative control for Complement factor 4d (C4d), B) Positive C4d staining of the placenta, C) Negative control for Complement factor 3a receptor (C3aR), D) Positive C3aR staining of the placenta. Scale bar = 50µm in all images, original magnification x10-20.

4.3.5 Antibody status

Antibody status was assessed pre-conception or in the first trimester in the SLE cohort, with serial measurements of double stranded DNA (dsDNA) utilised to indicate disease activity. For both PL and HP cohorts, antibody status was assessed at the time of recruitment to the study, i.e. 17 weeks' gestation (+/-2 weeks). All PL women had a negative antiphospholipid screen at the time of their index stillbirth (prior to pregnancy). Positive antiphospholipid (aPL) antibodies were defined as anticardiolipin (aCL) IgG≥5.7 U/ml, IgM≥10 U/ml and lupus anticoagulant (LAC) as a positive test (Russel Viper Venom Test). In cases of positive antibodies in this pregnancy in either the PL or HP cohorts, these were tested postnatally for persistence and referrals subsequently made for Rheumatology review. Results were revealed to the medical team, but all research data was anonymised, meaning the research team were blinded to the results.

4.3.6 Statistical analysis

All results were analysed with GraphPad Prism 6 (GraphPad Software Inc, USA). Analysis of variance (ANOVA, parametric data) or Kruskal Wallis (non-parametric data) was used to compare three or more groups. T tests or Mann Whitney tests were used for matched gestational comparisons between two groups. Chi squared test was used to compare proportions. Data are displayed as median and interquartile range (IQR) (non-parametric) or mean ± standard deviation (SD) (parametric). Statistical significance was set at $p<0.05$.

4.3.7 Outcome data

Outcome data was collated post-delivery from the medical notes. Individualised birthweight ratio (IBR) centile was calculated using GROW software from the Perinatal Institute (279,280), with an IBR <10 considered as FGR.

4.4 Results

4.4.1 Demographics

Patient demographics are shown in Table 13. As expected by study design there was a difference in parity, with women having previously suffered a stillbirth being exclusively parous (the primiparous woman had experienced a loss at 23+4 weeks gestation). Ethnicity differed across the cohorts with PL women predominantly of Caucasian origin, whereas in the SLE group there were a higher percentage of Asian women. Age and BMI were comparable between groups. There were no smokers in any of the cohorts.

As shown in Table 13, levels of autoantibody positivity were high in the PL cohort at 20.6%, compared to none seen in the healthy pregnancy participants. Of the autoantibodies that were detected, half were aPL antibodies (4/29), of which levels were between 10 and 20 U/ml for IgM and 9.4 for IgG. Whilst showing low level positivity and below the usual threshold of clinical significance, these aPL were considered to be abnormal. They did not, however, persist postnatally. Within the SP group, 25.5% of women were positive for aPL, including 2 women with dual aCL and LAC positivity (3.9%). 7 women in the SP cohort had a history of lupus nephritis (17.6%) as evidence of a more severe disease phenotype, which is typically associated with worse pregnancy outcomes.

Medication used in pregnancy is shown in Table 14; the one woman in the PL cohort using Hydroxychloroquine (HCQ) was also on prednisolone as a treatment regime for previous Chronic Histiocytic Intervillositis (CHI), a placental condition associated with a 67% chance of recurrence and highly associated with perinatal loss (reported rate of 77%) (281).

Table 13 – Cohort Demographics for Pregnant Population Studied.

Group	SLE Pregnant (SP), n=51	Previous Loss Pregnant (PL), n=29	Normal Pregnant (NP), n=44	P value
Age at conception (years)	31.9 (+/- 4.85)	31.07 (+/-5.9)	30.2 (+/-4.8)	0.26
BMI	25.9 (+/-6.1)	26.4 (+/-5.2)	24.6 (+/-3.5)	0.28
Ethnicity (n, percentage)	Caucasian 33 (64.7) Asian 15 (29.4) Black 3 (5.9)	Caucasian 23 (88.5) Asian 2 (7.7) Black 1 (3.8)	Caucasian 36 (81.8) Asian 4 (9.1) Black 6 (13.6)	**<0.0001**
Parity	Primiparous 32 (62.7) Multiparous 19 (37.3)	Primiparous 1 (4) Multiparous 26 (96)	Primiparous 26 (65) Multiparous 14 (35)	**<0.0001**
Gestation at delivery (days)	262.5 (+/-24)	266.8 (+/-5.1)	274.5 (+/-16)	**<0.01**
Induction of Labour Rate	24 (47%)	19 (65%)	9 (20.5%)	**<0.0001**
Mode of delivery	Vaginal delivery 62.8% Normal 44.2% Instrumental 18.6% Caesarean 37.2% Elective 25.6% Emergency 11.6%	Vaginal delivery 62.1% Normal 55.2% Instrumental 6.9% Caesarean 37.9% Elective 34.5% Emergency 3.4%	Vaginal delivery 67.5% Normal 50% Instrumental 17.5% Caesarean 32.5% Elective 17.5% Emergency 15%	0.77
Birthweight (grams)	2934 (+/-793.6)	3221 (+/-279.4)	3401 (+/-494.6)	**<0.01**
Individualised Birthweight Ratio (IBR, centile)	36.9 (+/- 28.3)	49.5 (+/-21.9)	45.38 (+/-31.37)	0.14
Neonatal Unit Admission	6 (11.8)	1 (3.44%)	3 (6.81%)	**<0.05**

Mean with standard deviation in parenthesis. Patient numbers given (n) with percentages of cohort in parenthesis, ANOVA for analysis.

Table 14 – Primary Disease, Antibody Status and Medication Use within the Study Cohorts

Group	SLE Pregnant (SP), n=51	Previous Loss Pregnant (PL) n=29	Normal Pregnant (NP), n=44
Antiphospholipid Syndrome	6 (11.8%)	0	0
Anticardiolipin Antibody Positivity **aCL IgG** **aCL IgM**	5 (9.8%) 6 (11.8%)	1 (3.4%) 3 (10.3%)	0 0
Lupus Anticoagulant	9 (15.7%)	0	0
SSA/Ro positive	7 (17.6%)	0	0
Other Autoantibodies	ANA	ANA speckled 1 (3.4%) ENA 1 (3.4%)	0
SLEDAI (whole pregnancy)	2 (+/- 2.7)	Not applicable	Not applicable
Aspirin	40 (78.4%), dose 75mg	12 (41.4%)	0
Hydroxychloroquine	25 (49%), median dose 200mg (IQR 200-400)	1 (3.4%)	0
Oral Steroids	20 (39.2%), median dose 5mg (IQR 5-10)	1 (3.4%)	0
Azathioprine	8 (15.7%), median dose 89.7mg (IQR 50.7-125)	0	0
Tacrolimus	5 (9.8%), median dose 2.5g (IQR 1-4g)	0	0
Antihypertensive	5 (9.8%)	0	0
Low Molecular Weight Heparin (LMWH)	20 (39.2%)	5 (17.2%)	0

Disease profiles and antibody positivity, expressed as n number (with percentage of cohort in parenthesis).

4.4.2 Pregnancy Outcomes

There were missing outcome data for 3 NP and 4 SP patients, due to participants giving birth at a different unit.

There were no stillbirths, fetal anomalies or chromosomal disorders in any of the pregnancies studied. Likewise, there were was no differences in Apgar scores at 1 or 5 minutes post-delivery. Rates of admission to neonatal intensive care were highest in SP, most likely due to lower gestational age at delivery. As shown in Table 13, this was lowest in women in the SP group, and highest in the NP group. The rate of induction of labour was highest in the PL cohort at 65%, and lowest in NP (20.5%), compared to 47% seen in SP ($p < 0.0001$). The vaginal delivery rate was comparable across all 3 cohorts, with approximately two thirds of each achieving a vaginal birth (SP=62.8%, PL=62.1%, NP=67.5%).

When considering the breakdown of preterm births by degree of prematurity, there were significantly more preterm births (defined as below 37 weeks) in SP (n=11, 23.4%) compared to NP (n=2, 4.9%) and PL (n=1, 3.4%) (p=0.002, Table 13). Of these, 4 in the SP group and 1 in the NP group occurred before 34 weeks. Women with a prior history of SLE nephritis (n=7) had significantly earlier gestational age at delivery (p=0.004) than SP women with no renal involvement, with a median gestational age at delivery of 249 days (IQR 204-259) vs 273 days (IQR 266-280) (Figure 23A).

When using birthweight as a marker of fetal wellbeing, the highest gross birthweight was seen in NP and was comparable in SP and PL (Table 13, Figure 23B); however, gestational age at delivery was also significantly lower in these cohorts. Customising birthweight, using IBR, showed no difference across the three cohorts, but a higher percentage of FGR babies (with a birthweight <10th centile) were defined in SP (17.6%) compared to NP (6.8%) and PL (0%) (p<0.0001). Gross birthweight was not significantly lower in PL women with positive autoantibodies (p=0.21). However, when considering the impact of autoantibody status on IBR in women with a previous stillbirth, the distribution curve shifted to the left, with women with positive autoantibodies having lower IBR than those without (mean 41.9 +/-17.8 vs 51.5+/-22.8). Numbers here were too small to determine statistical significance (Figure 23C). Similarly to the pattern seen for preterm birth, babies of SP women with a history of nephritis had a statistically significantly lower birthweight than in SP with no renal involvement, median 2530g (IQR 2082-2680) vs 3200g (IQR 2815-3558) (p=0.005)). This was reflected in a trend towards lower IBR in women with lupus nephritis (median 19.1 (IQR 12-32.1) vs 36.2 (IQR11.2-65.8), p=0.06) (Figure 23C). The presence of aPL (either aCL or LAC) did not significantly impact on fetal outcomes in either the SP or PL groups.

As shown in Table 14, disease activity was low within this cohort of SLE-affected pregnancies, with no significant change in SLEDAI across gestation. Table 15 shows that maternal complications were more prevalent in SP, although again remained at a low rate, with a total SLE disease flare of 9.8%. Renal flares were more common than non-renal flares, with an incidence of 7.8% vs 2%, occurring exclusively in women with pre-existing renal involvement.

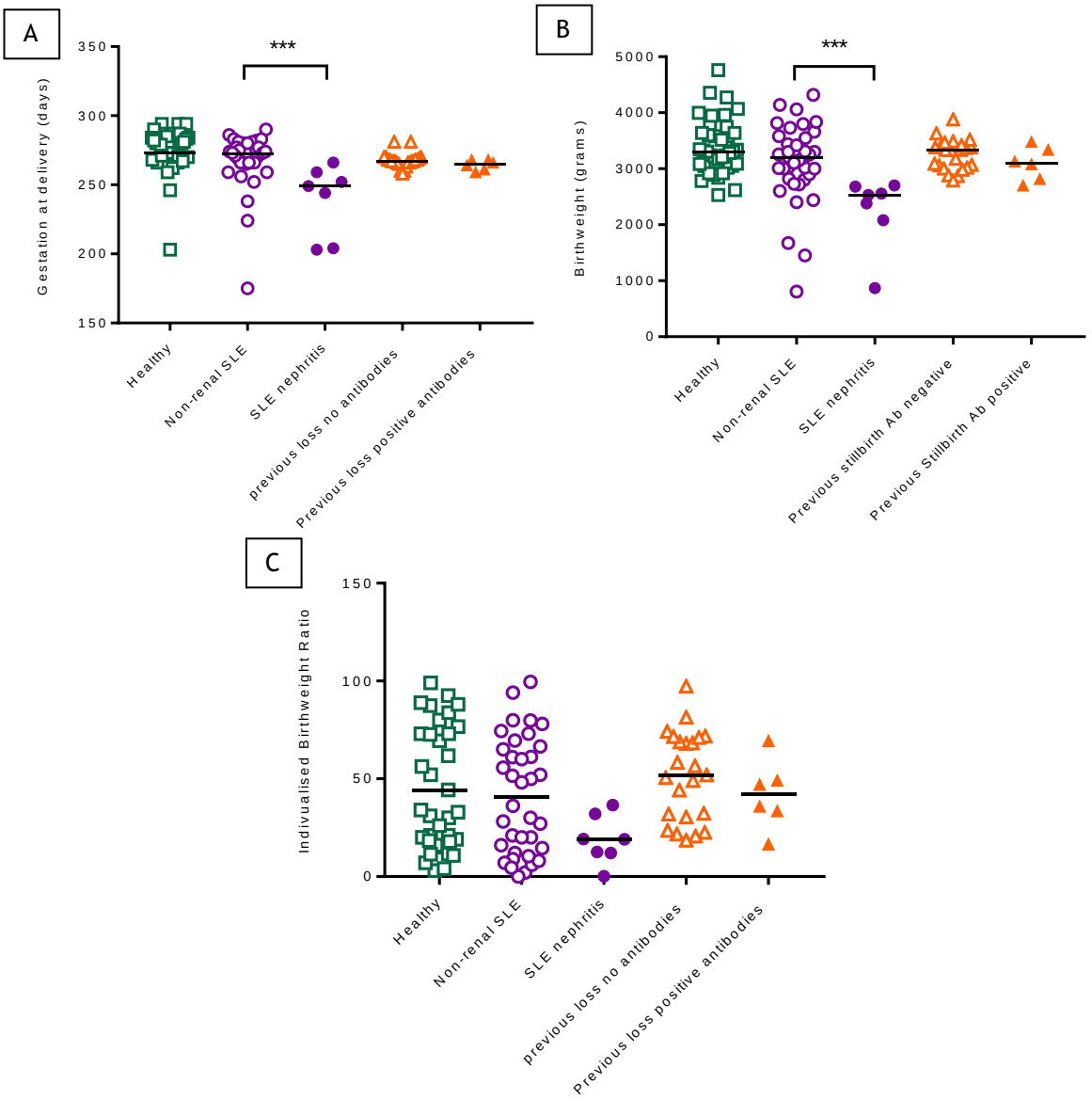


Figure 23 - Impact of disease phenotype on pregnancy outcomes. A) Gestational age at delivery in days, B) Birthweight and C) Individualised Birthweight Ratio at delivery. N numbers, Systemic Lupus Erythematosus (SLE) with no history of nephritis = 36, SLE with history of nephritis =7, healthy n=44. Previous loss, no antibodies n=23, with antibodies n=6. Line at median. Mann-Whitney U test, ***=p<0.001.

Table 15 - Maternal Complications (Pregnancy and Systemic Lupus Erythematosus (SLE) related)

Group	SLE Pregnant (SP), n=51	Previous Loss Pregnant, n=29	Normal Pregnant (NP), n=44
Hypertension (BP≥140/90)	4 (7.8%)	1 (3.4%)	0
Pre-Eclampsia	0	0	1 (2.2%)
Proteinuria (urine protein creatinine ratio >30).	4 (7.8%)	0	0
Non-renal flare	1 (2%)	0	0
Renal flare	4 (7.8%)	0	0
Thrombosis	0	0	0
Pulmonary hypertension	1 (2%)	0	0
Gestational diabetes	1 (2%)	0	0
Obstetric cholestasis	1 (2%)	0	0

Expressed as n number with percentage of cohort in parenthesis.

Table 16 – In Utero Placental Biometry, Placental Weight at Delivery and Fetal Placental Weight Ratio at Delivery.

Group	SLE Pregnant (SP)	Previous Loss Pregnant (PL)	Normal Pregnant (NP)	P value
Maximal width (cm) (17 weeks)	10.1 (+/-1.6)	11 (+/-1.5)	11.4 (+/-1.8)	**0.01**
Depth (cm) (17weeks)	2.68 (+/-0.63)	2.56 (+/-0.64)	2.53 (+/- 0.62)	0.52
Length:Thickness (17weeks)	3.99 (+/-1.2)	4.5 (+/-1.2)	4.8 (+/-1.7)	0.05
Maximal width (cm) (22 weeks)	13.3 (+/-2.1)	14.3 (+/-1.7)	14.5 (+/-1.7)	**0.03**
Depth (cm) (22 weeks)	3.16 (+/-0.9)	3.1 (+/-0.9)	2.9 (+/-0.6)	0.41
Length:Thickness (22 weeks)	4.6 (+/-1.5)	4.9 (+/-1.4)	5.5 (+/-1.8)	0.09
Placental weight at delivery (g)	457.8 (+/-137.6)	490 (+/-109)	496.5 (+/-135.6)	0.41
Fetal:Placental Weight ratio	6.8 (5.8-7.2)	6.6 (5.9-7.6)	7.4 (5.9-8)	0.57

Mean with standard deviation in parenthesis. Measurements in centimetres - weight in grams. Fetal placental weight ratio shown as median and IQR. SLE = Systemic Lupus Erythematosus.

4.4.3 *In-utero* Placental Biometry

Second trimester *in utero* biometry showed placental size (as defined by width) at both 17 and 22 weeks' gestation to be smallest in SP, largest in NP, and intermediate in PL (p=0.01 and 0.03 respectively, Table 16). This decreased width was represented as a trend towards decreased length to thickness ratio in SP, and intermediate values in PL (Table 16, Figure 24). This pattern persisted throughout pregnancy with placentas from SP women being lightest at delivery, and heaviest in NP, with intermediate values again in the PL group (Table 16), although these

differences were not statistically significant. Fetal placental weight ratio (gram fetus / per gram placenta) was highest for women in the NP group, and lowest in the PL group, although this was not statistically significant (p=0.57).

Neither SLE disease activity, nor PL autoantibody status, had an impact on placental biometry *in utero*. Placental weight at delivery was significantly lower in women with a history of nephritis (p=0.008) although this likely reflected reduced gestational age at delivery.

Uterine artery Doppler (UtAD) was also performed in these women (see chapter 5 Longitudinal Vascular Changes of Pregnancy in Women with Systemic Lupus Erythematosus or Prior Stillbirth, page 102 for full details, both UtAD resistance indices (RI) and pulsatility indices (PI) fell with increasing gestation in all cohorts. An abnormal UtAD waveform (notching) was seen in 2.2% SP and 13% NP at 22 weeks gestation. There was no association with outcome with UtAD waveform in NP, but in SP gestation at delivery was lower in SP with UtAD notching.

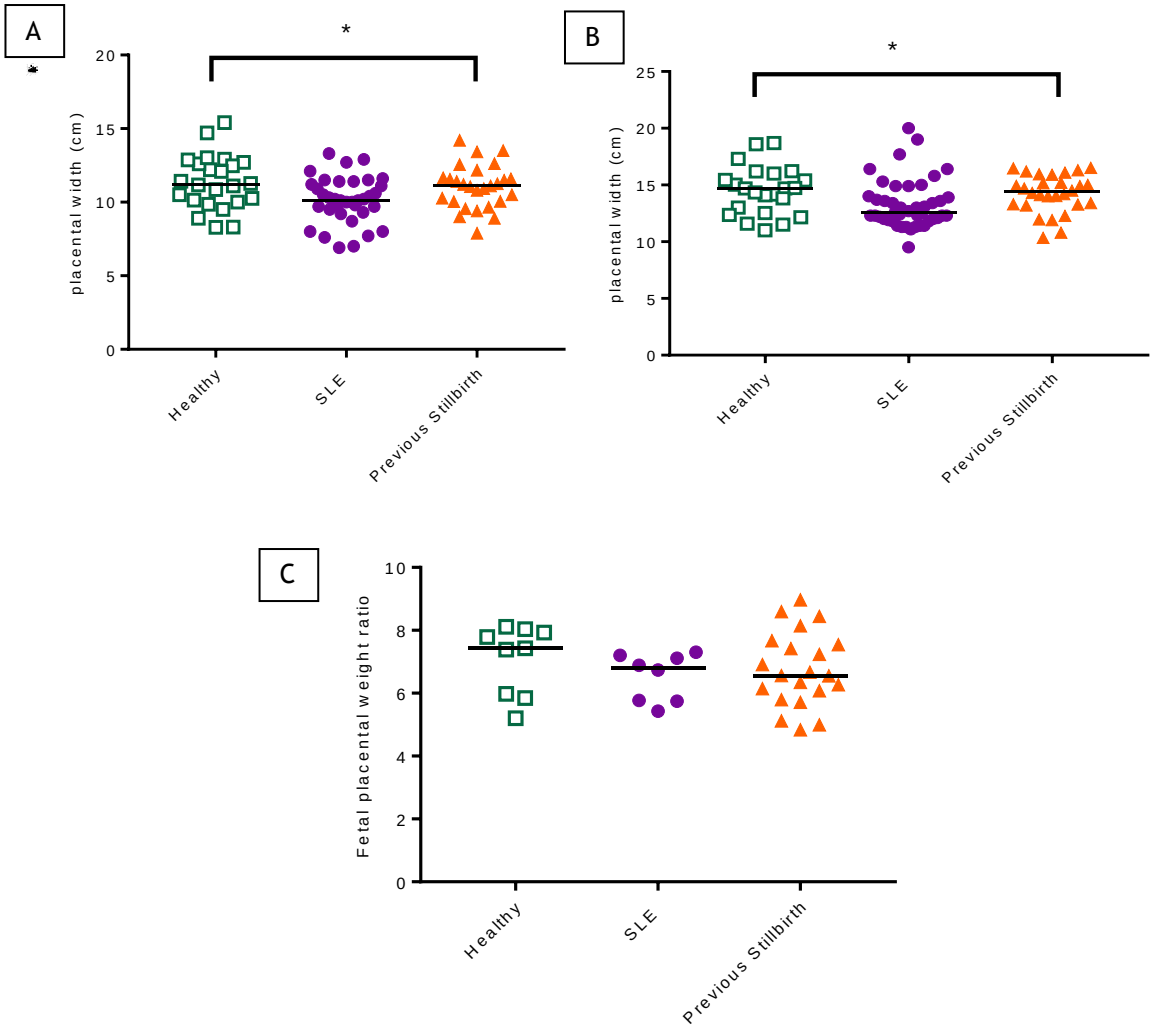


Figure 24 –Second trimester in utero placental dimensions. A) Maximal placental width at 17 weeks' gestation, B) Maximal placental width at 22 weeks' gestation, C) Fetal Placental Weight ratio at birth. One dot per placenta measured. Line at median. *p<0.05. SLE = Systemic Lupus Erythematosus.

4.4.4 Placental Histology and Complement Deposition Cohort Demographics

Table 17 shows the demographics of women with each pathology subtype. There were no statistically significant differences in maternal age across the cohorts, or when considered as two distinct groups (livebirth and stillbirth). Gestational age at delivery was lower in the group who had a stillbirth compared to livebirths ($p<0.001$). There was no difference in gestational age at delivery within the different histological groupings of stillbirth or livebirth.

Within the stillbirth group, there was a significant difference seen in birthweight ($p=0.02$), with the lowest birthweight seen in babies with placentas showing maternal vascular malperfusion (as defined by the 2014 Amsterdam Placental Workgroup Criteria: presence of distal villous hypoplasia, villous infarcts and accelerated villous maturation) (274,275), and the highest in those with no underlying pathology seen in their placentas. In live born pregnancies with placentas available for analysis, birthweight was lowest in SLE-affected pregnancies and highest in healthy pregnancies, although this difference was not statistically significant.

Placentas from stillbirths were smaller than their healthy counterparts, with a lower weight at delivery compared to livebirths ($p<0.001$). Placentas from stillbirths were shorter in both of their longest dimensions than those from livebirths ($p<0.0001$), depth was not statistically significantly different (p 0.06) but there was a trend towards increased depth in SLE placentas, and thinnest in healthy placentas. In line with fetal weight, placental weight was also lowest in those placentas with evidence of failure of maternal vascular adaptation ($p=0.04$). Consequently, the highest fetal placental weight ratio was also seen in those stillbirths with no underlying pathology and the lowest in placentas showing maternal vascular malperfusion.

4.4.5 Histological features of livebirth placentas

As shown in Table 18, there was a difference in the prevalence of differing histological features in the placentas of livebirths. The frequency of syncytial knots was significantly higher in SP than NP, and intermediate in PL. Conversely, the highest rate of normal morphology was seen in NP and lowest in SP.

Table 17 - Cohort Demographics of Placentas Examined. Median and interquartile range values shown. VUE = Villitus of unknown aetiology, SLE= Systemic Lupus Erythematosus

	Group	Maternal Age (years)	Gestation at Delivery (weeks)	Birthweight at Delivery (grams)	Placental Weight (grams)	Fetal Placental Weight Ratio
Adverse outcome	No Pathology (n=23)	30 (23.75-34)	29 (22-39)	2600 (637.4-3065)	320 (152-394)	7.1 (2.6-8.1)
	Perivillous Fibrin (n=15)	29 (23-33)	36.5 (30.75-39.25)	2220 (1510-3220)	358.5 (229.8-426)	5.9 (5.3-6.7)
	Villitus of Unknown Aetiology (VUE) (n=14)	32.5 (23-38.75)	34 (32-38)	2110 (876.6-2940)	331 (140-364)	6.5 (5.2-8.4)
	Maternal Vascular Malperfusion (n=28)	30 (28-33)	28 (27-35)	981 (552.5-1814)	224 (135-308.8)	5 (3.9-5.9)
Livebirth	Normal Pregnant (n=9)	34 (25-40)	38 (37-39)	3520 (3225-3936)	474.5 (399.5-699.5)	7.6 (6.2-8.0)
	SLE (n=13)	32 (26-36)	38 (38-39)	3100 (2775-3708)	475 (375-547)	6.8 (5.9-7.2)
	Previous Loss (n=19)	33 (28-35)	38 (37-38)	3338 (2915-3410)	454 (430-576)	6.6 (5.6-7.5)

Table 18 – Prevalence of Histological Features in the Placentas of Livebirths. N numbers shown with percentages in parenthesis. SLE= Systemic Lupus Erythematosus

	Increased syncytial knots	Thrombi	Perivillous Fibrin Deposition	Villitis	Immature Villi	Chorio-amnionitis	Infarcts	Calcification	Normal Morphology
SLE Pregnant, SP (n=13)	7 (53.8)	2 (15.4)	3 (23.1)	2 (15.4)	4 (30.8)	1 (7.7)	1 (7.7)	1 (7.7)	0
Previous Loss, PL (n=19)	6 (31.6)	1 (5.3)	4 (21.1)	5 (26.3)	9 (47.4)	2 (10.5)	0	0	2 (10.5)
Normal Pregnant, NP (n=9)	1 (11.1)	0	0	0	4 (44.4)	0	0	0	2 (22.2)

4.4.6 Placental Complement Deposition

Complement deposition (in the form of C4d and C3aR) was examined in placentas to investigate the level of deposition according to histological findings in stillbirth and the maternal disease state. As shown in Figure 25A, the percentage area of C4d deposition in stillbirth placentas was significantly different across the 4 histopathological groups examined (p=0.006). C4d deposition was lowest in women with no underlying pathology, and highest in those with evidence of poor vascular adaptation (p=0.01) or villitis (p=0.02). In livebirths, there was no difference in C4d deposition in the placenta between groups (Figure 25B). There was no difference in levels of C4d deposition seen when livebirth and stillbirth were compared as whole cohorts (Figure 25C). Examples of staining are shown in Figure 26, with staining predominantly localised to the trophoblast.

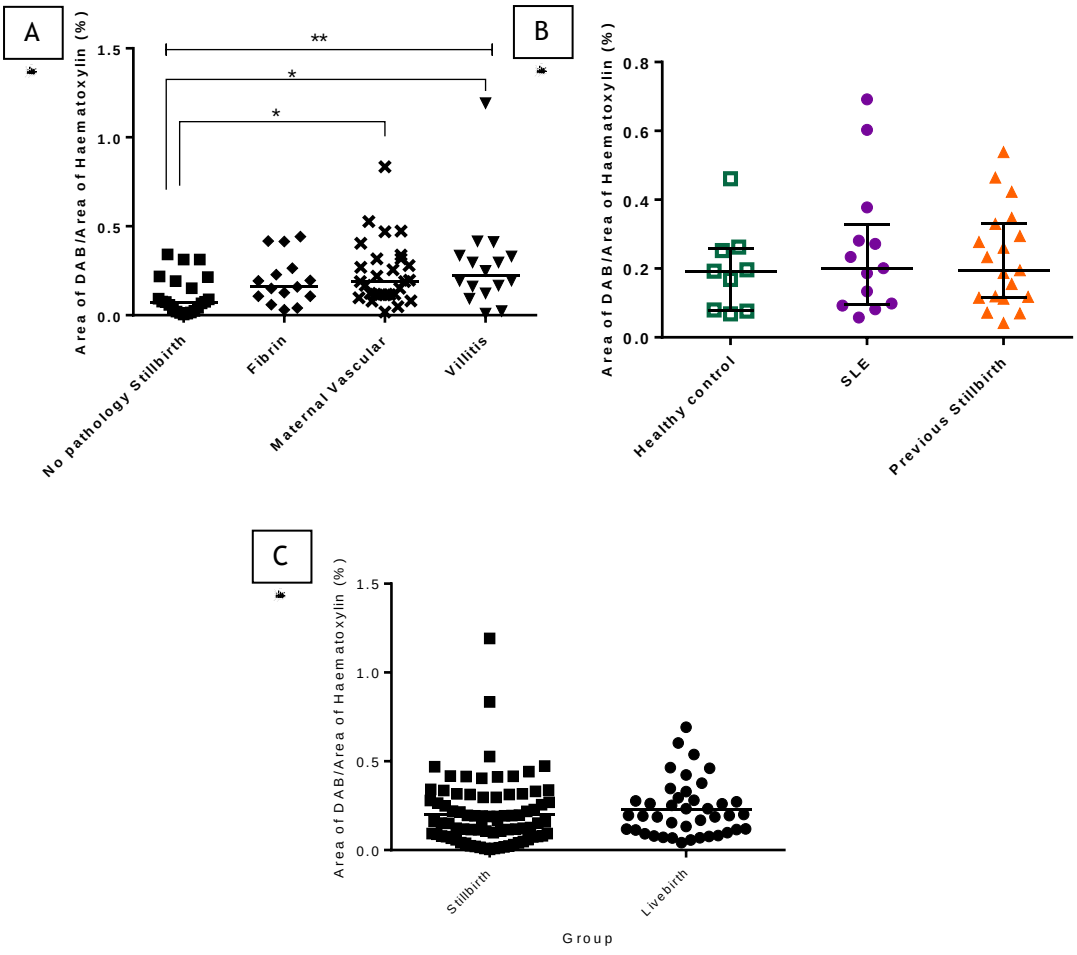


Figure 25 - Quantification of Complement factor 4d (C4d) staining (using HistoQuest image analysis software). A) C4d staining in stillbirth placentas, B) C4d staining in livebirth placentas, C) C4d staining in stillbirth vs livebirth placentas. Kruskal Wallis test with Dunn's post hoc, line at the median value. SLE= Systemic Lupus Erythematosus

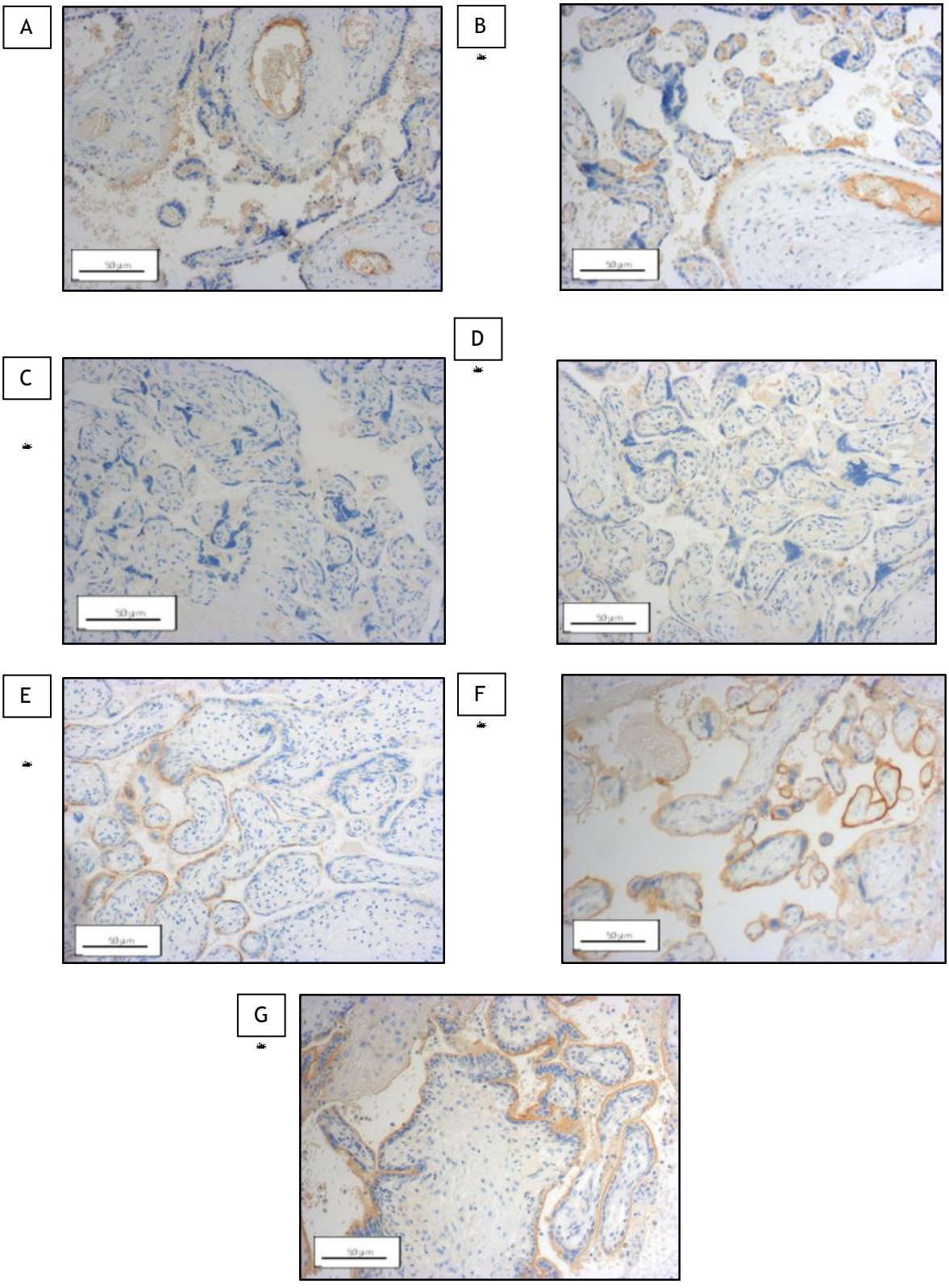


Figure 26 - Examples of Complement factor 4d (C4d) Staining in each Livebirth Group and Stillbirth Histological Subtype. A) Livebirth Systemic Lupus Erythematosus (SLE), B) Livebirth with Previous Stillbirth, C) Healthy Pregnant, D) No Histopathological Abnormality Stillbirth, E) Increased Perivillous Fibrin Stillbirth, F) Maternal Vascular Malperfusion Stillbirth, and G) Villitis Stillbirth. All images at x10 magnification. Scale bar 50µm.

As demonstrated in Figure 27A-C and Figure 28, the levels of staining for C3aR were significantly higher in the presence of maternal vascular malperfusion in the stillbirth placentas (p<0.0001). In a different pattern to that seen with C4d, there was a significant difference in the livebirth placentas, with the highest levels of staining seen in healthy livebirths, and lowest in lupus placentas (p=0.02). Levels of C3aR were significantly higher in livebirths than in stillbirth cases, (p<0.0001, Figure 27C). The C3aR staining was localised to the capillaries as opposed to the trophoblast localisation of C4d seen.

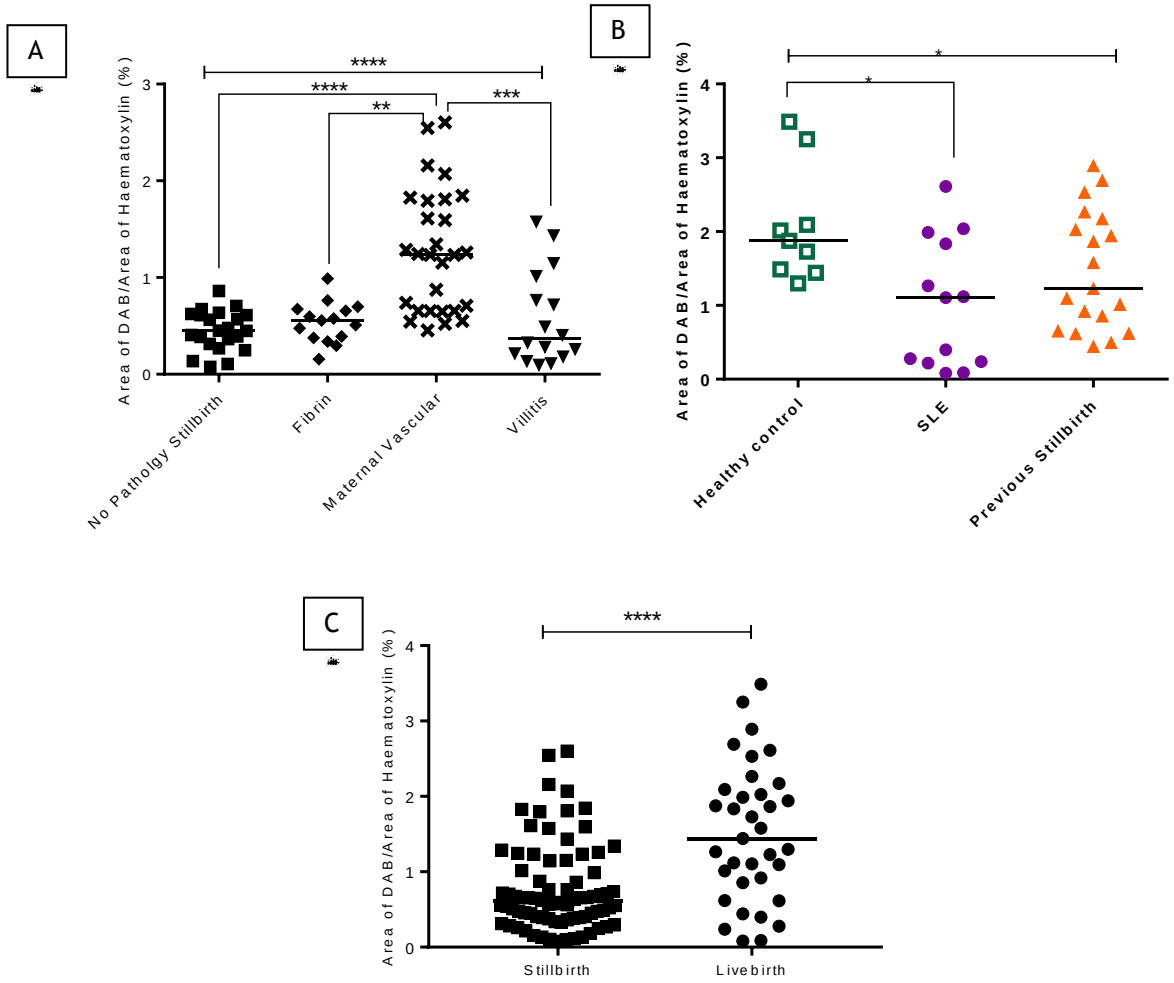


Figure 27 - Quantification of Complement factor 3a receptor (C3aR), Complement staining (using HistoQuest image analysis software). A)C3aR staining in stillbirth placentas, B) C3aR staining in livebirth placentas, C) C3aR staining in livebirth vs stillbirth placentas. Kruskal Wallis test with Dunn's post hoc, line at the median.

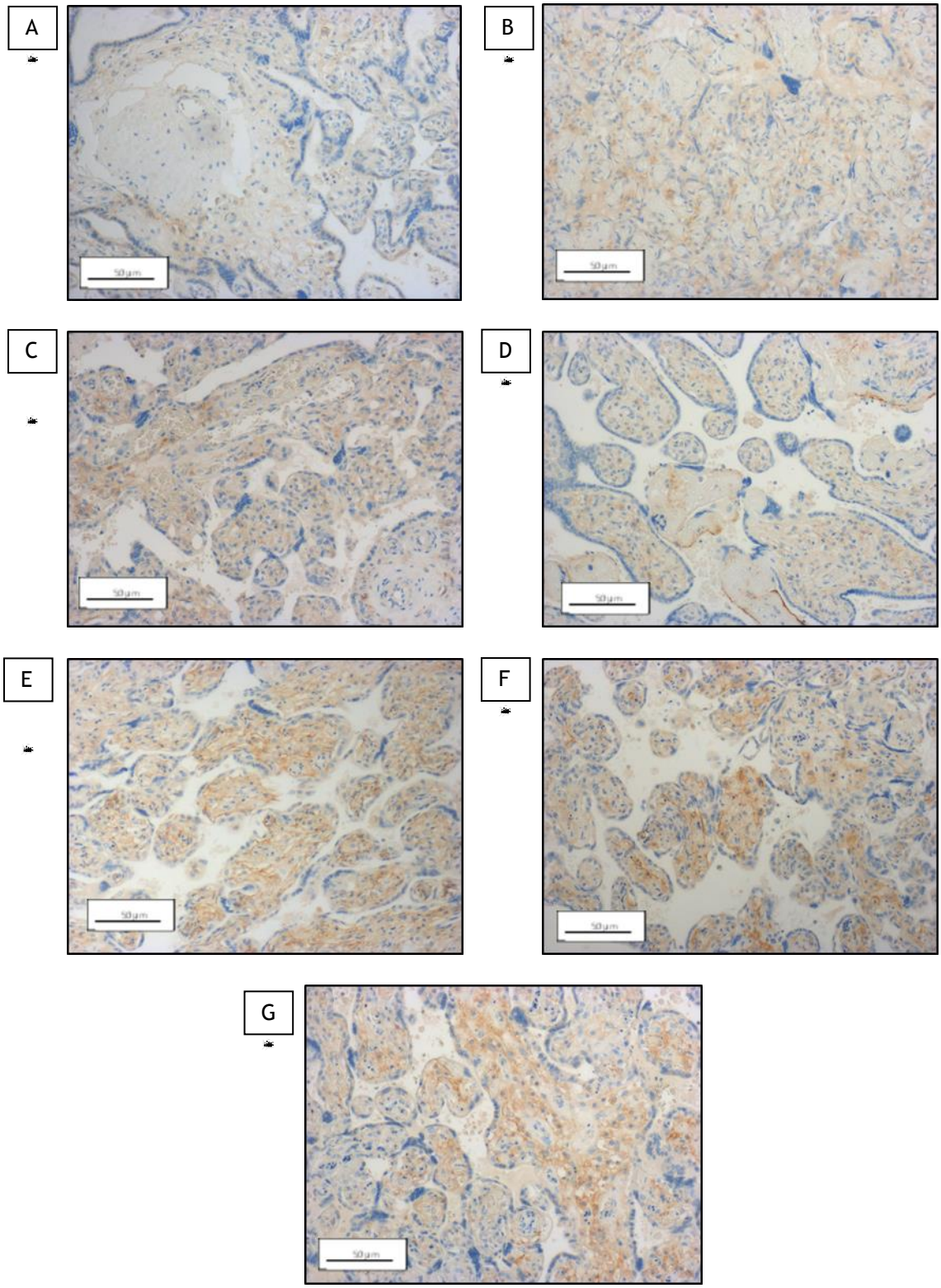


Figure 28 - Examples of Complement factor 3a receptor (C3aR). Staining in each Livebirth Group and Stillbirth Histological Subtype. A) Livebirth ystemic Lupus Erythematosus, B) Livebirth with Previous Stillbirth, C) Healthy Pregnant, D) No Histopathological Abnormality Stillbirth, E) Increased Perivillous Fibrin Stillbirth, F) Maternal Vascular Malperfusion Stillbirth, and G) Villitis Stillbirth. All images at x10 magnification. Scale bar 50µm.

4.5 Discussion

There is a body of evidence linking SLE and stillbirth, both by the increased risk of stillbirth seen in SLE and the increased prevalence of SLE diagnosis in women following a stillbirth (2,4,5). This study is the first to examine these two high risk populations of pregnant women (with SLE and pregnancy loss) and compare outcomes to healthy uncomplicated pregnancies. Women in both the SLE and previous pregnancy loss group had elevated levels of autoantibodies (20.6%) over those of healthy pregnancy. Although overall outcomes were favourable, worse pregnancy outcomes were seen in women with lupus nephritis, as a more severe phenotype of disease. Outcomes in women with a previous stillbirth were also favourable, with no adverse fetal outcomes recorded in their subsequent pregnancy. There was a trend towards a reduction in customised birth weight centile in women with previous stillbirth and positive autoimmune antibodies, even at low titres, perhaps suggesting a further means of risk stratifying this group and emphasising the need for retesting the autoantibody status. Additional surveillance of fetal growth for those with raised autoantibody titres when defined in their ongoing pregnancy may also be advised, rather than just pre- or post-natally.

This study recruited pregnant women from a tertiary centre, with specialist multidisciplinary team (MDT) clinics for both women with connective tissue diseases in pregnancy and women with prior stillbirth. This facilitated recruitment to this novel study, but may also be a limitation, as it might account for the low rate of adverse outcomes seen (flare rate 9.8% in this cohort vs literature rates of 13-68%) (2) . This may relate to optimal disease monitoring and management, resulting in better outcomes and lower disease flare rates. As a consequence this study may underestimate the complication rate seen in SLE and pregnancy in populations without such a service. The low rate of complications observed supports evidence that appropriate MDT management and optimal treatment reduces pregnancy complications in women with both SLE and previous stillbirth. Whilst this has previously been demonstrated and recommended in SLE (153,282,283), this is not the case for women with previous stillbirth. However, such management perhaps introduces potentially confounding effects. For example, use of low molecular weight heparin was high, which may influence the results as murine studies have demonstrated improved pregnancy outcomes in APS, presumed to be as a result of the anti-complement actions of heparin (213,214).

In addition to the potential differences in management as a result of MDT care, there were also differences in the demographics of this cohort from those typical of SLE. There was significant ethnic variation in the SLE cohort, although a lower than expected percentage of Afro-Caribbean women was seen (prevalence of SLE in Afro-Caribbean women 3 times that seen in women of Caucasian descent (284)). Typically, the prevalence of SLE is highest in women of Afro-Caribbean descent and lowest in Caucasian women (245), with Afro-Caribbean descent associated with an increased incidence of renal involvement (285,286). As such, the demographics of this study (5.9% Afro-Caribbean, 29.4% Asian and 64.7% Caucasian) may represent the low disease severity

seen, or be a reflection of the geographical area, which is predominantly of Asian/Caucasian origin.

Overall numbers in this study were low, however, given the rarity of both SLE in pregnancy and the relative infrequency of subsequent pregnancy following stillbirth they are likely representative.

In utero placental size was measured in the second trimester (17 and 22 weeks, +/1 2 weeks); for both these gestational ages placental width was smallest in the SLE cohort and largest in the healthy cohort, with those women who had prior stillbirths displaying intermediate values. Differing *in utero* placental biometry in the second trimester is consistent with data showing placentas from SLE-affected pregnancies to be smaller at delivery than their gestation-matched healthy counterparts (172). In considering the underlying histopathology of these placentas, histological analysis of the placentas of livebirths showed a distinct difference in the prevalence of markers of cell turnover, as demonstrated by increased levels of syncytial knots seen in SLE-affected pregnancies compared to their healthy counterparts (164–167,172). Given the high prevalence of placental abnormalities seen in the SLE and PL groups, and the observation that placental pathology can recur, it is therefore possible that these *in utero* similarities may reflect a similar underlying placental pathology, and as such opens up a potential area of further research as a therapeutic target.

Within the placentas analysed for complement deposition, the lowest placental weight and lowest fetal weight:placental weight ratio at birth was seen in stillbirths with evidence of maternal vascular malperfusion in the placenta, considered a marker of failure of adaptation of the maternal vasculature to pregnancy. This is in keeping with the wider literature regarding low birthweight and pre-eclampsia affected pregnancies (287). The absence of a reduced birthweight in stillbirths with no obvious pathological cause on placental examination suggests a role for a pathology separate to the typical “placental insufficiency” thought of in FGR and stillbirth, the nature of which is yet to be determined. Complement deposition (both C4d and C3aR) was highest in placentas with evidence of either maternal vascular malperfusion, or villitis. Placental villitis is considered to be an immune reaction in the absence of an infective cause, as such villitis is thought to represent autoimmunity at the fetal-maternal interface (288). The presence of increased complement deposition in these villitis placentas suggests that complement plays an important role in the pathology seen and as such offers a potential therapeutic target. Histologically, these may represent two groups of patients, those with PET, and those with an abnormal autoimmune response at the fetal-maternal interface.

Maternal complications were low in the women in this study, including low rates of SLE disease flare. This low flare rate and quiescent disease state may contribute to the positive pregnancy outcomes seen within the SLE cohort (289). Additionally, a low prevalence of LAC expression in this cohort (15.7%) may further explain the low fetal complication rate seen. Large cohort

studies of women with SLE and/or concurrent APS show APO to have an association with LAC but not aCL, with the presence of LAC associated with an odds ratio (OR) of 8.32 [CI, 3.59 to 19.26] for APO, in particular stillbirth (87,162). In considering the impact of disease activity on maternal and fetal outcomes, we utilised a history of lupus nephritis as a proxy for more severe disease, and in this subgroup of women the presence of maternal lupus nephritis (even if quiescent) was associated with preterm delivery and lower birthweight in line with other published data (87). In contrast to published literature, which shows an increased risk of caesarean section in women with SLE (290), this study showed no difference in mode of delivery for SLE pregnancies despite a higher induction of labour rate, suggesting SLE itself does not confer an increased risk of caesarean section (CS), nor is it a contraindication for vaginal delivery. Overall CS rates were, however, high in all groups in this study.

The prevalence of antibody positivity in women with a prior stillbirth was higher than expected at 20.6% compared to none in healthy women. The levels of these autoantibodies in subsequent pregnancy following stillbirth have not previously been reported. Similarly to nephritis in the SLE cohort, low level antibody positivity in women with previous stillbirth could potentially be used as a proxy for a more severe disease phenotype, and was linked to worsening fetal outcomes in this study (with a trend towards lower IBR at delivery seen in women with autoantibodies present). This contrasts with research into maternal health and the clinical importance of low level aCL, which showed that low levels of IgG or IgM aCL conferred no additional health risk (291). Anti-b2 glycoprotein I antibodies (anti-b2GPI) were not evaluated in this study as they were not part of routine clinical practice at the time, as such there is a potential for under-representation of aPL within these cohorts. This study demonstrates the utility in reassessment of maternal autoimmune antibody status in subsequent pregnancy and using this to further risk-stratify a high risk population alongside an opportunity to re-evaluate appropriate treatment interventions. For the 20.6% of PL women in this study with positive autoantibodies, these antibodies were present in the pregnancy following a stillbirth, and were previously not present at the time of stillbirth. There was no persistence of aCL antibodies on testing postnatally in these women. This fluctuation in aCL antibodies has not been demonstrated in the context of APS and or SLE in pregnancy, with large cohort studies showing minimal change in levels of aCL and LAC longitudinally throughout pregnancy, although here only aCL levels over 40 U/ml (292) were examined. In contrast, studies of pregnancies in a mixed autoimmune cohort showed that only 25% of women who initially had positive aPL continued to remain so over a period of 10 years, and short term fluctuation was not seen (293). Whilst the levels of aCL in this study were low level, the presence of these antibodies is associated with long term risk of maternal development of both vascular and thrombotic disease (294–296) and offers a potential public health role for extended screening of women with a stillbirth.

This study has shown that, with multidisciplinary management and low disease activity, women with SLE can have pregnancy outcomes better than those reported in the wider literature, including no increased risk of caesarean section. Risk stratification by disease phenotype could

classify these pregnancies into the highest risk groups, with potential for those women with SLE with no deep organ involvement to be managed in secondary rather than tertiary level care. The change in outcomes in women with a previous stillbirth and positive autoantibodies compared to those without, in both pregnancy outcomes and complement deposition in the placenta, suggests that a similar approach could be taken in these high risk women. This study also supports retesting for autoantibodies in subsequent pregnancies following stillbirth.

It has been shown here that women who have had a previous stillbirth have outcomes similar to those seen in women with SLE. This suggests that in some cases of stillbirth, there may be similar pathogenesis of these outcomes. The similarities seen in both C4d and C3aR deposition in the placentas and the frequency of abnormal placental histopathological features supports this hypothesis. In linking to known histological features seen in the placenta, the increased rate of deposition of complement factors associated with villitis, a known autoimmune condition of the placenta, suggests that this process may be autoimmune in origin.

In further investigation of this area, a more extensive examination of the placentas of women with previous stillbirth or SLE in both the presence and absence of treatment may show common histological features, and may help to further establish and explain the similarities seen between pregnancies affected by SLE and those with prior stillbirth. In unravelling these similarities it may be possible to identify a common aetiology that can be screened for in otherwise “healthy” pregnancies in order to reduce stillbirth prevalence. In extrapolating data from women with SLE and immunosuppressant usage in a larger study of placental changes and pregnancy outcomes, a novel therapy for prevention of recurrent stillbirth could be identified.

5 Longitudinal Vascular Changes of Pregnancy in Women with Systemic Lupus Erythematosus or Prior Stillbirth

5.1 Abstract

Introduction: Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease associated with hypertensive disease and increased vascular stiffness. Pregnant women with SLE face increased risks of pre-eclampsia and adverse fetal outcomes. In the absence of SLE, women who have experienced a stillbirth are also at increased risk of cardiovascular disease. In order to address the hypothesis that pregnant women with SLE or prior stillbirth will display an increased level of vascular stiffness in pregnancy, with altered circulating vascular factors (PlGF and sFLT-1) compared to healthy pregnant women, longitudinal changes in brachial blood pressure (BP), arterial stiffness and uterine artery Doppler (UtAD) were recorded alongside the circulating vascular factors placental growth factor (PlGF) and soluble Fms-like tyrosine-kinase 1 (sFLT-1) throughout pregnancy

Methods: Women with singleton pregnancies were recruited from St Mary's Hospital, Central Manchester University Hospitals Foundation NHS Trust, Manchester, UK , between December 2012 and January 2016 into 3 cohorts: pregnancies with SLE (SP, n=51), prior stillbirth (PL, n=29) or healthy uncomplicated pregnancies (NP, n=44). Arterial stiffness was measured using photoplethysmography (stiffness index (SI), and reflection index (RI)) and arteriography (pulse wave velocity (PWV)); BP was recorded with an automated BP monitor. Measurements were taken at 17, 22, 28 and 36 weeks' gestation (+/- 2 weeks). UtAD measurements were taken at 22 weeks gestation, while resistance index (UtRI) and pulsatility index (PI) were recorded. Maternal serum PlGF and sFlt-1 were measured at 28 weeks and 36 weeks gestation (+/- 2 weeks).

Results: Mean arterial BP was highest in PL and lowest in NP at all gestations (all $p<0.05$). Moreover, at all time points, systolic BP was significantly higher in PL women (all $p<0.001$). Both SP and NP displayed the expected second trimester drop in diastolic BP; this was not seen in PL. No difference in heart rate was seen across cohorts. Vascular stiffness was higher in SP women as shown by a higher PWV in SP at 17-28 weeks gestation (all $p<0.05$). In all cohorts, PWV increased with gestation. Similarly, RI increased from 28-36 weeks in SP ($p<0.05$), while SI was unchanged longitudinally. Locally to the uterus, both UtAD PI and RI fell with increasing gestation, and were lowest in PL at 17 weeks ($p=0.04$ and $p=0.0007$). Second trimester (22 weeks) UtAD showed notching in 2.2% SP, 0% PL and 13% NP. In SP women, gestation at delivery was lower in the presence of notching (median 252 days vs 271.5, $p=0.009$) compared to normal waveform SP. This relationship was not seen in NP. As expected, PlGF levels fell with increasing gestation in all cohorts. 10% of SP had PlGF <12pg/ml at 36 weeks, compared to nil NP/PL ($p<0.0001$). Levels between 12pg/ml and 100pg/ml were also highest in SP and PL compared to NP ($p=0.0002$). The same pattern was seen at 28 weeks ($p<0.0001$). At 36 weeks sFLT-1 was highest in SP and lowest in NP ($p=0.007$); no difference was seen at 28 weeks. There was no difference in the ratio of PlGF to sFlt-1 at either gestational age (28 and 26 weeks) in any cohort.

Conclusion: Women with SLE have higher PWV in pregnancy than both women with prior stillbirth and healthy women. Longitudinally, women with SLE undergo the same pattern of change in brachial BP seen in healthy women, with a drop in second trimester diastolic BP. Women with prior stillbirth maintain a higher BP throughout pregnancy, irrespective of heart rate and do not display a second trimester drop in diastolic BP. These vascular differences were not reflected in levels of circulating vascular markers. The changes in BP suggest an underlying vascular pathology to stillbirth, potentially akin to that seen in SLE, and warrants further investigation.

5.2 Introduction

Systemic Lupus Erythematosus (SLE) is a multisystem autoimmune disorder with varying clinical manifestations. The highest prevalence (1 in 500) occurs in women of childbearing age (297), therefore the impact of SLE on pregnancy (and vice versa) is an important clinical consideration.

Outside pregnancy, SLE is associated with exaggerated risk of cardiovascular disease (CVD) with up to 50 times increased risk compared to healthy women (126–128). This is predominantly linked to atherosclerosis, which is twice as common in SLE than in the healthy population (129), and acts as an independent risk factor for coronary artery disease (CAD) (125). Inflammation, hyperlipidaemia, hypertension and prolonged glucocorticoid use associated with SLE are considered a contributory factors for CAD, specifically impaired vascular endothelial function (136). Increased levels of vascular stiffness and higher rates of hypertension within this SLE group portend this suggestion (130,131), with increased vascular stiffness related directly to worsening disease activity (132,133).

Akin to SLE, pregnancy itself induces significant changes in maternal cardiovascular function, with shifting fluid volumes resulting from increased cardiac output from mid-first trimester and reduced second trimester blood pressure and arterial stiffness, with a gradual increase towards term (298,299). These changes occur both systemically and locally to the uterus. Overall, the effect of these changes in seen in maternal blood pressure (BP), with “normal” healthy pregnancies displaying a second trimester drop in diastolic BP. This is not seen in women who develop pregnancy induced hypertension/pre-eclampsia (PET) (300,301).

Failure of the maternal vascular system to adapt to pregnancy has been linked to maternal disease such as pre-eclampsia, which is associated with failure of the maternal spiral arteries to adapt to pregnancy (302,303). In SLE, this presumed failure of maternal vascular adaptation manifests with pregnancies at higher risk of complications and is associated with vascular disease (304). These vascular complications include increased rates of hypertension (16.3%) and pre-eclampsia (PET) (7.6%) compared to healthy pregnancies (2). In addition to the maternal vascular complications, there is an increased incidence of fetal complications, including early and late fetal loss (stillbirth rate of 3.6%), preterm delivery (39.4%), and fetal growth restriction (12.7%). The underlying pathology for the increased rates of fetal complications seen in SLE is unclear, and thought to have its basis in both maternal vascular dysfunction and abnormal maternal immunity and subsequent placentation.

Interestingly, even in the absence of autoimmune disease, women who suffer a stillbirth or miscarriage have an increased risk of CVD in later life (305,306). This risk is proportional to the number of losses, with a single stillbirth increasing CVD relative risk (RR) to 1.24 and multiple, early pregnancy losses, further exaggerating the risk, such that after 5 or more miscarriages the RR stands at 1.55 (305). Although widely acknowledged, links between these aetiologies and increased lifetime cardiovascular risk, are yet to be established.

In pregnancies at high risk for development of PET, the measurement of maternal circulating vascular markers is being developed for use as a predictive tool. Maternal circulating pro-angiogenic placental growth factor (PlGF) and the anti-angiogenic Soluble Fms-like tyrosine kinase-1 (sFlt-1) are both primarily formed in the placenta during human pregnancy (307,308). Acting reciprocally, PlGF and sFlt-1 can be considered in combination, with the ratio between PlGF and sFLT-1 used to predict development of PET, with a ratio of >38 (in the absence of SLE) considered predictive of subsequent development of PET (44). SLE complicates the diagnosis of PET, as the renal disease in SLE-related nephritis manifests similarly, and presents with the same clinical picture with proteinuria and maternal hypertension as hallmarks. In these cases, a PlGF/sFLT-1 ratio of >85 has been suggested to be diagnostic of PET, although this has yet to be fully confirmed in larger clinical studies (207).

In summary, both SLE and previous stillbirth are associated with long term cardiovascular disease, and with increased rates of pregnancy complications. We hypothesise that pregnant women with SLE or prior stillbirth will display an increased level of vascular stiffness in pregnancy, with altered circulating vascular factors (PlGF and sFLT-1) compared to healthy pregnant women. To address this hypothesis, we considered pregnancies complicated by either SLE or previous stillbirth and healthy pregnancies. Mothers who attended specialist multidisciplinary clinics for either connective tissue disease in pregnancy or previous stillbirth were recruited to determine longitudinal systemic changes in brachial blood pressure, arterial stiffness and compliance, and local changes in uterine artery Doppler parameters throughout pregnancy.

5.3 Methods

5.3.1 Recruitment

Pregnant women with singleton pregnancies were recruited from St Marys Hospital, Central Manchester University Hospitals Foundation NHS Trust, Manchester, UK, via either a specialist Rheumatology antenatal clinic for women with SLE (SLE Pregnant (SP), n=51), a specialist antenatal clinic for women who previously experienced a stillbirth (Previous Loss (PL), n=29), or routine antenatal clinics for healthy low risk women (Normal Pregnant (NP), n=44) between December 2012 and January 2016. The study was given a favourable opinion by ethics committee (13/NW/0158 and 14/NW/1149). All women gave informed written consent. All women were over age 18 at the time of recruitment, and could speak/read English (written information on the study and consent forms were only available in English). Multiple pregnancies were excluded. Women with a prior stillbirth were excluded if a chromosomal or physical abnormality had been observed in their previous pregnancy loss.

SLE activity was assessed longitudinally throughout pregnancy using the SLE Disease Activity Index (SLEDAI)(278). The SLEDAI system uses a numerical value (0-20) to represent disease activity, based on both clinical and laboratory features, with higher scores equating to higher disease activity.

5.3.2 Vascular measurements

All vascular assessments were undertaken by appropriately trained medical staff at 17, 22, 28 and 36 weeks' gestation (+/- 2 weeks). Systemic arterial stiffness was measured using photoplethysmography and arteriography, which were selected for their non-invasive nature, speed of performance, and previous use in separate and unconnected pregnancy and SLE studies (309,310).

An Arteriograph (TensioMed Ltd, Budapest, Hungary) was used to measure pulse wave velocity (PWV), a measure of aortic stiffness. Investigations were performed semi-recumbent in the left lateral position with a cuff placed on the right arm, over the brachial artery. Photoplethysmography was conducted with Pulse Trace PCA2 (Care Fusion, Chatham, UK), again in the left lateral position, with the probe attached to the left index finger. The stiffness index (SI), was considered to represent large arterial stiffness, and reflection index (PRI) small arterial tone (311,312). Photoplethysmography measurements were performed 3 times, at 2-5 minute intervals, with the mean value reported. In order to reduce observer bias, brachial blood pressure (BP) was recorded with an automated blood pressure monitor (Alere Microlife, Alere Ltd), validated for use in pregnancy (232,233). Brachial BP was recorded after fetal wellbeing had been established, to reduce false high readings due to anxiety; this was particularly relevant in PL women. Mean arterial pressure (MAP) was calculated for all women using diastolic pressure (DP) and systolic pressure (SP), MAP = DP+$1/3$(SP-DP).

Using colour Doppler ultrasound, the uterine artery was identified as it crossed the external iliac artery. Measurements were then taken on the medial aspect of the intersection (230). All ultrasound measurements were performed by a Royal College of Obstetricians and Gynaecologists (RCOG) accredited sonographer, using a Voluson E6 (GE Healthcare, Hatfield, UK) ultrasound machine with 3D, 4-8Hz curvilinear probe. Resistance index (UtRI) and Pulsatility index (PI) were recorded, as was a qualitative description of the waveform, with presence of notch considered abnormal.

5.3.3 Vascular function markers

Maternal circulating vascular markers were assessed in maternal serum at 28 weeks and 36 weeks' gestation (+/- 2 weeks). Blood samples were taken peripherally with a vacutainer system (Fisher scientific, Loughborough, UK) at the same time points as systemic vascular assessments. Each vacutainer was centrifuged at 4^{o}C for 10 minutes at 3000rpm. Aliquoted samples were then stored at -80^{o}c before further use.

PlGF was measured in these samples using the Triage PlGF test with Triage MeterPro point of care analyser (Alere, Massachusetts, USA). This assay reportedly measures a range of 12-3000picograms/millilitre (pg/ml), with coefficient of variation (CV) of 12.8-13.2% (313). Maternal circulating Soluble Fms-like tyrosine kinase-1 (sFlt-1) was measured using R&D Systems Quantikine ELISA (Bio-Techne, Minnesota, USA). Assay range was 31.2-2000pg/ml, with a sensitivity of 13.3 pg/ml, intra-assay CV of 2.6-3.8%, and an inter-assay CV 5.5-9.8% (239).

5.3.4 Outcome Data

Outcome data was collected from patient notes post-delivery. This included mode of delivery, gestation at delivery, birthweight and individualised birthweight ratio (IBR). IBR was calculated using GROW software (Perinatal Institute) to give a customised birthweight centile for fetal sex, maternal height, weight and ethnicity (156,279).

5.3.5 Statistical analysis

All results were analysed with GraphPad Prism 7 (GraphPad Software Inc, USA). All data were tested for normality. Repeated measures analysis of variance (ANOVA) or Kruskall Wallis test were used to compare longitudinal changes across pregnancy. T-tests or Mann Whitney U tests were used for gestational comparisons. For matched data sets, the Wilcoxon signed rank test was used. Fisher's exact test was used to compare percentages. Data are displayed as median and interquartile range (IQR) (non-parametric) or mean ± standard deviation (SD) (parametric). Statistical significance was set at $p \leq 0.05$. In the context of ANOVA, Dunn's post hoc test was used to correct for multiple comparisons.

5.4 Results

By definition, there was a significant difference in parity across cohorts, with PL all being parous (Table 19). Similarly, in line with the epidemiology of SLE, there was a higher proportion of non-Caucasian women in the SP group compared to NP and PL (78,258). Otherwise, cohorts were comparable in their demographics (Table 19). For SP, their primary disease, antibody positivity and medication are shown in Table 20. As discussed in chapter 4, page 79, disease activity was notably evident but low in this group, as was the use of immunosuppressant medication. There was one woman in the PL cohort taking a combination of oral steroids and hydroxychloroquine for previous stillbirth at 24 weeks in which placental histology demonstrated chronic histiocytic intervillositis (CHI) (an inflammatory condition seen in the placenta, with an 67% percentage chance of recurrence and 77% associated perinatal loss rate) (281). There were no smokers in any cohort.

In PL, the mean gestation of the previous pregnancy loss was 34.1 weeks (+/- 6.4 weeks) with mean birthweight of 2058g (+/- 1171g). Of these, 2 (7%) were attributed to fetal growth restriction, although full records for prior loss were not available in all cases. No stillbirths had fetal anomaly as a cause of death.

Table 19 – Demographics of pregnancy cohorts studies (current pregnancy)

Group	SLE Pregnant (SP), n=51	Previous Loss (PL), n=29	Healthy Pregnant (NP), n=44	P value
Age at conception (years)	31.9 (+/- 4.85)	31.07 (+/-5.9)	30.2 (+/-4.8)	0.26
BMI	25.9 (+/-6.1)	26.4 (+/-5.2)	24.6 (+/-3.5)	0.28
Ethnicity (n, percentage)	Caucasian 33 (64.7) Asian 15 (29.4) Black 3 (5.9)	Caucasian 23 (88.5) Asian 2 (7.7) Black 1 (3.8)	Caucasian 36 (81.8) Asian 4 (9.1) Black 6 (13.6)	**<0.0001**
Parity	Primiparous 32 (62.7) Multiparous 19 (37.3)	Primiparous 1 (4) Multiparous 26 (96)	Primiparous 26 (59.1) Multiparous 18 (40.9)	**<0.0001**
Previous Pregnancy Outcome	Livebirth 19 Stillbirth N/A Late Miscarriage N/A Early Miscarriage 19 (37.3) FGR – nil information	Livebirth 12 (41.4) Stillbirth 27 (93.1) Late Miscarriage 2 (6.9) Early Miscarriage 5 (17.2) FGR 7 (24.1)	Livebirth 18 (40.9) Stillbirth N/A Late Miscarriage N/A Early Miscarriage 5 (11.4) FGR N/A	
Birthweight	2934 (+/-793.6)	3221 (+/-279.4)	3401 (+/-494.6)	**<0.05**
Gestation at Delivery (days)	262.5 (+/-24)	266.8 (+/-5.1)	274.5 (+/-16)	**<0.05**
Individualised Birthweight Ratio (centile)	36.9 (+/- 28.3)	49.5 (+/-21.9)	45.38 (+/-31.37)	0.14

Mean values or percentages reported, with standard deviation or percentage of cohort in parenthesis. T test for parametric, Mann Whitney for non-parametric, Fishers exact test for comparison of percentages. BMI=Body Mass Index, SLE=Systemic Lupus Erythematosus

Table 20 - Disease profiles of pregnant cohorts studied

Group	SLE Pregnant (SP), n=51	Previous Loss (PL), n=29	Healthy Pregnant (NP), n=19
Antiphospholipid Syndrome*	6 (11.8%)	0	0
Anticardiolipin Antibody Positivity **aCl IgG > 5.7** **aCl IgM > 10**	 5 (9.8%) 6 (11.7%)	 1 (3.4%) 3 (10.3%)	 0 0
Lupus Anticoagulant	9 (17.6%)	0	0
SSA/Ro positive	7 (13.7%)	0	0
Other antibody	ANA 22 RNP 5	ANA 1 ENA (Anti Chromatin) 1	0
SLEDAI (whole pregnancy)	2 (+/- 2.7)	n/a	n/a
Aspirin	40 (78.4%), dose 75mg	12 (41.4%), dose 75mg	0
Hydroxychloroquine	25 (49%), dose 200mg (200-400)	1 (3.4%), dose 200mg	0
Oral Steroids	20 (39.2%), dose 5mg (5-10)	1 (3.4%), reducing regime (20mg initial dose) until 20 weeks gestation	0
Azathioprine	8 (15.7%), dose 89.7 (50.7-125)	0	0
Tacrolimus	5 (9.8%), dose 2.5g (1-4g)	0	0
Antihypertensive therapy	5 (9.8%)	0	0
Low Molecular Weight Heparin (LMWH)	20 (39.2%)	5 (17.2%)	0

Percentages expressed as n number with percentage of cohort in parenthesis. *Antiphospholipid syndrome defined using Sapporo criteria (98). SLEDAI was expressed as median value of whole pregnancy with standard deviation in parenthesis. Medication applied to pregnancy only. Median drug dose per day with IQR shown. SLE=Systemic Lupus Erythematosus

5.4.1 Systemic Cardiovascular Function

Brachial mean arterial pressure (MAP) was significantly different across the three cohorts at 16, 22 and 28 weeks' gestation, with PL having the highest MAP at all gestational time points (Figure 29), and HP the lowest values. The pattern of longitudinal change was different across the cohorts also, with both SP and NP displaying a second trimester drop in MAP (whole pregnancy RM-ANOVA p=0.005 and p=0.03 respectively). This drop was not seen in PL (whole pregnancy RM-ANOVA 0.1). The difference seen in MAP was predominantly driven by systolic blood pressure (SBP) which was significantly higher in PL at all gestations (all p<0.01). Diastolic blood pressure (DBP) was higher in PL at 22 and 28 weeks' gestation (p<0.001) (Table 21). There was no significant difference in BP between NP and SP at any time point, although SP did show higher values consistently. There was no significant difference in heart rate at any matched gestation across the cohorts and, in line with normal physiological changes of pregnancy, there was a non-significant increase in heart rate with increasing gestation in all three. None of the PL cohort developed hypertension or PET during this pregnancy, or in the immediate postnatal period, despite continuously elevated MAP observed in the antenatal period, at least from 16-36 weeks' gestation.

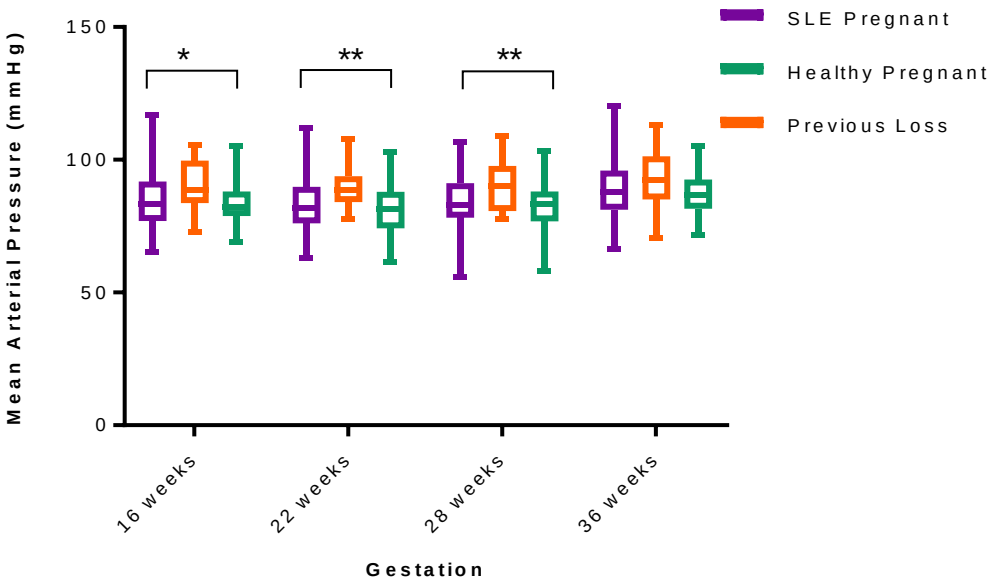


Figure 29 – Longitudinal changes in brachial mean arterial pressure throughout pregnancy. Data displayed as mean and standard deviation with minimum and maximum values. *p<0.05, **p<0.01, ANOVA used to compare 3 cohorts. SLE=Systemic Lupus Erythematosus

Table 21 - Brachial Blood Pressure

Gestation (weeks)		SLE Pregnant (SP), n=51	Previous Loss Pregnant (PL), n=29	Healthy Pregnant (NP), n=44	P value
16	Systolic BP	114.2 +/- 14.7 n=44	124.1 +/- 13.4 n=29	113.2 +/- 11.7 n=33	**<0.01**
	Diastolic BP	70.8 +/- 10.9 n=44	73.3 +/- 9.3 n=29	68.5 +/- 8.1 n=33	N/S
	MAP	85.1 +/- 11.5 n=44	90.1 +/- 9.4 n=29	83.8 +/- 8.8 n=33	**<0.05**
22	Systolic BP	112.5 +/- 14.6 n=49	120.2 +/- 9.7 n=27	109.5 +/- 14.6 n=32	**<0.01**
	Diastolic BP	68.9 +/- 8.8 n=49	74.4 +/- 8.9 n=27	65.8 +/- 8.9 n=32	**<0.01**
	MAP	83.3 +/- 10.1 n=49	89.5 +/- 8.1 n=27	80.9 +/- 9.4 n=32	**<0.01**
28	Systolic BP	114.1 +/- 11.8 n=42	122 +/- 13.9 n=22	110.8 +/- 10.65 n=32	**<0.01**
	Diastolic BP	68.5 +/- 10.4 n=42	75.3 +/- 9.8 n=22	67.3 +/- 9.9 n=32	**<0.05**
	MAP	83.5 +/- 10 n=42	90.7 +/- 9.8 n=22	82 +/- 9.2 n=32	**<0.01**
36	Systolic BP	116 +/- 15.2 n=35	124.7 +/- 13.1 n=22	112.7 +/- 9.1 n=27	**<0.01**
	Diastolic BP	74.3 +/- 11.6 n=35	77.3 +/- 11.3 n=22	72.9 +/- 7.7 n=27	N/S
	MAP	88.1 +/- 11.8 n=35	92.9 +/- 10.9 n=22	86.8 +/- 7.9 n=27	N/S

Brachial blood pressures, all measurements in mmHg. Mean and standard deviation shown. ANOVA with Dunn's post hoc correction for analysis. BP = Blood pressure, MAP = mean arterial pressure. N/S = non-significant. SLE=Systemic Lupus Erythematosus

Aortic arterial stiffness (pulse wave velocity, PWV) gradually increased with increasing gestation in all 3 cohorts, with highest PWV values seen in SP and lowest in NP (Figure 30). PL had intermediate PWV values to those seen in SP and NP. Despite the differences seen in aortic arterial stiffness, aortic blood pressure (SBPAo) was not significantly different across the three groups.

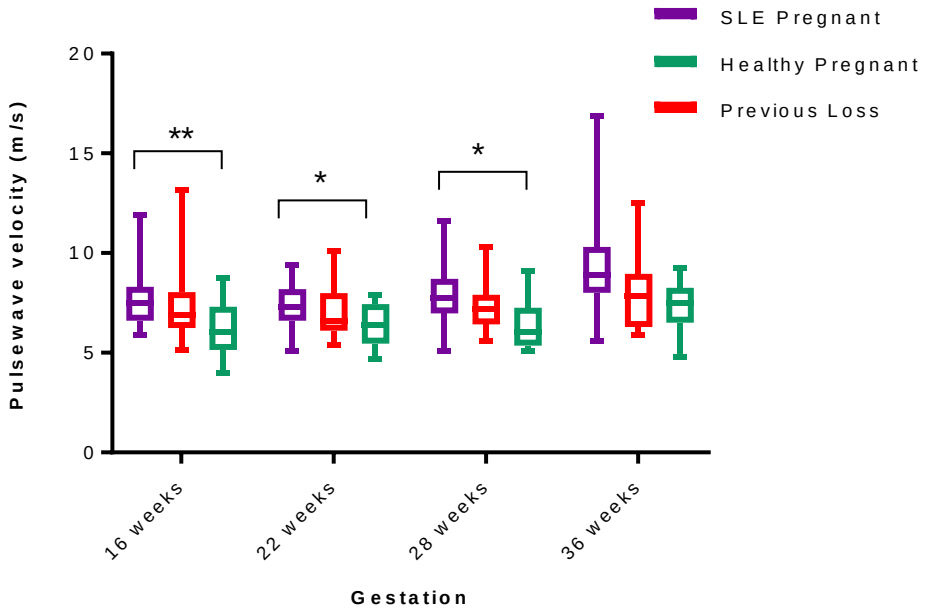


Figure 30- Longitudinal changes in Pulsewave Velocity throughout pregnancy, as measured by Arteriograph. Data displayed as mean and standard deviation with minimum and maximum values. *p<0.05, **p<0.01. ANOVA for 3 cohort comparison. SLE=Systemic Lupus Erythematosus

In an alternative method of assessment of vessel stiffness, photoplethysmography-based measurements were not significantly different across the study groups at any considered gestation (Figure 31). However, values were highest in SP at every time point, indicating a trend for higher PWV in these women. Large artery stiffness (SI) remained stable throughout pregnancy (Figure 31A), but small artery stiffness (RI) increased from 28-36 weeks' gestation in SP (p=0.02), but remained stable in both NP and PL (Figure 31B).

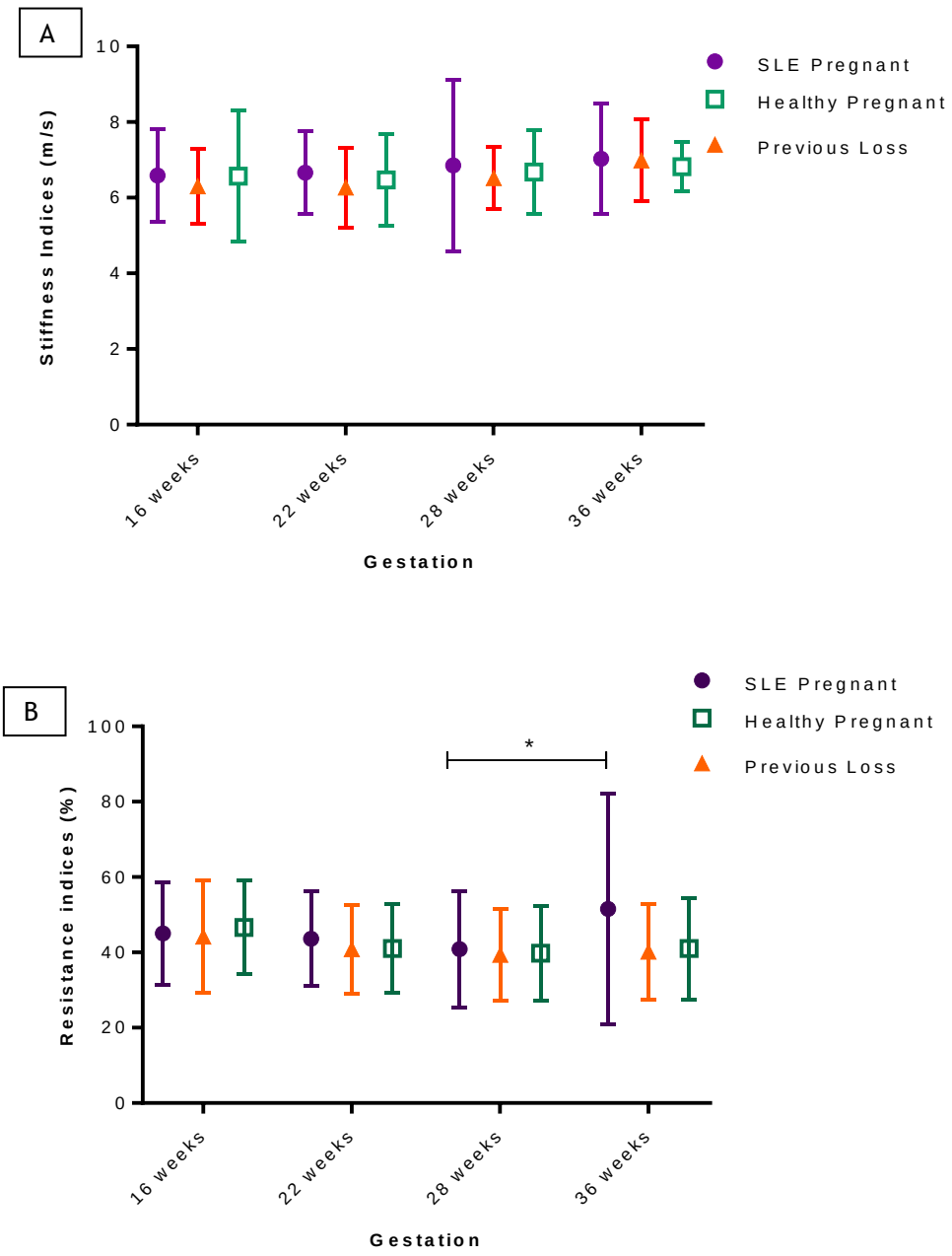


Figure 31 - Change in photoplethysmography measurements longitudinally throughout pregnancy. A) Large artery stiffness - Stiffness Indices, B) Small artery stiffness - Resistance Indices. Mean and standard deviation shown. *p <0.05 (SP RI at 28 vs 36 weeks' gestation). SLE=Systemic Lupus Erythematosus

There was a lower mean uterine artery pulsatility index (PI) and resistance index (UtRI) at 16 weeks' gestation in PL compared to both SP and NP (p=0.04 and p=0.0007, respectively). In all cohorts' uterine PI and UtRI fell significantly with increasing gestation, as shown in Table 22 (A and B). In considering the waveform pattern of the Uterine Artery at 22 weeks' gestation (in line with studies using UtAD to predict adverse outcome (314)), the abnormal presence of uterine artery notching was only seen in SP and NP, present in 2.2% and 13.0%, respectively.

Table 22 – Changes in Uterine Artery Doppler measurements with increasing gestation. A) Pulsatility Index B) Resistance Index.

A

Gestation	SLE Pregnant (SP), n=51	Previous Loss Pregnant (PL), n=29	Healthy Pregnant (NP), n=44	P value
16	1 (0.8-1.3) n=34	0.79 (0.69-0.99) n=29	0.98 (0.76-1.29) n=23	**<0.05**
22	0.9 (0.7-1) n=49	0.83 (0.69-0.9) n=29	0.86 (0.69-0.99) n=22	0.44
28	0.8 (0.6-0.9) n=43	0.69 (0.64-0.83) n=24	0.71 (0.64-0.87) n=18	0.23
36	0.6 (0.5-0.9) n=19	0.63 (0.5-0.84) n=24	0.77 (0.67-0.91) n=9	0.67
P value	**<0.001**	**<0.01**	**<0.001**	

B

Gestation	SLE Pregnant (SP), n=51	Previous Loss Pregnant (PL), n=29	Healthy Pregnant (NP), n=44	P value
16	0.6 (0.5-0.63) n=34	0.55 (0.48-0.59) n=29	0.67 (0.52-0.77) n=23	**<0.001**
22	0.5 (0.5-0.6) n=49	0.54 (0.48-0.57) n=29	0.53 (0.47-0.59) n=22	0.4
28	0.5 (0.4-0.6) n=43	0.48 (0.45-0.54) n=24	0.49 (0.44-0.55) n=18	0.14
36	0.4 (0.4-0.5) n=19	0.45 (0.39-0.53) n=24	0.47 (0.42-0.55) n=9	0.67
P value	**<0.001**	**<0.001**	**<0.0001**	

Data shown as median and IQR, individual n numbers shown per gestation. ANOVA for analysis. SLE=Systemic Lupus Erythematosus

In SP, the gestational age at delivery was significantly lower in women with uterine artery notching (either unilateral or bilateral) compared to SP with a normal uterine artery waveform (median 252 days vs 271.5, p=0.009) (Figure 32A). In line with published research on the predictive use of uterine artery Doppler in low risk populations (230,315), this was not seen in NP and PL. IBR centile was not significantly affected by the presence of uterine artery notching at 22 weeks, although there was a trend towards lower IBR in all cohorts in the presence of uterine artery notching (Figure 32B).

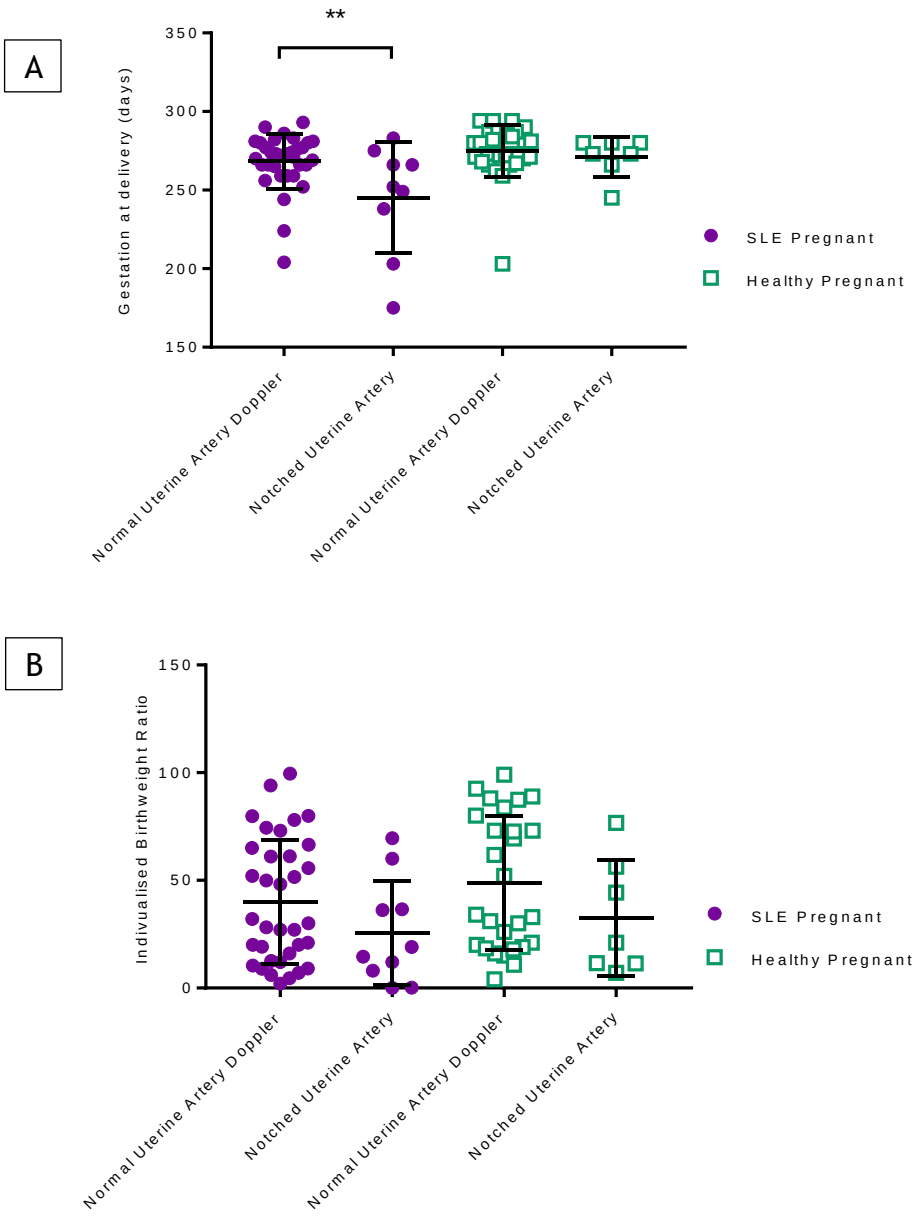


Figure 32 - Pregnancy outcome divided by the presence of a notched uterine artery Doppler in the second trimester. A) Gestation at delivery in days, B) Individualised Birthweight ratio (IBR) centile, Median and IQR shown. **p<0.05. SLE=Systemic Lupus Erythematosus n numbers, Healthy pregnant (NP) normal uterine artery Doppler = 26-7, notched uterine artery Doppler= 4, SLE pregnant (SP) normal uterine artery Doppler = 57, notched uterine artery Doppler=6-8.

5.4.2 Circulating Maternal Vascular Function Markers

Data for both PlGF and sFLT-1 was log transformed for analysis due to large heterogeneity of results. There was no difference in circulating PlGF levels between groups at matched gestations (Figure 33A). As expected, circulating PlGF fell with increasing gestation (175,177), with PlGF levels significantly lower at 36 weeks compared to 28 weeks in all groups (SP p<0.0001, NP p<0.001, PL p<0.01) (Figure 33A). When considered as a categorical variable, 2 SP (10%) had circulating PlGF levels <12pg/ml at 36 weeks, compared to none in PL or NP cohorts (p<0.0001). Similarly at 36 weeks, the proportion of intermediate PlGF levels (>12,<100pg/ml) (237,238) was

significantly higher in both SP and PL, n=8 (40%) and n=7 (47%) respectively, compared to n=3 (21%) in NP (p=0.0002). There were no PlGF values <12pg/ml at 28 weeks gestation in any of the cohorts. At 28 weeks, the number of intermediate PlGF values was again highest in SP, with 4 (15%) intermediate levels, compared to 1 (4.5%) in NP and 0 in PL (p<0.0001).

Circulating sFLT-1 levels were highest in SP and lowest in NP at 36 weeks gestation (p=0.007) (Figure 33B). No differences were seen at 28 weeks gestation. Equally, no differences seen in the ratio of PlGF to sFlt-1 were observed at either gestational age in any of the three cohorts.

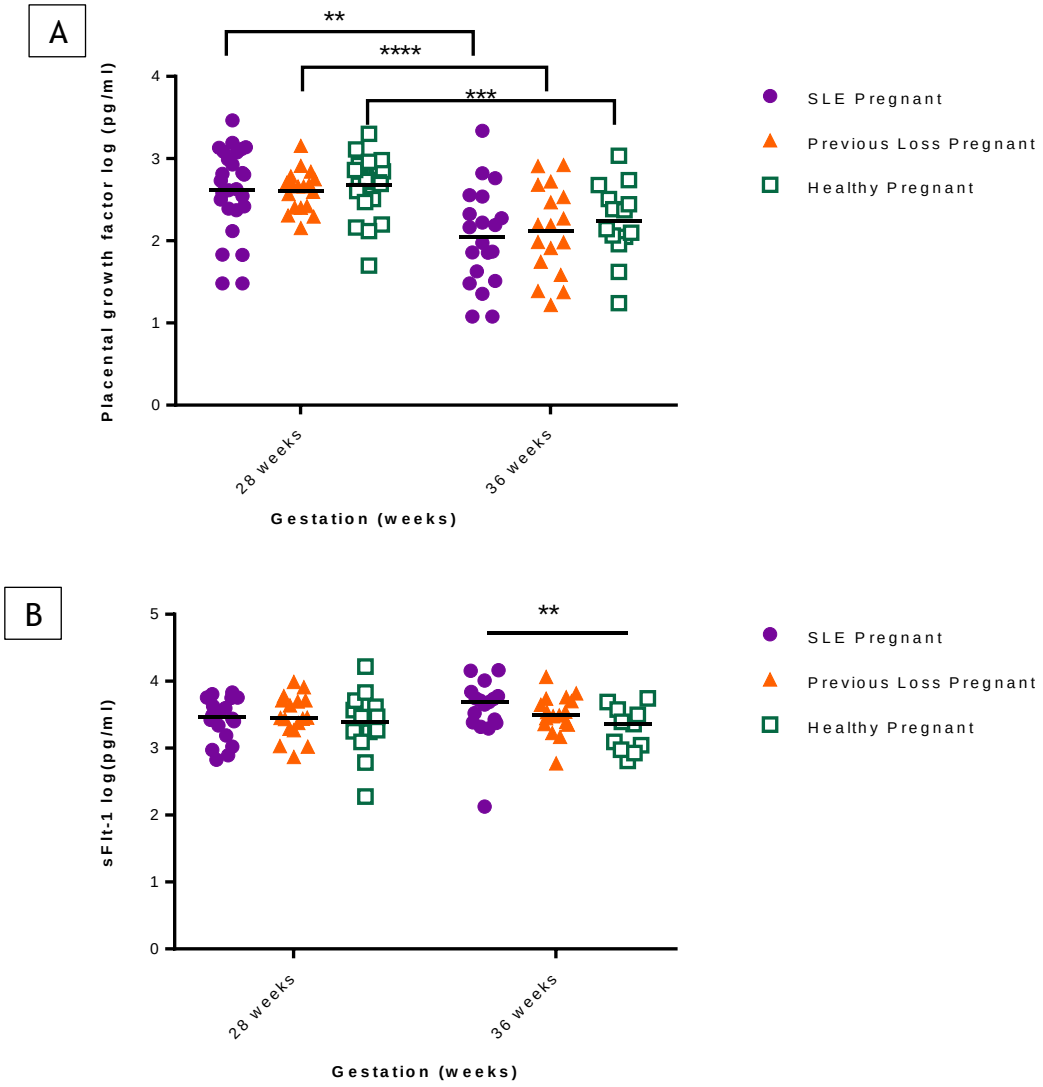


Figure 33 -Third trimester circulating vascular markers in maternal serum.A) Maternal Circulating Placental Growth Factor, B) Maternal circulating Soluble Fms-Like Tyrosine Kinase-1. 28 weeks SP n=26, HP n=24, PL=18, 36 weeks, SP n=20, HP n=16, Pl=17. b, 28 weeks SP n=18, HP n=13, 36 weeks, SP n=16, HP n=11. Line at median, **=p<0.01, ***=p<0.001, ****=p<0.0001. SLE=Systemic Lupus Erythematosus

5.5 Discussion

Women with SLE, and women who experience a stillbirth have an increased lifelong risk of cardiovascular disease (33–35). Moreover, women with SLE have an increased risk of experiencing

stillbirth (2). This study is the first comparison of longitudinal vascular changes in pregnancies in women with SLE, women in a subsequent pregnancy following a stillbirth, and healthy uncomplicated pregnancies. These longitudinal data have demonstrated that SLE-affected pregnancies broadly follow the same vascular changes seen in healthy pregnancies, including a second trimester drop in brachial BP. There is, however, evidence of compromised vasculature seen in SLE affected pregnancies in the form of an elevated PWV throughout pregnancy. The presence of raised PWV is known to be predictive of long term cardiac morbidity in an otherwise healthy population (316). As such, this change in pattern in PWV seen in pregnant women with SLE is consistent with their long term risk of developing cardiovascular disease, as previously defined in these women in their non-pregnant state (126–128), and consistent with evidence of increased arterial stiffness outside of pregnancy (130,131). In non-pregnant women, raised PWV in SLE has been associated with an increased risk of atherosclerosis of the carotid artery, and been demonstrated to be related to disease severity in women (133). It has, however, only had a weak association with high blood pressure in non-pregnant women with SLE (317). This study adds evidence to this, as the pregnant women with SLE in this study only displayed a non-significant increase in brachial BP compared to their healthy counterparts despite an increased PWV. The low disease activity, and use of immunosuppressant medication seen within this SLE cohort, may explain the relatively low levels of PWV elevation seen, as this has previously been shown to be related to increased inflammatory markers and disease activity, albeit in a mixed gender cohort of older men and non-pregnant women with SLE (318).

In contrast to that seen in women with SLE, a different vascular pattern was seen in women in a subsequent pregnancy following a stillbirth, with a loss of the “healthy” second trimester drop in diastolic brachial BP alongside consistently higher MAP, in the absence of an elevation in heart rate, or a change in PWV. This lack of second trimester drop in diastolic BP has previously been described in pregnancies associated with PET and hypertensive disorders of pregnancy (300) but not in pregnancies with normotensive outcomes. The changes seen in brachial BP in women with a prior stillbirth, while subtle, is evidence that these women display a different vascular phenotype to that seen in their healthy counterparts, and also to that seen in women with SLE. The lack of difference in heart rate between the groups is supportive of the fact that the difference in blood pressure seen relates to maternal vessel disease, not anxiety. Elevation of blood pressure during pregnancy has been linked to long term maternal health outcomes, with women displaying increased blood pressure in pregnancy having an increased lifetime risk of developing long term CV disease (319). This links with data on increased CV disease in women who have had a stillbirth, and offers a potential common underlying pathogenesis.

These changes in systemic maternal vascular response did not extend locally to the uterine arteries, which displayed no difference in either resistance indices or pulsatility indices across the three cohorts at any matched gestation. In line with other published data, both uterine PI and RI fell with increasing gestation (320). In women with SLE, the proportion with an abnormal UtAD waveform (presence of a diastolic notch at 22 weeks gestation) was 12.2%; this is lower

than that seen in the wider literature in SLE pregnancies (reported rates of 14-18%) (159,321). Whilst overall levels of uterine artery notching in this cohort were low, the relationship between abnormal second trimester UtAD was maintained, with an association between abnormal waveform and earlier gestation at delivery, alongside a trend towards lower IBR centile at birth. This is in line with the literature for both SLE-affected pregnancies and other high risk pregnancies, such as pre-eclampsia (201). To date, there have been no studies looking at uterine artery Doppler in women with a previous stillbirth, and so the data here is novel. It is, however, reassuring that in this cohort there were no recurrent stillbirths, cases of pre-eclampsia or fetal growth restriction. These complications would usually be associated with a notched uterine artery in the second trimester (201), meaning a larger sample size may show this relationship, as such the value of uterine artery Doppler in this cohort remains uncertain.

In considering the biochemical aspects of vascular changes and pregnancy, levels of anti-angiogenic sFlt-1 were elevated in women with SLE at 36 weeks, with women with a prior loss having values intermediate between those of SLE and those of healthy women. Levels of pro-angiogenic PlGF were unaffected by maternal history or disease state, they did however fall from 28 to 36 weeks gestation in line with other published research (177,322,323). This is similar to the finding by Qazi et al (324), and echoes that of Leanos-Miranda et al (325) in whose larger cohorts abnormal levels of angiogenic factors were only seen in cases of SLE complicated by pre-eclampsia. These biochemical changes have not previously been described in the context of previous stillbirth. The low prevalence of adverse pregnancy outcome for either mother or fetus may mean that the results seen in this study are merely a reflection of the healthy pregnancy, close monitoring and appropriate medical intervention in SLE or prior stillbirth, rather than reflective of the whole population.

This study considered the longitudinal changes in vascular measurements of pregnancy in two high risk cohorts (SLE and previous pregnancy loss) compared to healthy uncomplicated pregnancies. The relatively small numbers included due to the rarity of the disorder, meant that the study had insufficient statistical power to report adverse pregnancy outcome. However, given the rarity of both these high risk pregnancy states, it was felt that the numbers were sufficiently large for an observational study, and to demonstrate longitudinal changes in PWV, BP and photoplethysmography based measurements, alongside the importance of further research in this area. This was particularly evident when uterine artery Doppler was considered, as there was a low rate of notching seen, and none in those with prior stillbirth. As such, only trends in outcomes could be assessed. A larger, multi-centre study would enable this relationship to be further explored.

The group of women with SLE studied here are comparable in their demographics to those in the wider literature, with a mean age of 31.9 consistent with a peak age of diagnosis between the ages of 20-40 (326). The prevalence of aPL (either aCL or LAC) was lower at 21.5 and 17.6% respectively compared to that seen by Love et al. (44% and 34% respectively) in their analysis of 29 studies (327). Overall, disease activity in this cohort was low, as was the prevalence of end

organ damage. As such, the lower levels of aPL seen in this cohort may reflect lower disease activity, and therefore lower rates of pregnancy complications and less severe vascular disease.

Women were recruited for this study from MDT specialist clinics, either for SLE or for prior stillbirth; as such they were on treatment and being closely monitored. Whilst the benefits of this approach in SLE have been widely reported, there is little data about the benefits of a multidisciplinary team (MDT) approach in the antenatal care of women with a prior stillbirth. Both of the high risk cohorts in this study were managed in a tertiary centre with a MDT; the resultant effect is that the women studied, especially those with SLE, had well controlled disease and many had received pre-conception counselling (see chapter 4.3.4, page 83). Women were monitored serially for changes in urine protein excretion and serum biochemical markers to identify any evidence of worsening disease, and treatment was optimised accordingly. This is both a strength and weakness of the study, as it demonstrates that with good disease control adequate vascular adaptation can take place, but also means that any failure of vascular adaptation that may be seen in highly active disease was not observed. There is no published evidence for an MDT approach in women with previous stillbirth but there is evidence of benefit in women with recurrent miscarriage (328) and, as such, similar benefits may be seen in women with previous stillbirth.

Pregnancy outcomes in this study were good, however there were differences seen across the cohorts. Gross birthweight was lower in SLE affected pregnancies, as was age of gestation at delivery. When birthweight was customised to account for gestation using IBR, this significance was lost, suggesting that the differences seen in birthweight can be explained by an increased frequency of preterm birth rather than a true lowering of birthweight (see chapter 4, page 79). This is similar to the wider literature which reported both lower birthweights and earlier gestation at delivery in SLE pregnancies compared to healthy women (153,329). The lack of individualisation of birthweight to maternal characteristics in previous studies looking at pregnancy outcomes in SLE makes these studies hard to directly compare to this study. Women with a prior stillbirth showed no change in either IBR or birthweight when compared to healthy women, gestation at delivery was however significantly lower in women with a prior stillbirth when compared to healthy women, although this is potentially iatrogenic as outlined in chapter 4.5, page 98. Previous studies of subsequent pregnancy following stillbirth have shown lower birthweight, and lower gestation at delivery in women with prior stillbirth compared to healthy counterparts (27,28,31). The retrospective nature of these studies and lack of customisation for maternal characteristics makes comparison with these cohorts difficult. The nature of these high risk pregnancies and their previous poor outcome also means that a proportion of these “early” deliveries may be iatrogenic and, as such, they may skew the data that is available.

This study has highlighted the differing patterns of longitudinal vascular change of pregnancy in two separate high risk populations. Both these conditions are associated with lifelong increased cardiovascular risk (33–35). It may be that the differences seen in this study suggest a difference in the underlying pathology in the causes of that cardiac risk. If it is truly representative of

higher blood pressure and arterial stiffness, then this could predispose towards elevated rates of hypertension in later years. In considering pregnancy as a stress test of the maternal vasculature, these subtle changes could indicate the need for health surveillance, and offer potential time for intervention in order to reduce lifelong cardiovascular risk. As outlined in Chapter 3 (page 65), stillbirth and adverse pregnancy outcomes are associated with an increased lifelong risk of development of maternal autoimmune disease, including SLE, further increasing cardiovascular risk. Early recognition of these women as high risk and stratification of them into a high risk cardiovascular disease screening programme through primary care has a potential to optimise and treat maternal cardiovascular disease early, and as such reduce end organ damage and disease burden. In addition to the cardiovascular risks identified, this study further adds to the body of evidence for the use of second trimester uterine artery Doppler in the development of individualised care in women with SLE, with a role as a tool in these high risk pregnancies and subsequent pregnancy management. Further assessment of vascular stiffness and BP in the postnatal period would further delineate this relationship between pregnancy outcome and maternal CV disease.

In summary, pregnancy provides an additional challenge to the maternal vasculature and a different pattern of maternal adaptation is seen, dependent on maternal disease and obstetric history. This study shows lower levels of both fetal and maternal complications than expected, which may be due to the specialist tertiary care received. Women with SLE have higher PWV than healthy women, and women with a prior stillbirth do not display a second trimester drop in brachial blood pressure and maintain higher BP throughout pregnancy, irrespective of heart rate. In order to unpick the relationship between maternal vascular changes of pregnancy and previous stillbirth or SLE, more prolonged monitoring would be beneficial, with consideration given to 24 hour monitoring of blood pressure to assess the diurnal variations, including nocturnal changes, as the absence of diurnal drop in BP has been demonstrated to be associated with long term cardiovascular risk and development of pre-eclampsia (330–332). A retrospective study of women who experience a stillbirth may offer further insight into whether the change in maternal blood pressure pattern seen in a subsequent pregnancy is, in fact, present in the index pregnancy, thus providing the potential for the development of a predictive model for stillbirth.

6. Pregnant Women with Prior Stillbirth Display a Systemic Immune Response Similar to those with Systemic Lupus Erythematosus

6.1 Abstract

Introduction - Systemic Lupus Erythematosus (SLE) is an autoimmune condition associated with an increased rate of adverse pregnancy outcomes including stillbirth. Underlying the autoimmune response in SLE is a pro-inflammatory T cell response with elevation in T helper 17 cells (T17) and a reduction in T regulatory cells (Treg). Similarly circulating microparticles (MPs) have been shown to be elevated in SLE and reduced with treatment efficacy. We sought to examine these changes in T17 and Treg cells in pregnancy in women with SLE, women with a prior stillbirth and healthy pregnant women, alongside changes in circulating microparticles throughout pregnancy.

Methods - Women with singleton pregnancies were recruited from St Mary's Hospital, Central Manchester University Hospitals Foundation NHS Trust, Manchester, UK between December 2014 and January 2016. Three cohorts were recruited, pregnant women with SLE (SP), pregnant women with prior stillbirth (PL) and healthy pregnant women (NP). MPs were measured longitudinally in 10 women per group, and T cells in 5 per group. Maternal circulating Treg cells and Th17 cells were measured at 17 weeks' gestation (+/- 1 week) using fluorescence activated cell sorting analysis (FACS). Treg positivity was defined as $CD4^+$, $CD25^+$ and $FoxP3^+$. Th17 cell determinations were performed on (i) directly isolated PBMCs and (ii) following subsequent stimulation in culture. For FACS analysis, dual $CD3^+$ and $IL17^+$ positivity were considered confirmatory of Th17 cells. Maternal circulating MPs in peripheral venous blood were measured longitudinally at 22, 28 and 36 weeks' gestation (+/- 2 weeks).

Results – Baseline numbers of Th17 cells were higher in PL women with autoantibodies compared to PL with no autoantibodies (p=0.0006); after culture, this difference remained (p=0.02). There was no difference across the three cohorts in baseline or cultured Th17. There was no difference seen in Treg cells at 17 weeks gestation. The most abundant MP subset was endothelial (CD62e) and erythrocyte (CD235a). CD62e MPs gradually fell throughout pregnancy whereas CD235a MPs increased with increasing gestation. Constitutive endothelial MPs increased significantly longitudinally in PL (p=0.02), and at 28 weeks were significantly higher in PL than NP and SP (p 0.02). Activated EMP MPs (CD62e) were significantly higher in NP compared to SP at 22 weeks (p=0.03), and were significantly higher in PL compared to SP at 28 weeks (p=0.02). Erythrocyte MPs (CD235a) were higher in NP at 28 weeks compared to SP (p 0.04). Leucocyte derived MPS (CD45) were lowest in PL compared to NP at 22 weeks' gestation (p=0.04); at 36 weeks, levels of CD45 MPs were highest in NP and lowest in SP (p=0.04). Tissue factor MPs (CD42) were highest in PL at 28 weeks compared to both NP and SP (p=0.03 and p<0.01 respectively).

Conclusion – Women with PL had a different Treg/Th17 profile to that seen in SP and NP, with the presence of autoantibodies associated with an elevated Th17 response, consistent with a systemic pro-inflammatory response that has not previously been reported. Similarly the highest levels of MPs were seen in PL. Endothelial MPs (EMPs) were highest in PL; this was in the absence of pre-eclampsia. The overall pro-inflammatory change seen in PL together with the elevation in EMPs suggest a link between stillbirth, systemic inflammation and vascular dysfunction, similar to that seen in SLE outside of pregnancy.

6.2 Introduction

SLE is an autoimmune disease which typically affects women of child-bearing age (145). It is associated with increased rates of pregnancy complications, including stillbirth, which has a reported incidence 5 times greater than that of healthy women (2). The relationship between SLE and stillbirth remains unclear, with a 6-fold relative risk of developing SLE in the first four years after at stillbirth (4,5,224). The pathogenesis of SLE is undoubtedly complex, particularly when superimposed on pregnancy. In the non-pregnant state, the development of SLE, as an adverse immune response, is considered to be related to aberrant clearance of apoptotic cells from the circulation, culminating in an excess of circulating nuclear constituents (122). These nuclear constituents are recognised as foreign by antigen presenting cells (APCs) and there is subsequent B cell activation, culminating in the development of pathogenic autoantibodies to self DNA and/or RNA (101). The most common autoantibodies seen are antinuclear antibodies (ANA) (84) and the most specific to SLE are autoantibodies against double stranded DNA (dsDNA), which are found in 60% of patients with SLE (86). Antibodies to nuclear antigens are also common, including anti-Smith (anti-Sm) and anti-ribonucleoprotein (anti-RNP). SLE patients also commonly have either one or both of the antiphospholipid antibodies (aPL), with Lupus anticoagulant (LAC) present in 25-38% of cases and anticardiolipin (aCL) in 13-31% (86). The third marker of aPL Beta2-glycoprotein 1 (beta2GP1) is also commonly seen in SLE (17-49%), although is less commonly measured (333).

It is purported that this exaggerated response of B cells is equally marked by alterations in T cells in SLE, specifically an imbalance of T helper (Th) cell production, from an anti-inflammatory Th2-phenotype, to that of the more pro-inflammatory Th1 and Th17 based responses (103,104). It is this shift to a more pro-inflammatory environment which may underpin the pathology of pregnancy complications, including fetal loss. Certainly the pathogenic nature of such a T cell shift has been demonstrated in otherwise healthy women suffering recurrent miscarriage in early pregnancy (334). More specifically, these pregnancies are impacted by reduced regulatory T cell levels (Treg) and elevated Th17 cells (51,209,335). Widely considered to be part of the immune tolerogenic process, Treg cells are considered fundamental in accommodating fetal acceptance, increasing progressively in both maternal peripheral blood and decidua in the first and second trimesters before returning to pre-pregnancy levels in late gestation (52–54).

T cells are formed as a response to exposure to self-antigens that are recognised as “foreign” by the immune system. One theory on the formation of these autoantibodies relates to excess formation and/or reduced clearance of microparticles (MPs) and their subsequent triggering of an immune reaction. MPs are small bound vesicles 200-1000nm in size (118–120), formed as a result of cell surface shedding and apoptosis. Containing both RNA and/or DNA of the cells from which they were derived (121), it is considered that recognition of the RNA/DNA held within these MPs is a stimulus for subsequent autoantibody production, as seen in SLE, and further promotion of a patient’s inflammatory cycle (122,123). This phenomenon certainly appears

evident in patients with SLE, in whom levels of endothelial MPs (EMPs) within their peripheral circulation are raised in line with disease symptoms and reduced with treatment efficacy (139). From a pregnancy perspective, links between excess EMPs and both miscarriage and development of preeclampsia (PET) have been made (217–219). However, the levels and appearance of EMPs in women prior to, or after, a stillbirth have never been investigated, nor have they been assessed in SLE affected pregnancies.

This study investigated whether heathy women who have already experienced stillbirth have either a similar underlying autoimmune process to that defined in SLE, and specifically whether these issues are manifest in their subsequent pregnancy, i.e. whether their T cell phenotypes are aberrant and mismatched and/or their MPs (levels and profiles) are significantly attenuated. To investigate this, both Treg and Th17 cells were quantified in maternal blood of women at 17 weeks' gestation in their current pregnancy, following a stillbirth in their prior pregnancy. Moreover, their circulating MPs have been fully profiled for all major cell lineage markers, at three time points spanning their second and third trimesters (specifically, antenatal weeks 22, 28 and 36). These time points were chosen to best reflect SLE-related features of pregnancy pathogenesis, as outlined in the literature and our own past studies. To emphasise similarities, SLE-associated data and that of healthy pregnant women have been included at these relevant times points for comparison.

6.3 Methods

6.3.1 Participant recruitment

Pregnant women with singleton pregnancies were recruited via the antenatal clinics of St Mary's Hospital, Central Manchester University Hospitals Foundation NHS Trust, Manchester, UK between December 2014 and January 2016. Favourable opinions from an Ethics Committee (13/NW/0158 and 14/NW/1149) were in place before recruitment, and all women gave informed written consent. Women were recruited to three cohorts, pregnant women with SLE (SLE Pregnant, SP), previous stillbirth/second trimester loss (previous loss, PL) and healthy pregnancies (normal pregnant, NP). Women were excluded from the PL or NP cohorts if they had prior diagnosis of an autoimmune condition. They were also excluded from the PL cohort if their previous stillbirth was related to a fetal anomaly, genetic cause or intra-partum event. For serial microparticle measurements (see section 6.3.4), 10 women were recruited per group. For T cell measurements (see section 6.3.2), at least 5 women were recruited to each cohort [6 SP, 5 NP and 11 PL (4 with autoantibodies, 6 without)]. Peripheral blood samples were taken with a vacutainer system (Fisher scientific, Loughborough, UK).

6.3.2 Autoantibodies

Antibody status (LAC, aCL, ANA, dsDNA, anti-Sm, anti-RNP, anti-Ro and anti La) was assessed pre-conception or in the first trimester in the SLE cohort, with serial measurement of double stranded DNA (dsDNA) utilised to assess disease activity. Beta2GP1 was not measured as it is not

routinely used or tested at the hospital patients were recruited from. For both PL and NP cohorts, the antibody status was assessed at 17 weeks gestation (+/-2 weeks). All PL women had a negative autoantibody screen at the time of their stillbirth (previous pregnancy). In cases of positive antibodies in either the PL or NP cohorts, these were tested postnatally for persistence and referral made for Rheumatology review. Results were revealed to the medical team but all research data was anonymised meaning the research team were blinded to results.

6.3.3 T cell recognition and quantification

Maternal circulating Treg cells and Th17 cells were measured at 17 weeks' gestation (+/- 1 week) using fluorescence activated cell sorting analysis (FACS). Treg cells were identified with a commercial kit (BioLegend, London, UK). Peripheral blood mononuclear cells (PBMCs) were first isolated from whole blood using a Ficoll gradient (see chapter 2.7.1, page 49). Once isolated, PBMCs were labelled according to the manufacturer's instructions with fluorescent-conjugated primary antibodies: CD4PE-Cy5, CD25PE and FoxP3-FITC. Treg positivity was defined as $CD4^+$, $CD25^+$ and $FoxP3^+$ (103,336–339). Th17 cell determinations were performed on (i) directly isolated PBMCs and (ii) following subsequent stimulation in culture. The stimulated cells underwent a 16 hour incubation at 37°C with medium (RPMI60, 10%(v/v) heat inactivated FBS, 2mmol Glutamine, 200u/ml Penicillin, 100µl/ml Streptomycin, 5µl Phorbol 12-Myristate 13-Acetate (PMA, 50mg/ml) and 5µl Ionomycin (200ng/ml), before the addition of Monensin Sodium (5mmol) (all supplied by Sigma-Aldrich Company Ltd. Gillingham, UK). Th17 measurements were again made with a commercial assay (R+D Systems, Abingdon, UK), in accordance with instructions. For FACS analysis, dual $CD3^+$ and $IL17^+$ positivity were considered confirmatory of Th17 cells.

6.3.4 Microparticles

Maternal circulating MPs in peripheral venous blood were measured longitudinally at 22, 28 and 36 weeks' gestation (+/- 2 weeks). Citrated samples of maternal blood were used for MP analysis. Biodipy FL maleimide (FL N-(2-aminoethyl)) (Life Technologies, Warrington, UK) was used as a cellular marker (240), alongside antibodies (or control) as detailed in Table 23. All antibodies were FITC conjugated for optimal fluorescent assessment. Fluorospheres (Beckman-Coulter, High Wycombe, UK) were used to identify MPs at 10µm in diameter with a fluorescence range of 525nm to 700nm, using a forward scatter (FS) and side scatter (SS) log scale. A gate was then set up below this size range to capture MPs. All antibodies used were by Becton Dickinson (Oxford, UK). All measurements were taken using a BD Accuri C6 Flow Cytometer (BD Biosciences, San Diego, California). Colour correction was applied, in line with manufacturer's instructions, and confirmed using individual antibody conjugates.

Table 23 – Monoclonal antibodies for flow cytometric identification of maternal peripheral blood microparticles.

Type of Microparticle	Monoclonal Antibody
Platelet	CD41a
Constitutive Endothelial Cell	CD146
Activated Endothelial Cell	CD62e
Leucocyte	CD45
Erythrocyte	CD235a
Tissue Factor Expressing Cell	CD142
Isotype Control	-

6.3.5 Statistical analysis

Results were analysed with GraphPad Prism 7 (GraphPad Software Inc., San Diego, USA). Analysis of variance (ANOVA, parametric) or Kruskall Wallis (non-parametric) were used to compare changes across pregnancy. T-tests (parametric) or Mann Whitney U tests (non-parametric) were used for gestational comparisons (as applicable). A Chi-squared test was used to compare cohort percentages. Data is displayed as median and interquartile range (IQR), unless otherwise specified. Significance was set at $p<0.05$. All data was tested for normality (D'Agostino-Pearson test), prior to analysis.

6.4 Results

6.4.1 Cohort demographics

Relevant patient demographics are reported in Table 24 and Table 25 BMI = body mass index at time of booking for antenatal care. Median and ranges shown. N number with percentage in parenthesis. n/s = not significant. SLE=Sytemic Lupus Erythematosus

Table 25. By study design there was a difference in parity. Moreover, as previously outlined in chapter 4.4.2 (page 87) there was a marked difference in fetal outcomes across cohorts, with lowest birthweight, individualised birthweight ratio (IBR), and gestation at delivery attributed to women with concomitant SLE. Medication usage was higher in women with SLE.

Table 24 - Cohort demographics for T cell measurement.

	Healthy Pregnant (NP, n=6)	SLE Pregnant (SP, n= 7)	Previous Loss Pregnant (PL, n= 10)		P value
			Autoantibody Positive (n=4)	Autoantibody Negative (n=6)	
Maternal age at conception (years)	32.5 (24-38)	31 (26-35)	33 (28-34)	29.5 (19-43)	n/s
Maternal Body Mass Index (BMI, kg/m^2)	23.6 (18.9-29.4)	28.9 (17.0-37.8)	28.6 (26.5-35.3)	23.1 (20.6-36.7)	n/s
Gravidity	1 (1-3)	2 (1-3)	2.5 (2-4)	3 (3-7)	**0.004**
Primiparity n,(%)	4 (67)	5 (71)	0	0	
Birthweight (g)	3350 (3085-4355)	2478 (868-3160)	2949 (2700-3480)	3470 (3020-3880)	**0.004**
Gestation at delivery (days)	282 (268-292)	252 (203-274)	263 (259-268)	270 (268-281)	**0.03**
Individualised Birthweight Ratio (Centile)	55.7 (11.4-8.1)	9.75 (0.2-20.3)	42.55 (16.5-69.4)	71.5 (21.7-97.3)	**0.02**
Antiphospholipid Syndrome	0	1	0	0	
Anticardiolipin Antibody Positivity **aCl IgG** **aCl IgM**	0 0	1 0	1 2	0	
Lupus Anticoagulant	0	0	0	0	
SSA/Ro positive	0	1	0	0	
Other Autoantibodies	0	Anti RNP 1	ENA 1	0	
Aspirin	0	7 (100)	3 (75)	2 (33)	
Hydroxychloroquine	0	4 (57)	0	0	
Oral Steroids	0	4 (57)	0	0	
Azathioprine	0	2 (29)	0	0	
Tacrolimus	0	3 (43)	0	0	
Antihypertensive	0	2 (29)	0	0	
Low Molecular Weight Heparin (LMWH)	0	5 (71)	1 (25)	0	

BMI = body mass index at time of booking for antenatal care. Median and ranges shown. N number with percentage in parenthesis. n/s = not significant. SLE=Sytemic Lupus Erythematosus

Table 25 - Cohort demographics of pregnant women for Microparticles measurement.

	Healthy Pregnant (NP, n=10)	SLE Pregnant (SP, n= 10)	Previous Loss Pregnant (PL, n= 10)	P value
Maternal age at conception (years)	30 (24-42)	30.5 (20-40)	30.5 (19-37)	n/s
Maternal Body Mass Index (BMI)	24.6 (21-29.9)	24.5 (20-36)	25.3 (20.9-40.4)	n/s
Gravidity (n)	2 (1-5)	1 (1-8)	2.5 (2-5)	n/s
Primiparity (%)	6 (60)	8 (80)	0 (0)	
Birthweight (g)	3273 (2620-4272)	3110 (1450-3680)	3355 (2880-3880)	n/s
Gestation at delivery (days)	282 (266-294)	273 (238-287)	267 (258-287	n/s
Individualised Birthweight Ratio (Centile)	28.5 (4-87.4)	18 (0+81)	62.5 (18.6-97.3)	n/s
Antiphospholipid Syndrome	0	1	0	
Anticardiolipin Antibody Positivity **aCl IgG** **aCl IgM**	 0 0	 2 1	 1 1	
Lupus Anticoagulant	0	0	0	
SSA/Ro positive	0	2 (20)	0	
Other Autoantibodies	0	ANCA 3 (30) Anti-RNP 1(10)	ANA 1(10)	
Aspirin	0	6 (60)	4 (40)	
Hydroxychloroquine	0	3 (30)	1 (10)	
Oral Steroids	0	6 (60)	1 (10)	
Azathioprine	0	1 (10)	0	
Tacrolimus	0	0	0	
Antihypertensive	0	0	0	
Low Molecular Weight Heparin (LMWH)	0	4 (40)	3 (30)	

BMI = Body Mass Index at time of booking for antenatal care. Median and ranges shown. N number with percentage in parenthesis. n/s = not significant. SLE=Sytemic Lupus Erythematosus

6.4.2 T cells

As illustrated in Figure 34A, there was no significant difference in percentage of Treg cells at 17 weeks gestation across all three cohorts, including PL, defined as either seropositive or seronegative for autoantibodies within their current pregnancy. Baseline peripheral blood Th17 cells were notably, although not statistically significantly, higher in PL. There was a clear and significant distinction between those with positive autoantibodies in this current pregnancy compared to those with negative seropositivity ($p=0.006$, Figure 34B). Similarly, this distinction remained in Th17 cells stimulated in culture (Figure 34C, $p=0.02$), and likewise, although variable, no differences were recorded between NP, SP and PL, currently without auto-antibodies. When T cells were considered as a ratio of Treg to Th17 (D and E) the ratio was lowest in PL and highest in SP, although this was not statistically significant. This suggests that the women with a prior stillbirth have a systemic pro-inflammatory response.

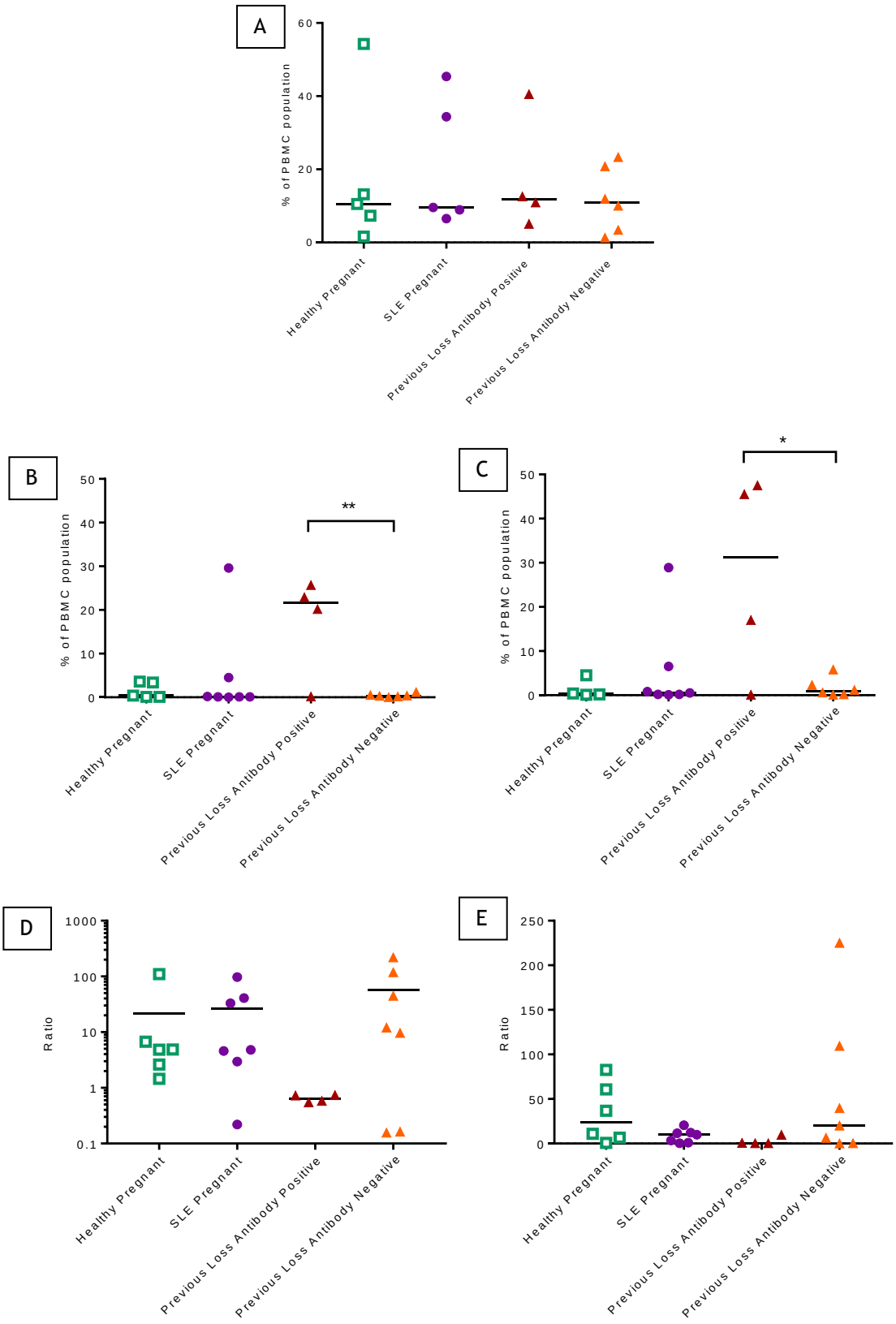


Figure 34 - Treg and Th17 cells in maternal peripheral circulation at 17 (+/-2) weeks' gestation in pregnant women with SLE, pregnant women with prior stillbirth and healthy uncomplicated pregnancies. (A) Treg cells CD4+CD25+FoxP3+ expressed as % of peripheral blood mononuclear cells (PBMC). (B) Th17 cells (IL17+CD3+). (C) Stimulated Th17 cells. (D) Ratio of Treg to unstimulated Th17 cells. (E) Ratio of Treg to stimulated Th17 cells. Median indicated. *=p<0.05, **=p<0.005. SLE=Sytemic Lupus Erythematosus

6.4.3 Microparticles

As previously described, maternal circulating MPs were measured longitudinally in pregnancy in all three cohorts of women (n=10 per cohort). Study characteristics are outlined inTable 25. As previously described, medication usage was higher in SP women. Measurements were taken at 22, 28 and 36 weeks' gestation (+/- 2 weeks).

6.4.3.1 Total Microparticles

Total circulating MPs were generally highest at all time points in PL women (Figure 35) compared to SP or NP. This difference was most marked at 36 weeks gestation, when levels of total circulating MPs were significantly higher ($p<0.001$ and $p<0.05$, SP and NP, respectively).

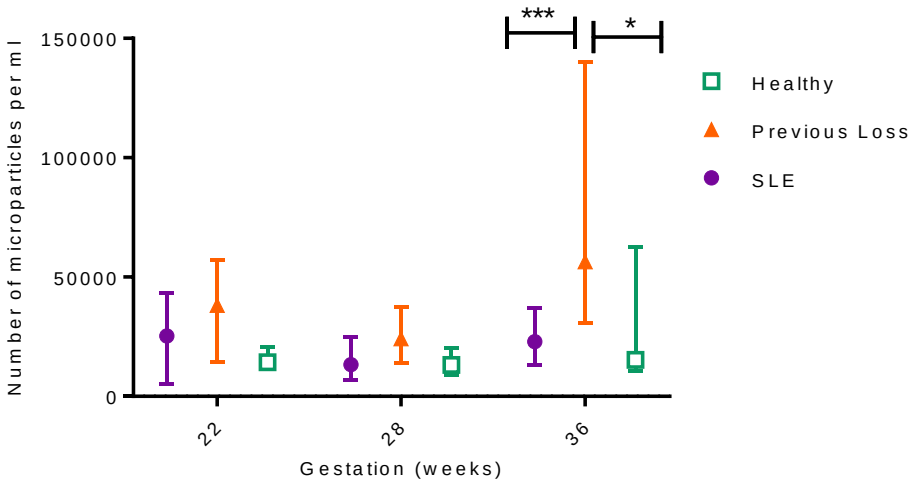


Figure 35 – Change in total circulating plasma microparticles with increasing gestation. Median and IQR shown. ***p<0.001, *p<0.05. SLE=Sytemic Lupus Erythematosus

6.4.3.2 Microparticle subsets

Longitudinally, each subgroup of MPs (representative of their cells of origin) underwent change throughout pregnancy (Figure 36A-E), with the majority of MPs increasing with gestation. The most abundant MP subsets seen was endothelial (CD62e) and erythrocyte (CD235a); of these CD62e MP levels gradually fell throughout pregnancy whereas CD235a MPs increased with increasing gestation.

Constitutive endothelial MP (defined as CD146 positive) levels increased significantly with increasing gestation in PL ($p=0.02$). There was no longitudinal change seen in SP or NP. At 28 weeks' gestation, the levels of CD146 positive MPs were significantly higher in PL compared to SP ($p=0.02$) and higher than in both women with SLE and healthy women at 36 weeks ($p<0.01$ and $p=0.02$ respectively) (Figure 36A).

Activated EMP marker (defined as CD62e positive) was stable at a low level throughout pregnancy in SP and NP (Figure 36B), in contrast in PL there was a non-significant rise from 22-28 weeks then a fall from 28-36 weeks ($p=0.002$). Levels of circulating CD62e MPs were significantly

higher in NP compared to SP at 22 weeks (p=0.03), and were significantly higher in PL compared to SP at 28 weeks (p=0.02).

Platelet MPs (defined as CD41a positive) showed a non-significant rise throughout pregnancy in SP, while in both NP and PL a non-significant fall in levels was seen from 28-36 weeks (Figure 36C). There was no significant difference in levels at any matched gestational time point.

Erythrocyte MPs (defined as CD235a positive) were lowest in SP throughout pregnancy, where it fell steadily from 22-36 weeks' gestation (Figure 36D). Levels were highest in PL at all time points. In NP, the levels of CD235a MPs gradually increased with gestation, so that at 28 weeks the level of CD235a MPs was significantly higher in NP when compared to SP (p=0.04).

Leucocyte derived MPS (defined as CD45 positive) were lowest in PL compared to NP at 22 weeks' gestation (p=0.04), with SP intermediate in their values (Figure 36E). Levels then increased from 22-28 weeks' gestation in PL and remained stable from 28-36 weeks; this rise was not statistically significant. The opposite pattern was seen in NP where CD45 MPs fell from 22-28 weeks, then rose from 28-36 weeks. At 36 weeks, levels of CD45 MPs were highest in NP and lowest in SP (p=0.04).

Tissue factor MPs (defined as CD42 positive) were seen at their lowest level in SP, with a fall from 22-28 weeks. In contrast, levels of CD42 MPs rose from 22-28 weeks in PL and were significantly higher than those seen in both NP and SP at 28 weeks (p=0.03 and p<0.01 respectively); levels then fell in PL such that there was no difference in levels between the three cohorts at 36 weeks' gestation (Figure 36E).

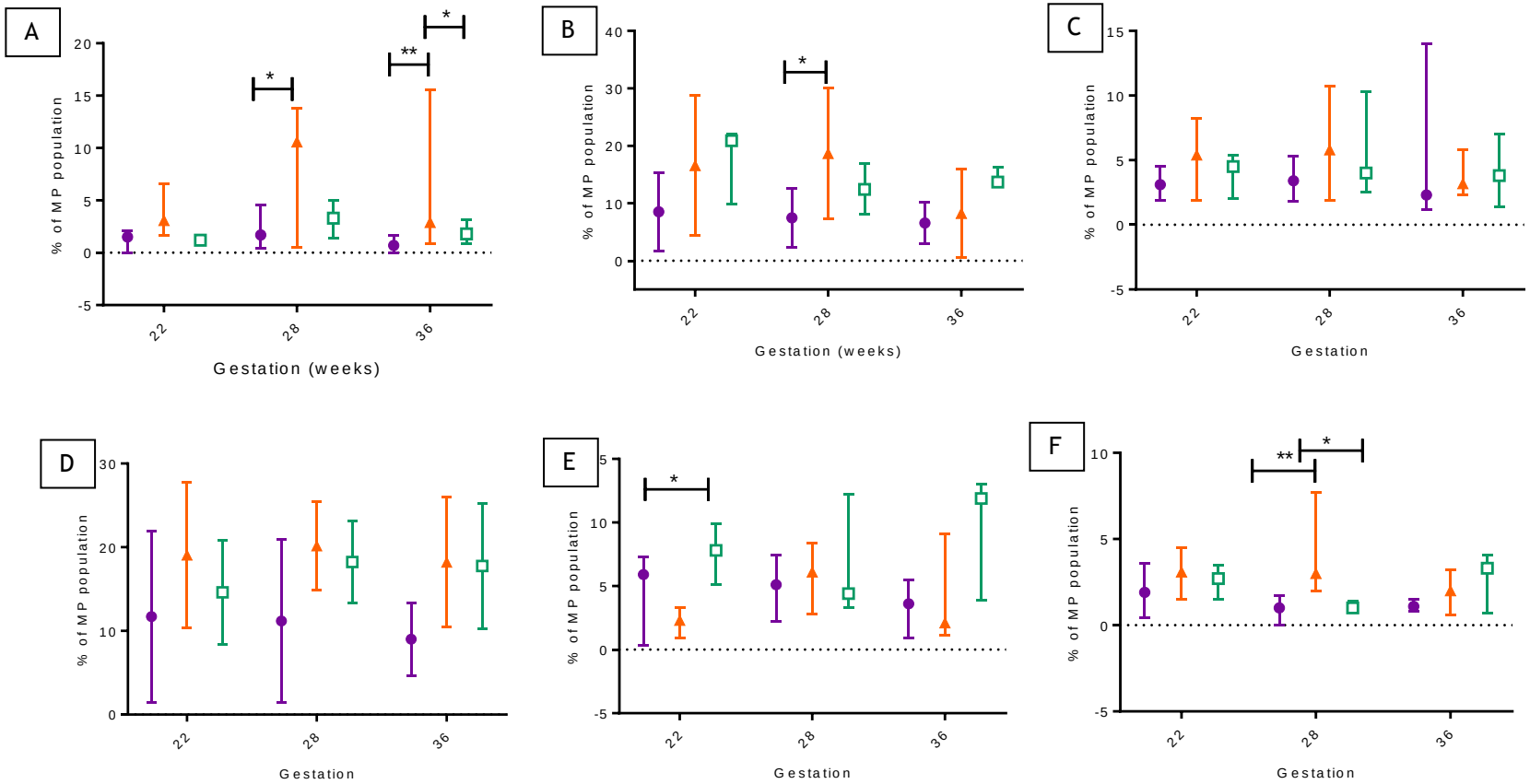


Figure 36 – Longitudinal Changes in Subsets of Microparticles throughout pregnancy A) CD146, Constitutive Endothelial B) CD62e, Activated Endothelial C) CD41a, Platelet D) CD235a, Erythrocyte E) CD45, Leucocyte F) CD42, Tissue Factor. Median and IQR shown. Green square/line for healthy women, Orange triangle/line for previous stillbirth, and purple circle/line for SLE (Sytemic Lupus Erythematosus). ***p<0.05, **p<0.01**

6.5 Discussion

This study has demonstrated a different profile of Treg and Th17 cells in pregnancies complicated by both SLE and prior stillbirth, when compared to uncomplicated pregnancies. In women with prior pregnancy loss, the presence of autoantibodies was associated with an elevated Th17 response at 17 weeks' gestation, both at baseline levels and after stimulation, consistent with a pro-inflammatory systemic response; this has not been reported previously.

When considering vascular disease and T cells, studies of SLE and vascular disease have demonstrated a reduction in Treg cells and an elevation of Th17 in patients with SLE complicated by atherosclerosis compared to SLE alone, suggesting a pathological role for Th17 and Treg cell imbalance in the development of atherosclerosis (104). Mimicking the differences seen in EMPs and recurrent pregnancy loss, elevated Th17 cell levels have been previously reported in women with recurrent miscarriage (335,340). In this study, there was a significant elevation of levels of circulating Th17 at 17 weeks' gestation in women with autoantibodies and previous stillbirth (in this subsequent pregnancy, no autoantibodies present at time of stillbirth) compared to healthy pregnancies, SLE pregnancies and pregnancies to women with prior stillbirth but no autoantibodies. It is therefore likely that this elevation in Th17 represents an increased systemic inflammation in women with prior stillbirth and autoantibodies. This increased inflammation may, in part, explain the association with long term development of SLE in women with a prior stillbirth or adverse pregnancy outcome (4,5), and be symptomatic of an, as yet undiagnosed, evolving maternal disease. This maternal inflammation also links with pregnancy outcomes as described in chapter 4 (page 79), with women with a prior stillbirth and autoantibodies having a trend towards lower individualised birthweight ratios.

Alongside this pro-inflammatory shift demonstrated by T cells, a change in circulating microparticles was also seen, with highest levels of total MPs being observed in women with previous stillbirth. This increase in MPs is supportive of the theory that immune dysfunction may underlie some of the pathology of stillbirth. Levels of MPs were generally lowest in women with SLE, which may reflect treatment efficacy and low disease activity, as EMP levels have been demonstrated to lower with treatment efficacy (139). Other MP subtypes have not previously been looked at in SLE longitudinally, either in the non-pregnant or pregnant state.

In women with a prior stillbirth, the most marked elevation of MPs seen was that of CD146, constitutive endothelial cell MPs. These have previously been demonstrated to be elevated in patients with SLE associated with vascular dysfunction, and have been shown to be elevated with active disease (341). The pathology underlying this elevation in EMP levels is not known but is presumed to relate to EMPs as a marker of endothelial damage (138,342,343). Elevation of EMPs is also seen in hypertensive disease in the absence of SLE (344,345). As shown in chapter 5 (page 102), women with a prior stillbirth had elevated brachial blood pressure throughout pregnancy compared to both women with SLE and healthy women; as such, this elevation in CD146 MPs may

be reflective of this increase in blood pressure. In this cohort, numbers were too low to comment on vascular changes that may be associated with the observed changes in MPs.

This study used Biodipy FL maleimide as a cell surface marker. Whilst this has been demonstrated as a valid tissue marker for microparticles (240), most studies into MPs have used annexin V as a marker. In this study, we encountered significant problems when using this reagent, with clumping and cellular aggregation in samples, a known potential problem due to the pro-coagulant effects seen with annexin V. Biodipy FL maleimide, on the other hand, reduced the likelihood of clumping and cellular aggregation in samples, and as such provided a more reliable, consistent result. It has been shown in other studies to be a valid marker of MPs (240), and as such these results are comparable to those seen in other studies.

In this study, overall levels of MPs were low in women with SLE, which may reflect optimal disease management and low disease activity. As described in Chapter 4.4 (page 83), overall disease activity and disease flare was low in this cohort of women, and management was via a specialist multidisciplinary team, As such, the levels of MPs seen here may not be truly reflective of SLE in pregnancy, but instead may be the result of optimally managed disease in pregnancy. Work by Parker et al has previously demonstrated a reduction in EMPs with treatment (139), which would support these findings. Similarly, women with SLE showed no difference in levels of Th17 despite an elevated autoantibody positivity rate, which likely reflects treatment effects. In our cohort of SP, 4 of 7 women were treated with with Hydroxychloroquine which has previously been demonstrated to reduce levels of Th17 in women with SLE, and so may confound these results (346).

Only small numbers of patients were included in this study, and there was a large heterogeneity in results, however these numbers are comparable to those in the wider literature examining MPs and pregnancy (347). Radu et al have previously showed a non-significant increase in all subtypes of MPs throughout pregnancy in healthy women (216). Similarly, in this study, total MPs rose with increasing gestation. When looking at individual MP subsets, both platelet and EMP levels fell with increasing gestation, which may reflect the physiological anaemia seen in pregnancy. In this cohort, total MPs were highest in women with a prior stillbirth throughout pregnancy; this has not been previously reported in the wider literature. In considering the effect of pregnancy pathology on MPs, Bretelle et al (221) showed a decrease in total MPs in pre-eclamptic pregnancies alongside an elevation in platelet (CD41) MPs compared to healthy pregnancies, suggesting a different underlying pathology in women with prior stillbirth to that seen in pre-eclampsia. Their cohort was, however, of mixed gestational range samples, and there was no information included in their study of previous pregnancy outcomes.

EMPs have been shown to be a marker of endothelial pathology in cardiac disease (348). Correlating this link between endothelial dysfunction and pregnancy, studies have shown an elevation in EMPs in pre-eclampsia (349,350). In this study, EMPs were elevated throughout pregnancy in women with a prior stillbirth, this was in the absence of pre-eclampsia. This echoes

findings by Carp et al who demonstrated increased levels of EMPs in non-pregnant women with recurrent pregnancy loss, in the absence of APS, compared to non-pregnant parous women (351). This may suggest an underlying vascular phenotype common to pregnancy loss that as yet is not understood.

In this study, the new presence of autoantibodies in women with a prior stillbirth was associated with increased levels of Th17 in pregnancy, and previous stillbirth itself was associated with increased levels of total circulating MPs, alongside increased EMPs throughout pregnancy. These two factors in combination suggest a link between stillbirth, systemic inflammation and vascular dysfunction. Despite this association the pregnancies in this study had good outcomes, although only small numbers included. The low levels of both Th17 and EMPs seen in SLE pregnancies is supportive of the efficacy of treatment. This suggests that treating women with a prior stillbirth with immunosuppression, such as Hydroxychloroquine, akin to the treatment of SLE-affected pregnancies may reduce some of the increased rates of recurrent pregnancy loss seen in this high risk group of women. As such, further investigation is warranted into whether these immunological markers can be used to develop screening tests or to risk-stratify high risk groups to allow further personalisation of care. In considering the similarities seen in an exaggerated pro-inflammatory response between untreated SLE outside of pregnancy in other studies and women with prior stillbirth in this cohort, alongside the increased rates of stillbirth seen in SLE, it is reasonable to consider a common pathological process underlying some pregnancy losses outside of the context of SLE. As such, further studies with bigger numbers, including prospective work, is called for to further unpick this relationship and potentially develop a screening test for stillbirth prevention.

7 Discussion and Future Work

Pregnancy acts as a window into a woman's lifelong health by challenging both the maternal immune system and cardiovascular system. Thus, by extension, pregnancy pathologies are associated with the development of disorders affecting these systems. The studies reported in this thesis have addressed the overarching hypothesis that unexplained antepartum stillbirth has a bi-directional association with autoimmune-mediated processes. Firstly, population level epidemiological approaches were used to demonstrate that women with a previous stillbirth are at an increased risk of subsequent development of autoimmune connective tissue disease (CTD). While the cohort studies demonstrated that, even in the absence of overt autoimmune CTD in a subsequent pregnancy after stillbirth, these women display evidence of reduced systemic maternal vascular adaptation to pregnancy, smaller *in utero* placental size and increased incidence of abnormal placental histology, and a pro-inflammatory shift in maternal immunology. As such, this thesis provides evidence that stillbirth and adverse pregnancy outcomes share a common aetiology of public health significance. At the very least, it could be argued that a stillbirth should trigger increased surveillance for long-term maternal development of either autoimmune or cardiovascular disease.

The original hypothesis was that stillbirth or late miscarriage is related to an autoimmune-mediated process similar to that found in underlying adverse pregnancy outcomes in SLE. As such, it was hypothesised that women with a previous fetal loss will display similarities in vascular and immune dysfunction to that identifiable in SLE-affected patients and pregnancies. The generated findings, conclusions and discussions are now considered below.

7.1 Women with a Previous Fetal Loss have an Increased Risk of Developing SLE

This thesis demonstrated the validity and utility of a primary care database in the UK to explore both normal and adverse pregnancy outcomes (APOs) and the subsequent development of maternal autoimmune CTD. Successful identification of both these pregnancy outcomes within this database and subsequent longitudinal follow up for maternal autoimmune CTD (defined as development of autoimmune antibodies, antiphospholipid antibodies (aPL), antiphospholipid syndrome (APS) or formal autoimmune CTD diagnosis (including SLE)) demonstrated an increased risk of subsequent autoimmune CTD, and more specifically SLE, in women with stillbirth compared to women who had an uncomplicated livebirth. A relatively short time frame was used in this study (mean 7.5 years follow up) and as such may underestimate the strength of the relationship seen between APO and autoimmune CTD, especially given the known lag time between antibody positivity and disease diagnosis, and therefore further cases of SLE or autoimmune CTD may later be formally diagnosed (82,90). These findings are similar to those seen in large Danish secondary care based studies, and demonstrate that APO remains a risk factor for development of autoimmune CTD in a genetically different population to those previously studied (4,5).

Through sub-analysis, two distinct chronological groupings of events were noted in this study: firstly those with higher rates of SLE, autoimmune CTD diagnosis, and non aPL antibody positivity preferentially appearing within the first 4 years after stillbirth and, secondly, those subsequently diagnosed with APS, 5 or more years after their index-pregnancy loss. Although this could represent undiagnosed active disease at the time of stillbirth, it may be that stillbirth itself is the trigger for development of autoimmune disease.

7.2 Women with a Previous Fetal Loss Display Similarities in Vascular and Immune Dysfunction to SLE-Affected Patients and their Pregnancies

From a systemic perspective, the data from Chapter 4 showed that 21% of women in a pregnancy following a stillbirth had autoantibodies present in their subsequent pregnancy. These autoantibodies were not recognised at the time of stillbirth, nor did these antibodies persist into the postnatal period of this subsequent pregnancy, and therefore would not routinely be used to inform future post- or ante-natal care. Of the autoantibodies detected, 67% were antiphospholipid (aPL) antibodies. This study is the first to describe the presence of these low levels of aPL antibodies in a subsequent pregnancy. Interestingly, no antibodies were evident at the time of their previous loss (tested at time of delivery of the stillborn baby). This may be due to the increased rate of development of autoantibodies over time, as demonstrated in the epidemiological study in chapter 3. Although numbers in this study were too small to evaluate statistical significance, in this cohort of women the presence of low level autoantibodies was associated with a trend towards reduced IBR centile compared to women without these autoantibodies. This finding is similar to studies of pregnancy and SLE in which rates of APO were highest in women with aPL, these studies showed the greatest risk to be associated with lupus anticoagulant (LAC) (87,162), the rates of aPL in their multicentre study group ((Predictors of pRegnancy Outcome: bioMarkers In antiphospholipid antibody Syndrome and Systemic lupus Erythematosus, PROMISSE) were 12.5% and LAC positivity 8.8%, although the study included 385 women, and as such was powered to detect the influence on adverse pregnancy outcome (87). The rates of aPL seen were, however, comparable to the rates seen in this study, and therefore the absence of effect in this study in the SLE cohort is likely due to sample size.

With regard to systemic changes in the maternal immune system, no difference was seen in levels of anti-inflammatory T regulatory (Treg) cells at 17 weeks gestation between the 3 pregnancy cohorts (healthy, SLE and past stillbirth). A trend towards increased peripheral pro-inflammatory T helper 17 (Th17) cells was seen in past stillbirth cases. When autoantibody status was considered, women with a previous stillbirth and autoantibodies present had an increased level of Th17 cells compared to their non-autoantibody positive counterparts. This difference remained present in both *in vitro* stimulated or unstimulated T cell conditions.

It may therefore be possible that a subset of women, with a previous stillbirth, display a systemic pro-inflammatory reaction, akin to that previously described in non-pregnant SLE patients, although not seen in this pregnant SLE population (352). It could also be possible, but

not provable here, that lower than expected levels of Th17 seen in our own SLE cohort, are a feature of either low disease activity or immunosuppressant therapy, i.e. hydroxychloroquine usage, both of which are reportedly associated with reduced circulating Th17 levels (346,353).

Microparticles (MPs), markers of cell breakdown, turnover and apoptosis were assessed longitudinally throughout pregnancy. With the exception of endothelial MPs, the levels of MP subsets in SLE in either the pregnant or non-pregnant state has not been previously reported. In line with other published data on MPs in healthy women, MPs increased throughout pregnancy (216). The most significant difference seen in this study related to EMPs, which were significantly elevated in women with a previous stillbirth at 28 and 36 weeks gestation, and rose with increasing gestation. In support of the pro-inflammatory changes already described in women with previous stillbirth, total circulating MPs were higher in these women, demonstrating increased levels of cell breakdown products or inefficient handling. Intriguingly, the lowest levels of MPs in this study were seen in the SLE pregnant cohort. Although this finding seems paradoxical, data on endothelial MPs (EMPs) outside of pregnancy, show reductions in parallel to disease activity and treatment (139). Given the low disease activity, reduced Th17 levels and a dearth of maternal complications within the SLE pregnant group here, it can only be surmised that judicious treatment is impacting on these previously recognised markers of SLE.

The longitudinal changes in blood pressure (BP) over gestation, for both pregnant women with SLE and those with prior stillbirth described in chapter 5, are novel. As anticipated, healthy women displayed the established pattern of a second trimester drop in diastolic BP, similarly, this pattern was also seen in women with SLE. However, this longitudinal change was not seen in women with a prior stillbirth in whom mean arterial pressure (MAP) was higher at all gestational time points and no drop in MAP was seen in the second trimester. The elevated BP seen in women with a prior stillbirth was associated with an increase in circulating constitutive (CD146 positive) endothelial cell microparticles. This marker is associated with endothelial dysfunction and cardiovascular (CV) disease (138,342,343); the combination of elevated blood pressure and elevated CD146 in this cohort of women is suggestive of a pathological vascular process in these pregnancies. This further supports the role of stillbirth as a predictor of adverse long term maternal cardiovascular health (33–35). Other markers of systemic vascular function showed increased pulse wave velocity (PWV, a marker of aortic arterial stiffness) in pregnant women with SLE, and lower values in healthy women from 16-28 weeks gestation; this again has not been reported previously. PWV underwent a non-significant increase with increasing gestation. Consistent with these changes in PWV was an increase in photoplethysmography-based measurements, with small artery stiffness (RI) undergoing a significant increase with gestation in women with SLE. This increase was not seen in the other two cohorts. In contrast to changes with PWV, there was no difference seen in photoplethysmography-based large artery stiffness (SI) across either the cohorts or gestation. This difference in the impact seen on small vs. large vessels may represent early stage changes associated with CVD that precede evidence of end organ damage (354,355). Linking to long term CV disease, elevation in PWV has been shown to

relate to the presence of atherosclerosis (133), an established risk factor for CV disease. All told, these data highlight notable maternal vascular issues in pregnancy in those women with a prior stillbirth. According to past studies, these issues could not only impact on their current pregnancy, they may also highlight the role a stillbirth could play as a predictor of long term maternal cardiovascular health.

Linking maternal systemic vascular function with placental-derived vascular markers, changes seen in circulating maternal placental growth factor (PlGF) and in soluble Fms-like tyrosine kinase-1 (sFlt-1) were considered. Consistent with published data, PlGF fell from 28-36 weeks' gestation, and levels of sFlt-1 remained stable (177,322,323). Overall, no differences in PlGF were seen across the pregnant cohorts. However, levels of anti-angiogenic sFlt-1 were higher in women with SLE at 36 weeks gestation compared to healthy women - a finding which echoes the wider literature on SLE, in which abnormalities are only seen in pregnancies complicated by PET (324,325). Changes in these circulating placental-derived vascular factors have not been previously described in women with previous stillbirth.

Previous studies have shown that a smaller *In utero* placental size is been associated with increased risk of APO, in both uncomplicated pregnancies and pregnancies with low plasma protein-A (PAPP-A) (261,262). In this study, *in utero* gestational age- matched placental measurements in the second trimester showed placentas in women with SLE had a significantly smaller width than their healthy counterparts. Of note, intermediate measurements were seen in women with prior stillbirth. This trend was also apparent for fetal:placental weight ratio, with the highest seen in healthy women, lowest in SLE and those with prior stillbirth again in between. Low fetal:placental weight ratio has been shown to be associated with reduced nutrient transfer compared to placentas with an age appropriate fetal:placental weight ratio (356), and as such may explain some of the adverse pregnancy outcomes seen in SLE. When examining the placentas from stillborn offspring, significantly smaller weights were recorded than their livebirth equivalents, despite similarities in gestational age at delivery. This difference in placental dimensions is consistent with published data on reduced *in utero* placental size and poor pregnancy outcome (261,262), and suggests that the differences seen between the stillbirth and livebirth placentas in this study reflect the spectrum of placental disease, with a more severe phenotype markedly associated with stillbirth. Furthermore, changes were also evident in placental architecture, with increased numbers of syncytial knots seen in SLE placentas, and fewest in healthy women. Syncytial knots are considered to be a marker of placental hypoxia, oxidative stress and ageing (168,357,358). The increased prevalence of syncytial knots in SLE, and to a lesser extent in women with previous stillbirth, supports the theory that an underlying pathology related to maternal vascular malperfusion underpins the pregnancy pathology seen in these women (30). Interestingly, villitis of unknown aetiology (VUE), an inflammatory process which occurs in the absence of a pathogen (274), was most markedly present in women with a prior stillbirth (present in 26.3% of placentas), and not seen in healthy women. This acts as evidence to support the theory of an immune pathology at

the fetal-maternal interface. The intermediate prevalence of villitis (15.4%) seen in women with SLE may be due to confounders such as concurrent immunosuppressant usage and duration of pregnancy, as the incidence of villitis increases with pregnancy duration. In a systematic review of VUE, Derricott et al. showed a 15.6% prevalence of VUE in healthy term pregnancies, with rates as high as 28.6% seen in FGR (359). As such, the rates seen in this study differ from the published literature, with lower rates seen in healthy women, and higher rates seen in the high-risk populations. Low numbers of placentas were collected for healthy women in this study, and as a result may explain the low levels of VUE seen in this healthy population. Unfortunately, this study was not powered to determine this possibility.

To investigate further evidence of immune involvement, complement deposition was utilised as a histological marker of tissue rejection, or at the very least aberrant maternal (and even fetal) immune recognition. Complement factor 4d (C4d) levels in the placenta were unchanged across the livebirth cohorts, with no increase in C4d deposition seen in SLE-related placentas. This finding was in contrast to previous research, which demonstrated an increased level of C4d in placentas of women with SLE (187–190). A potential explanation is the use of immunosuppressant medication and low molecular weight heparin (LMWH), which reduces complement activation (360). Additionally, the low level of disease activity in this cohort may also explain these results. For stillborn offspring, placental C4d deposition was highest in those associated with villitis and, to a lesser degree, in samples with evidence of maternal vascular malperfusion. As stated above, in the absence of an infective agent, villitis in the placenta is presumed to be the result of an autoimmune reaction (361–363), as such the changes seen in this study, which have not previously been reported, support this and provide tantalising evidence for a breakdown in maternal immune tolerance of the placenta, with perhaps failure to adapt to the antigenically disparate elements of the feto-placental unit at a local or systemic level.

In contrast, the levels of deposition of the complement receptor for complement factor 3a (C3aR) were significantly higher in placentas from healthy women, and lowest seen in placentas from women with SLE and who had a previous stillbirth. This lowering of C3aR in the placenta is consistent with data on pre-eclampsia (PET), in which lower levels of C3aR were seen compared to healthy pregnancies (270); this change is presumed to relate to aberrant systemic complement function with downregulation of C3aR, presumed to be an adaptive mechanism (270). The observed changes indicate a link between inflammatory and vascular changes, with the placenta failing to adapt to the immune challenges of pregnancy, as C3aR deposition was highest in placentas with evidence of maternal vascular malperfusion and villitis, and lowest in those with no obvious pathology. Although the role and importance of this in the breakdown of maternal tolerance is again speculative, the possibility that this underlies the catastrophic collapse of the placenta and fetus in stillbirth cannot, however, be disregarded.

This study has demonstrated that the mode of delivery was unaffected by maternal disease state, a finding in contrast to that seen in the wider literature which reports increased risk of caesarean section in women with SLE (364). Overall rates of caesarean section were comparable

to national data on mode of delivery (365). It should be noted that complication rates (specifically FGR and extreme prematurity) were also curtailed in these cohorts compared to expectations, as these factors are known to be associated with increased risk of caesarean section (366,367). In considering the low rates of complications (both maternal and fetal), this study suggests a positive role for multidisciplinary team (MDT) care in such high risk pregnancies. Whilst the benefits of MDT care have been widely reported in pregnant women with SLE, this has yet to be demonstrated or reported in women with a previous stillbirth (153,282,283).

In women with SLE, the presence of lupus nephritis, whether active at the time of conception or quiescent, was associated with significantly lower gestational ages at delivery, and lower birthweight than healthy controls. Although no statistical change in individualised birthweight ratio (IBR) centile was demonstrated. The lower birthweight seen reflects published data on pregnancy outcomes and low birthweight in SLE (2,142). The lack of effect on IBR, despite differences in birthweight and gestation at delivery, perhaps emphasises the need for careful interpretation of pregnancy outcomes in SLE. Arguably, the absence of data on maternal characteristics, even with gestation matching and use of customised centiles, makes over diagnosis of FGR a distinct possibility. As such this may account for the outcome discrepancies seen with SLE in this study.

Screening methods for APO in this study included the use of uterine artery Doppler (UtAD). In women with SLE, the presence of an abnormal UtAD (as defined by the presence of a diastolic notch) was associated with a significant reduction in gestational age at delivery and a trend towards lower final IBR centile. In line with other published data, this pattern was not seen in healthy, low risk women (368,369). There were no cases of abnormal UtAD in women with a previous stillbirth, but the prevalence of UtAD abnormalities in this cohort has never previously been reported. This low rate of pregnancy complications seen in this study is likely the cause for the absence of UtAD abnormalities seen in this cohort. However, this undoubtedly demonstrates the need to develop a better model of risk prediction in these women, than currently applied. Uterine artery Doppler has been demonstrated to be of utility in the risk stratification of women with SLE, although its relationship to SLE disease activity means it is not purely a predictor of fetal wellbeing but also maternal disease state.

7.3 Limitations

The key limitations of this thesis relate to sample size and the low rate of complications seen within the study cohorts, not least those with SLE which reflect the study being undertaken within specialist clinics with pre-conception disease optimisation in a single UK-tertiary maternity centre.

The relative rarity of SLE as a disease meant that the number of study participants was low, although comparable to those seen in the wider literature, in which study numbers range from 11-203, the mean number of study participants being 52 (2). Women were required to speak and read English in order to be eligible for the study; given the demographics of women attending

the maternity unit, this reduced the number of suitable participants. An additional complication in recruiting to the previous stillbirth arm of this study, was that several women found the emotional challenges of appointments significant, and as such could not contemplate additional time spent in the hospital for the extra measures required.

Both cohorts of high-risk women were recruited from specialist MDT antenatal clinics; paradoxically, this is both a strength and weakness of the study. The MDT nature of the management of these women meant that disease activity and complications were optimally managed and, potentially as a consequence, levels of maternal and fetal complications were lower than those reported in the wider literature (2). Additionally, the access to pre-conception counselling for women with SLE meant, where possible, medication and other maternal risk factors were optimised and mitigated prior to conception, which would further reduce the risk of poor outcomes in SLE.

With regard to clinical investigations, the measurement of UtAD at 36 weeks was technically challenging through fetal positioning. Specifically, the low fetal head caused difficulties in accessing adequate views of UtAD at the appropriate anatomical landmarks. Ultimately, this impacted on UtAD observations at these later gestations, with low n numbers available for analysis. A potential solution in the future may be to use a transvaginal route rather than transabdominal, but this was not permitted here, and may prove less acceptable to participants, thus making recruitment into subsequent studies harder.

From an epidemiological perspective, changes to data recording in CPRD meant that the highest quality data were from the year 2000, thus limiting the time available for follow up for women in the study, and potentially missing cases of autoimmune CTD that would develop later. In addition, poor quality data on ethnicity in this dataset also meant it was not possible to consider the impact of ethnic group, and therefore potential underlying genetic risk factors that would impact on the risk for development of autoimmune CTD. The absence of data on ethnicity is important as non-White ethnicity is an established risk factor for both stillbirth and autoimmune disease (21,370,371). Consequently, the findings should be explored in a database with as much information about confounding factors as possible.

7.4 Future Work

Undoubtedly, more work is needed to unravel the relationship between stillbirth, SLE and autoimmune disorders. Of note, there is a growing interest in inflammatory disorders of the placenta including villitis of unknown aetiology and chronic histiocytic intervillositis and their relationship to stillbirth (359,372). In order to assess more accurately the immunological changes seen in subsequent pregnancy and their relationship to pregnancy loss or APO, measurements including assessment of maternal circulating Th17 and Treg cells would ideally be performed at the time of stillbirth or APO. These could then be related to assessment of complement deposition and the presence of inflammation in the placenta of the stillborn fetus. This additional information would allow connection to be made between any maternal disease,

placental histology and identifiable causes of stillbirth with these immunological markers, enabling the potential development of a screening test to identify women at increased risk of stillbirth.

This thesis suggests that retesting all women following stillbirth for the presence of autoimmune antibodies, including antiphospholipid antibodies, in the first trimester of a subsequent pregnancy would enable further risk stratification. As well as stratifying these women as high risk, the clinical target and relationship between these antibodies and adverse pregnancy outcomes would also inform current and novel immunosuppressive therapies.

From a vascular perspective, this study has shown a clear difference in the longitudinal pattern of BP in pregnant women with a previous stillbirth, most notably, the absence of a mid-trimester drop in diastolic BP. Further assessment of the vasculature in these women would therefore be warranted. Firstly, the diurnal variation in BP (utilising 24 hour BP) could be assessed for the presence or absence of a nocturnal BP drop which would further characterise the vascular profile of these women. Secondly, more extensive evaluation of their vasculature, including carotid artery Doppler, would enable identification of atherosclerosis and carotid intima narrowing - features strongly associated with the long term cardiac morbidity seen in SLE (125,127,129). Combined, these might tie together the cardiac morbidity seen following stillbirth with the vascular pathology of SLE. Long term follow up of BP in women after a stillbirth would inform on a public health level as to whether these vascular changes seen during pregnancy and in the puerperium are pre-existing, permanent, or a pregnancy phenomenon, and what that means for long term cardiovascular disease risk and the role of primary prevention in primary care.

In terms of vascular assessment and stillbirth prevention, a retrospective study of BP in women whose pregnancies end with stillbirth, to look at longitudinal changes in BP throughout pregnancy, is suggested. This would inform as to whether the changes seen in maternal BP profile across a subsequent pregnancy following stillbirth were already present in the index pregnancy. If so, these changes could be used to form part of a predictive model of stillbirth, and identify women in their index pregnancy before fetal death occurs.

7.5 Summary

The work presented in this thesis has compared pregnant women with SLE, prior stillbirth and healthy uncomplicated pregnancies using multiple methodologies. It has demonstrated similarities between women with SLE and those with a previous stillbirth at both the vascular and immunological level. From an epidemiological perspective, this work has demonstrated a link between stillbirth and adverse pregnancy outcomes and development of autoimmune CTD, including SLE. Given these similarities, it is therefore plausible that SLE and some cases of otherwise unexplained stillbirth share a common aetiology. Quantifying this further could potentially lead to the development of a screening test to identify women at increased risk of stillbirth, and a targeted treatment to reduce stillbirth risk. The vascular similarities seen in these two high risk groups, alongside their common risk of lifelong cardiovascular disease,

highlight the need to identify women with a stillbirth for increased health surveillance and screening for modifiable CVD risk factors, to allow for timely prophylactic interventions.

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Appendix 1 – Patient Information (Undiagnosed Lupus-Like Autoimmunity and unexplained stillbirth)

Central Manchester University Hospitals NHS
NHS Foundation Trust

Participant information sheet (Pregnant Patients)

Undiagnosed Lupus-Like Autoimmunity and unexplained stillbirth

Background

The reasons why women have a miscarriage or stillbirth are in many cases still unknown. One possibility is that some of these affected women have unrecognised immune issues (such as Lupus) before and during their pregnancy. At present, the number of these affected women is unclear, along with the form these immune issues can take. For these reasons we wish to research the immune and circulatory systems of women who have had a previous stillbirth or late miscarriage (i.e. beyond 15 weeks gestation). We believe that these measurements will help us understand how a women's immune system may predispose them to pregnancy loss. This knowledge may help save babies by providing more appropriate pregnancy care and medicines.

Why have I been chosen to take part?

You have been asked to participate because you fall into one of two groups:

1. You are currently pregnant and experienced a miscarriage (after 15 weeks gestation) or stillbirth in your previous pregnancy.

2. You are a healthy pregnant woman with no previous adverse pregnancy issues.

What do I have to do?

On the days you have been invited, attend the Maternal and Fetal Health Research Centre on the 5th floor in St Mary's Hospital. These visits will be at around 12, 16, 22, 28, and 36 weeks gestation. For convenience, most of these visits will be arranged at the same time as your usual visits to antenatal clinic, but some will be extra and not part of your routine antenatal care. Because caffeine in drinks such as coffee or cola can affect some of the measurements, we ask that you don't have any drinks containing caffeine for 4 hours before the appointment.

At each visit, clinical examination will be performed that will include measuring your blood pressure using a standard blood pressure cuff, measuring the elasticity (stretchiness) of your blood vessels using a special cuff around your finger. Ultrasound scans of the baby and placenta will also be performed, to monitor the growth of your baby, size of placenta and blood flow to and from the baby. We will also take some blood, approximately 25mls (5 teaspoons). These will be used to look at important elements associated with your immune system.

After delivery of your baby, with your prior consent, we will collect your placenta (afterbirth) from the delivery suite and store pieces of the tissue for monitoring interactions with your immune system. The placenta is usually destroyed after delivery and unused tissue will be disposed of.

Following delivery (around 6 weeks after the birth), we ask you return one last time for similar measurements to be performed.

Does this affect my treatment?

Your involvement in this study will probably not affect the treatment you receive throughout your pregnancy. However, in the unlikely case that evidence of an auto-immune condition is suggested - a situation known to influence the course of pregnancy - your clinical team will be informed immediately, so that your pregnancy care can be tailored, accordingly.

Will information about me be used?

In addition to the measurements made during your antenatal visits, we would like your permission to use your clinical information such as your age, past medical problems and previous pregnancy problems, as well as the outcome of your current pregnancy. All information and samples will be kept anonymously both during and after the study has finished. At all times this information, and any collected biological samples, will be kept confidentially.

What are the possible risks of taking part?

For all patients, potential risks are those associated with all clinical visits, i.e. distress, feeling faint, nausea etc. There are no additional physical risks involved with participating in this study, aside from the usual discomfort when a blood sample is taken and the potential for a bruise to develop at the site. There have been no adverse events in similar studies and some women taking find these additional procedures reassuring. In cases of distress, the information and emotional support provided will be derived from the specialist care team within our high-risk antenatal clinics.

Are there any possible benefits?

There no direct benefits from taking part in this study. Women who participate in this study will either already be receiving close clinical care within the high risk pregnancy clinics at St Mary's Hospital, or will be recruited into these clinics for the purposes of this study. As implied, if in the course of this research evidence of an auto-immune condition is identified, the clinical teams will be informed and will act on these findings in the best interests of you and your baby.

Will any samples be kept?

There may be some blood samples and placental tissue remaining after the tests in this study are complete. With your permission, we would like to be able to store these for future research. These will be kept within our department tissue biobank and with your consent will be treated as a gift to medical research. However, you may prefer that we do not store these samples. In this case, please inform the researcher and indicate this on the consent form.

What will happen to my clinical and personal information?

All the clinical information you provide will be encoded (so that your personal details such as name and address are secure) and stored securely. This information will not be revealed to anyone other than the research team.

What if I change my mind?

You can change your mind about taking part in the research at any point without having to give a reason. Feel free to discuss the research with other doctors in clinic, your GP, or the Patient Advisory Liaison Service (PALS) on 0161 276 4124.

Do I have to sign any forms?

If you decide to take part in this research, please read and sign the consent form. You can still change your mind, even after the form is signed. In this case your data will be removed and biological samples destroyed.

Where will the research take place?

The Maternal and Fetal Heath Research Centre on the 5th floor in St Mary's Hospital, Oxford Street, Manchester M13 9WL, Tel: (0161)276 6916, Fax: (0161)701 6971. This is part of the Central Manchester Foundation NHS Trust.

What will happen to the results?

Your collected data will be stored securely at all times on NHS premises, separated from your secure personal details. In line with University policy, this will be for minimum of 10 years. Results will be presented anonymously at research meetings or published in medical journals. No individuals will be identified. Unless an autoimmune condition is defined and clinical team informed, the findings from the study will not be recorded on your hospital records and they will only be available to people involved directly in the study.

Have the ethics committee approved this study

This study has been approved by the NRES Committee North West - Greater Manchester West

What if I have a complaint?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If they are unable to resolve your concern or you wish to make a complaint regarding the study, please contact a University Research Practice and Governance Co-ordinator on 0161 275 7583 or 0161 275 8093 or by email to research.complaints@manchester.ac.uk.

In the event that something does go wrong and you are harmed during the research you may have grounds for a legal action for compensation against the University of Manchester or Central Manchester NHS Foundation Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Travel Expenses

You may claim for reasonable travel expenses for visits to the hospital that are outside the usual times that you would need to come to hospital. Please ask the researchers if you wish to do this.

Thank you very much for considering taking part in our research.

If you wish to obtain further advice about this research you may contact the researchers on the details below:

Researchers

Dr Ian Crocker

Dr Hannah Kither

Dr Clare Tower

Professor Ian Bruce

Contact details*:*

Dr Ian Crocker

Maternal and Fetal Health Research Centre,

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Manchester M13 9WL

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Research Midwife:

Louise Stephens: 0161 701 6958

Appendix 2 – Consent Form (Undiagnosed Lupus-Like Autoimmunity and unexplained stillbirth)

CONSENT FORM

Title of Project: Undiagnosed Lupus-Like Autoimmunity and unexplained stillbirth

Researchers: Dr Hannah Kither, Ms Louise Stephens, Dr Clare Tower, Dr Ian Bruce, Dr Ian Crocker

Please read and initial each relevant box

All participants:

1. I confirm that I have read and understood the information leaflet (version 3) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without my medical care or legal rights being affected. ☐
3. I understand that sections of my medical notes may be looked at by responsible individuals from the University of Manchester or Central Manchester Trust where it is relevant to my taking part in the research. I give permission for these individuals to have access to my records. ☐
4. I agree that my clinical details, relevant to this study, can be recorded for use by the research team. ☐
5. I give permission for measurements of my blood pressure and circulation to be taken and recorded. ☐
6. I agree to have blood samples taken and analysed for this study ☐
7. I give permission for my blood to be stored so that it may be used for this and future studies, including genetic studies, and will be regarded as a gift to medical research. I understand that these samples will be anonymous (i.e. do not have my name on and cannot be identified) and will be moved, on completion of the study, to a University designated biobank. ☐
8. I agree to take part in this study ☐

Pregnant patients only:

9. I agree to have additional ultrasound scans on my baby and placenta ☐
10. I agree to have my placenta collected and analysed for this study ☐

☐

11. I give permission for my placental tissue to be stored so that it may be used for this and future studies, including genetic studies, and will be regarded as a gift to medical research. I understand that these samples will be anonymous (i.e. do not have my name on and cannot be identified) and will be moved, on completion of the study, to a University designated biobank.

_____	_____	_____
Name of participant	Date	Signature

_____	_____	_____
Name of person taking consent	Date	Signature

Appendix 3 - American College of Rheumatologists Criteria for diagnosis of SLE

Criteria	Definition
1.Malar Rash	Fixed erythema, flat or raised, over the malar eminences,tending to spare the nasolabial folds
2.Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3.Photosensitivity	Skin rash as a result of unusual reaction to sunlight by patient history or physician observation
4.Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5.Nonerosive Arthritis	Involving ≥ 2 peripheral joints, characterized by tenderness, swelling, or effusion
6.Pleuritis or pericarditis	**a.** Pleuritis-convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion **b** .Pericarditis-documented by ECG or rub or evidence of pericardial effusion
7.Renal disorder	**a.** Persistent proteinuria ≥ 0.5 grams per day or ≥ 3+ if quantitation not performed **b.** Cellular casts-may be red cell, hemoglobin, granular, tubular, or mixed
8.Neurologic disorder	**a.** Seizures-in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance **b.** Psychosis-in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9.Hematologic disorder	**a.** Hemolytic anemia-with reticulocytosis **b.** Leukopenia-less than 4,000/mm^3 total ≥ 2 or more occasions **c.** Lymphopenia-less than 1 ,500/mm^3 ≥ 2 or more occasions **d.** Thrombocytopenia-less than 100,000/mm^3 in the absence of offending drugs
10.Immunologic disorder	**a.** Anti-DNA: antibody to native DNA in abnormal titer **b.** Anti-Sm: presence of antibody to Sm nuclear antigen **c.** Positive finding of antiphospholipid antibodies based on **1)** an abnormal serum level of IgG or IgM anticardiolipin antibodies **2)** A positive test result for lupus anticoagulant using a standard method **3)** A false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.
11. Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with “drug-induced lupus” syndrome

Appendix 4 – FARSITE Search terms

Pregnancy Loss

Single stillbirth
AND/OR H/O: stillbirth
AND/OR Recurrent miscarriage
AND/OR Complete miscarriage
AND/OR Incomplete miscarriage
AND/OR Inevitable miscarriage
AND/OR Fetal intrapartum death
AND/OR Intrauterine fetal death
AND/OR (Birth: [hypoxia] &/or [asphyxia]) or (fetal distress) or (death - fetal asphyxia)
AND/OR Intrauterine fetal death
AND/OR (Birth: [hypoxia] &/or [asphyxia]) or (fetal distress) or (death - fetal asphyxia)
AND/OR Ectopic pregnancy
AND/OR Ectopic pregnancy
AND/OR Other ectopic pregnancy
AND/OR Fresh stillbirth
AND/OR [X]Fresh stillbirth
AND/OR Fetal death due to prelabour anoxia
AND/OR Intrauterine fetal death

Autoimmune Codes

Discoid lupus erythematosus
AND/OR Lupus erythematosus migrans
AND/OR Lupus erythematosus nodularis
AND/OR Lupus erythematosus profundus
AND/OR Lupus erythematosus tumidus
AND/OR Lupus erythematosus unguium mutilans
AND/OR Subacute cutaneous lupus erythematosus
AND/OR Lupus erythematosus NOS
AND/OR [X]Other local lupus erythematosus
AND/OR Systemic lupus erythematosus
AND/OR Nonbacterial verrucal endocardiosis
AND/OR Systemic lupus erythematosus with organ/system involvement
AND/OR Systemic lupus erythematosus with pericarditis
AND/OR Cerebral lupus
AND/OR Drug-induced systemic lupus erythematosus
AND/OR Scleroderma (& [acrosclerosis] or [systemic sclerosis])
AND/OR Scleroderma (& [acrosclerosis] or [systemic sclerosis])
AND/OR (Sicca - Sjogren
AND/OR Dermatomyositis (& [Poikilodermatomyositis])
AND/OR Polymyositis
AND/OR Antiphospholipid syndrome
AND/OR (Systemic fibrosclerosis) or (other specified diffuse collagen diseases)

AND/OR Inflammatory polyarthropathy: (& [rheumatoid arthritis] or [other])
AND/OR Rheumatoid arthritis
AND/OR (Polyarthropathy: [unspecified] or [NEC]) or (polyarthritis)
AND/OR [X]Systemic connective tissue disorders
AND/OR [X]Other forms of systemic lupus erythematosus
AND/OR [X]Other forms of systemic sclerosis
AND/OR [X]Other systemic diseases of connective tissue
AND/OR [X]Systemic disorders of connective tissue in other diseases classified elsewhere
AND/OR
[X]Dermatopolymyositis unspecified
AND/OR Pm-1 - Polymyositis-scleroderma antibody level
AND/OR Anti-tissue transglutaminase level
AND/OR Lupus antibody activity
AND/OR IgA anticardiolipin level
AND/OR Fluid anti-nuclear factor level
AND/OR Anti-nuclear (speckled) antibody level
AND/OR Double stranded deoxyribonucleic acid antibody detection using Crithidia
AND/OR Lupus anticoagulant screening test
AND/OR Aspergillus fumigatus 1 precipitin level
AND/OR Aspergillus fumigatus 2 precipitin level
AND/OR Cysticercosis EIA
AND/OR Anti-nuclear factor positive
AND/OR Anti-nuclear factor weakly positive
AND/OR Ro antibody positive
AND/OR La antibody positive
AND/OR Sm antibody positive
AND Polyneuropathy in collagen vascular disease
AND/OR Polyneuropathy in rheumatoid arthritis
AND/OR Myopathy due to rheumatoid arthritis
AND/OR Myopathy due to systemic sclerosis
AND/OR Myopathy due to Sj+Ã,gren's disease
AND/OR Lung disease with systemic sclerosis
AND/OR Lung disease with diseases EC
AND/OR (Nephrotic syndrome in systemic lupus erythematosus) or (lupus nephritis)
AND/OR Lupus erythematosus

Appendix 5 – READ codes and Medcodes used within the Clinical Practice Research Datalink (CPRD) search criteria

		VAGINAL DELIVERY
5868	Ly0..00	Spontaneous vertex delivery
22491	Ly1..00	Spontaneous breech delivery
83535	Lyu5000	[X]Other single spontaneous delivery
12052	Z257.00	Delivery normal
9383	Z257.11	Normal delivery
11194	Z257.12	Spontaneous vaginal delivery
25681	Z257.13	SVD - Spontaneous vaginal delivery
17492	Z257.14	FTND - Full term normal delivery
9791	Z257.15	ND - Normal delivery
9160	Z257100	Spontaneous vertex delivery
48230	Z254800	Deliveries by spontaneous breech delivery
11530	Z254900	Vaginal delivery
22613	Z248.00	Normal labour
11532	7F14.00	Breech extraction delivery
105276	7F14000	Breech extraction delivery with version
73048	7F14y00	Other specified breech extraction delivery
54591	7F14z00	Breech extraction delivery NOS
33378	7F15.00	Other breech delivery
29070	7F15000	Spontaneous breech delivery
23994	7F15100	Assisted breech delivery
15926	7F15y00	Other specified other breech delivery
64464	7F15z00	Other breech delivery NOS
4780	L20..00	Normal delivery in a completely normal case
239	L20..11	Spontaneous vaginal delivery
31743	L200.00	Normal delivery but ante- or post- natal conditions present
58721	L20z.00	Normal delivery in completely normal case NOS
28726	7F19100	Water birth delivery
33773	7F19y00	Other specified normal delivery
8967	7F19z00	Normal delivery NOS
60256	Z254B00	Brow delivery
48742	Z254C00	Face delivery
65819	7F18y00	Cephalic vagin deliv abnorm pres head without instrument OS
64367	7F18z00	Cephalic vagin deliv abnorm pres head without instrument NOS
167	7F19.00	Normal delivery
		INSTUMENTAL VAGINAL DELIVERY
40744	Z254A00	Abnormal delivery
1279	7F16.00	Forceps cephalic delivery
20002	7F16000	High forceps cephalic delivery with rotation
5033	7F16100	High forceps cephalic delivery NEC
61077	7F16200	Mid forceps cephalic delivery with rotation
23394	7F16300	Mid forceps cephalic delivery NEC
5213	7F16400	Low forceps cephalic delivery

65940	7F16500	Trial of forceps delivery
34628	7F16600	Failed forceps delivery
70242	7F16700	Barton forceps cephalic delivery with rotation
21554	7F16900	Kielland forceps cephalic delivery with rotation
33480	7F16y00	Other specified forceps cephalic delivery
14686	7F16z00	Forceps cephalic delivery NOS
2458	7F17.00	Vacuum delivery
688	7F17.11	Ventouse delivery
105419	7F17000	High vacuum delivery
51736	7F17100	Low vacuum delivery
57945	7F17200	Vacuum delivery before full dilation of cervix
62318	7F17300	Trial of vacuum delivery
86399	7F17y00	Other specified vacuum delivery
14892	7F17z00	Vacuum delivery NOS
20738	7F19000	Manually assisted vaginal delivery
47508	7F1A.00	Other methods of delivery
6452	L395.00	Forceps delivery
17684	L395.11	Keilland's forceps delivery
20425	L395.12	Neville - Barnes forceps delivery
17704	L395.13	Simpson's forceps delivery
53937	L395000	Forceps delivery unspecified
50675	L395100	Forceps delivery - delivered
30274	L395200	Low forceps delivery
42275	L395300	Mid-cavity forceps delivery
57789	L395400	Delivery by combination of forceps and vacuum extractor
40710	L395z00	Forceps delivery NOS
25223	L396.00	Vacuum extractor delivery
6141	L396.11	Ventouse delivery
58844	L396000	Vacuum extractor delivery unspecified
49446	L396100	Vacuum extractor delivery - delivered
37699	L396z00	Vacuum extractor delivery NOS
69780	Lyu5100	[X]Other and unspecified forceps delivery
28903	Z254200	Delivered by low forceps delivery
34411	Z254300	Delivered by mid-cavity forceps delivery
98059	Lyu5400	[X]Other manipulation-assisted delivery
66315	Lyu5500	[X]Other specified assisted single delivery
100675	Lyu5700	[X]Assisted single delivery, unspecified
		CAESAREAN DELIVERY
740	7F12.00	Elective caesarean delivery
61259	7F12000	Elective upper uterine segment caesarean delivery
9668	7F12100	Elective lower uterine segment caesarean delivery
10049	7F12111	Elective lower uterine segment caesarean section (LSCS)
47546	7F12y00	Other specified elective caesarean delivery
3085	7F12z00	Elective caesarean delivery NOS
4638	7F13.00	Other caesarean delivery
15514	7F13000	Upper uterine segment caesarean delivery NEC

17744	7F13100	Lower uterine segment caesarean delivery NEC
364	7F13111	Lower uterine segment caesarean section (LSCS) NEC
72513	7F13200	Extraperitoneal caesarean section
12118	7F13300	Emergency caesarean section
15073	7F13y00	Other specified other caesarean delivery
35190	7F13z00	Other caesarean delivery NOS
720	L398.00	Caesarean delivery
52875	L398000	Caesarean delivery unspecified
33477	L398100	Caesarean delivery - delivered
863	L398200	Caesarean section - pregnancy at term
9800	L398300	Delivery by elective caesarean section
618	L398400	Delivery by emergency caesarean section
28861	L398500	Delivery by caesarean hysterectomy
50847	L398600	Caesarean delivery following previous Caesarean delivery
7670	L398z00	Caesarean delivery NOS
7916	Z254500	Delivered by caesarean section - pregnancy at term
47863	Lyu5200	[X]Other single delivery by caesarean section
		MISCARRIAGE
3082	L04..00	Spontaneous abortion
42458	L040.00	Spontaneous abortion unspecified
1737	L02..00	Missed abortion
		STILLBIRTH
47338	633..12	Stillbirth [prevention record]
31203	6332	Single stillbirth
8776	Q48D.00	[X] Stillbirth
20573	Q48D000	[X]Fresh stillbirth
8295	Q48D100	[X]Macerated stillbirth
974	Q4z..15	Stillbirth NEC
8906	ZV27.12	[V]Stillbirth
18369	ZV27100	[V]Single stillbirth
1413	L264.00	Intrauterine death
8147	L264.11	Fetal death in utero
59452	L264000	Intrauterine death unspecified
44345	L264100	Intrauterine death - delivered
69254	L264200	Intrauterine death with antenatal problem
37831	L264z00	Intrauterine death NOS
20320	Q210.00	Fetal death due to prelabour anoxia
52308	Q211.00	Fetal death due to labour anoxia
		IUGR
48784	62O4.00	Fetal maturity: dates not=size
19575	Q10z.00	Fetal growth retardation NOS
4697	Q10z.11	Intrauterine growth retardation
15563	Q11..00	Short gestation and unspecified low birthweight problems
50704	Q113.00	Light for gestational age
43004	Q13..00	Light for gestational age
102246	L265200	Small-for-dates with antenatal problem

36640	L265300	Maternal care for poor fetal growth
9542	L265311	Maternal care for intrauterine growth retardation
34777	Q10..00	Slow fetal growth and fetal malnutrition
56744	Q10..11	Fetal malnutrition
22032	Q100.00	Fetus small-for-dates, without mention of malnutrition
36894	Q100.11	Fetus small-for-dates (SFD), without mention of malnutrition
63290	Q101.00	Fetus small-for-dates with signs of malnutrition
95638	Q101.11	Fetus small-for-dates (SFD) with signs of malnutrition
23495	L265.00	Small-for-dates fetus in pregnancy
		APH
63648	Q021000	Fetus/neonate affected by antepartum haemorrhage unspecified
42661	Q021011	Fetus affected by APH - antepartum haemorrhage
66335	L260.00	Fetal-maternal haemorrhage
104829	L260000	Fetal-maternal haemorrhage unspecified
69788	L260200	Fetal-maternal haemorrhage with antenatal problem
102894	L260z00	Fetal-maternal haemorrhage NOS
		PIH/PET
11248	R110.00	[D]Proteinuria
36394	L16C.00	Pregnancy induced oedema+proteinuria without hypertension
34265	L16C000	Gestational proteinuria
37201	L16C100	Gestational oedema with proteinuria
9170	L12..00	Hypertension complicating pregnancy/childbirth/puerperium
12604	L120.00	Benign essential hypertension in pregnancy/childbirth/puerp
37344	L120000	Benign essential hypertension in preg/childb/puerp unspec
44912	L120100	Benign essential hypertension in preg/childb/puerp - deliv
97781	L120200	Benign ess hypert in preg/childb/puerp - deliv with p/n comp
61408	L120300	Benign essential hypertension in preg/childb/puerp-not deliv
73633	L120400	Benign essential hypertension in preg/childb/puerp +p/n comp
34136	L120z00	Benign essential hypertension in preg/childb/puerp NOS
20439	L123.00	Transient hypertension of pregnancy
55338	L123000	Transient hypertension of pregnancy unspecified
54942	L123100	Transient hypertension of pregnancy - delivered
67035	L123200	Transient hypertension of pregnancy - deliv with p/n comp
71730	L123300	Transient hypertension of pregnancy - not delivered
70342	L123400	Transient hypertension of pregnancy + postnatal complication
38882	L123600	Transient hypertension of pregnancy
35646	L123z00	Transient hypertension of pregnancy NOS
9429	L124.00	Mild or unspecified pre-eclampsia
14867	L124.11	Mild pre-eclampsia
57233	L124000	Mild or unspecified pre-eclampsia unspecified
49893	L124100	Mild or unspecified pre-eclampsia - delivered
66860	L124200	Mild or unspecified pre-eclampsia - delivered with p/n comp
27800	L124300	Mild or unspecified pre-eclampsia - not delivered
48347	L124400	Mild or unspecified pre-eclampsia with p/n complication
16879	L124500	Mild pre-eclampsia

8744	L124600	Pre-eclampsia, unspecified
59124	L124z00	Mild or unspecified pre-eclampsia NOS
9067	L125.00	Severe pre-eclampsia
40686	L125000	Severe pre-eclampsia unspecified
42088	L125100	Severe pre-eclampsia - delivered
62919	L125200	Severe pre-eclampsia - delivered with postnatal complication
67447	L125300	Severe pre-eclampsia - not delivered
62916	L125400	Severe pre-eclampsia with postnatal complication
40730	L125z00	Severe pre-eclampsia NOS
12090	L126.00	Eclampsia
55986	L126000	Eclampsia unspecified
16421	L126100	Eclampsia - delivered
70487	L126200	Eclampsia - delivered with postnatal complication
3364	L126300	Eclampsia - not delivered
70891	L126400	Eclampsia with postnatal complication
39117	L126500	Eclampsia in pregnancy
44306	L126600	Eclampsia in labour
67287	L126z00	Eclampsia NOS
43664	L127.00	Pre-eclampsia or eclampsia with pre-existing hypertension
47741	L127000	Pre-eclampsia or eclampsia with hypertension unspecified
42947	L127100	Pre-eclampsia or eclampsia with hypertension - delivered
90577	L127200	Pre-eclampsia or eclampsia with hypertension - del+p/n comp
53160	L127300	Pre-eclampsia or eclampsia with hypertension - not delivered
105933	L127400	Pre-eclampsia or eclampsia with hypertension + p/n comp
93055	L127z00	Pre-eclampsia or eclampsia + pre-existing hypertension NOS
17805	L129.00	Moderate pre-eclampsia
33715	L12A.00	HELLP - Syndrome haemolysis, elev liver enzyme low platelets
34173	L12B.00	Proteinuric hypertension of pregnancy
40723	L12z.00	Unspecified hypertension in pregnancy/childbirth/puerperium
41949	L12z000	Unspecified hypertension in preg/childb/puerp unspecified
44068	L12z100	Unspecified hypertension in preg/childb/puerp - delivered
89561	L12z200	Unspecified hypertension in preg/childb/puerp -del +p/n comp
3421	L12z300	Unspecified hypertension in preg/childb/puerp - not deliv
35698	L12zz00	Unspecified hypertension in preg/childb/puerp NOS
		METERNAL CV OUTCOMES
69686	L417000	Cerebral venous thrombosis in pregnancy
55974	L417100	Cerebral venous thrombosis in the puerperium
105500	L41yz00	Other venous complication of pregnancy and puerperium NOS
73311	L41z.00	Venous complications of pregnancy and puerperium NOS
66649	L41z500	Venous complication of pregnancy, unspecified
44404	L43z400	Obstetric pulmonary embolism NOS with postnatal complication
47607	L440.11	CVA - cerebrovascular accident in the puerperium
56279	L440.12	Stroke in the puerperium
23667	L413.00	Antenatal deep vein thrombosis
26873	L413.11	DVT - deep venous thrombosis, antenatal
65725	L413000	Antenatal deep vein thrombosis unspecified

94405	L413100	Antenatal deep vein thrombosis - delivered
100502	L413200	Antenatal deep vein thrombosis with antenatal complication
61203	L413z00	Antenatal deep vein thrombosis NOS
4607	L414.00	Postnatal deep vein thrombosis
18830	L414.11	DVT - deep venous thrombosis, postnatal
69921	L414000	Postnatal deep vein thrombosis unspecified
23588	L414200	Postnatal deep vein thrombosis with postnatal complication
61204	L414z00	Postnatal deep vein thrombosis NOS
		MATERNAL RENAL DISEASE
X	L393.00	Acute renal failure following labour and delivery
72458	L393000	Post-delivery acute renal failure unspecified
96179	L393100	Post-delivery acute renal failure - delivered with p/n prob
69433	L393200	Post-delivery acute renal failure with postnatal problem
47080	L162.00	Unspecified renal disease in pregnancy
38312	L162.12	Nephropathy NOS in pregnancy without hypertension
		PREMATURITY
164	635..13	Premature baby
29074	6351	Baby premature 36-38 weeks
27662	6352	Baby v. premature 32-36 weeks
29996	6356	Baby premature 26-28 weeks
45702	6357	Baby premature 24-26 weeks
45693	6358	Baby premature 39 weeks
45688	6359	Baby premature 38 weeks
19598	635A.00	Baby premature 37 weeks
17968	635B.00	Baby premature 36 weeks
20809	L14..00	Early or threatened labour
3004	L14..11	Premature labour
28928	L140.00	Threatened premature labour
15167	L142.00	Early onset of delivery
3365	L142.11	Premature delivery
104873	L143.00	Premature labour and delivery
105032	L143000	Premature labour without delivery
104872	L143100	Premature labour with premature delivery
20429	Q11..11	Baby born premature
26683	Q110.00	Very premature - less than 1000g or less than 28 weeks
23670	Q111.00	Premature - weight 1000g-2499g or gestation of 28-37weeks
35209	Q116.00	Premature infant 28-37 weeks
1211	Q11z.00	Born premature NOS
		PLACENTAL PROBLEMS
39175	63D4.00	Placental infarct
96843	L267600	Placental infarction
69814	Q022000	Fetus or neonate affected by placental dysfunction
46724	Q022200	Fetus or neonate affected by placental insufficiency
88892	Z262300	Small placenta
100516	Z262H00	Placenta calcified
96739	Z262I00	Placenta infarcted

38782	L265.11	Placental insufficiency
		ABRUPTION
14762	L11..00	Antepartum haemorrhage, abruptio placentae, placenta praevia
8067	L112.00	Placental abruption
65397	L112000	Placental abruption unspecified
23583	L112100	Placental abruption - delivered
41588	L112200	Placental abruption - not delivered
102737	L112300	Premature separation of placenta with coagulation defect
42213	Q021100	Fetus or neonate affected by abruptio placentae
52633	Q021111	Fetus affected by placental abruption
55761	Q021500	Fetus or neonate affected by premature placental separation
68169	Q021.00	Fetus/neonate affect other placental separation/haemorrhage
33940	Z262C00	Retroplacental clot
60604	Z262D00	Fresh retroplacental clot
46822	Z262E00	Old retroplacental clot
64626	Z262E11	Stale retroplacental clot
60398	L112z00	Placental abruption NOS
		NEONTAL LUPUS
99435	N000500	Neonatal lupus erythematosus

		Immunology Codes
4502	43F1.00	Rheumatoid factor positive
27043	43G1000	Anti-nuclear factor positive
14064	43G1011	Anti-nuclear antibody positive
27045	43G1200	Anti-nuclear factor weakly positive
27044	43G1211	Anti-nuclear antibody weakly positive
14467	43G8.00	Serum IgM anticardiolipins level
3251	43G9.00	Serum IgG anticardiolipins level
14471	43GD.00	Lupus circulating anticoagulant index
14478	43GE.00	Lupus anticoagulant screen
4547	43GT.00	Serum anti-cardiolipin level
19870	43Gv.00	dsDNA binding autoantibody level
27235	43Gw.00	ssDNA binding autoantibody level
14500	43mB.00	Anti-cardiolipin antibody level
19871	43mE.00	Anti-nuclear antibody level
27232	43mN.00	Anti-nuclear IgM antibody level
14486	43mO.00	Anti-nuclear IgG antibody level
34606	43N0100	Ro antibody positive
34607	43N1100	La antibody positive
57975	43N2100	Sm antibody positive
		Autoimmune disease codes
44095	F371000	Polyneuropathy in disseminated lupus erythematosus
62401	F371200	Polyneuropathy in rheumatoid arthritis
31209	F396400	Myopathy due to rheumatoid arthritis
55601	F396600	Myopathy due to scleroderma
21022	F396700	Myopathy due to Sjogren's disease

94996	H572.00	Lung disease with systemic sclerosis
42940	H57y100	Lung disease with polymyositis
47364	H57y300	Lung disease with Sjogren's disease
31564	H57y400	Lung disease with systemic lupus erythematosus
47672	K01x400	Nephrotic syndrome in systemic lupus erythematosus
22205	K01x411	Lupus nephritis
4125	M154.00	Lupus erythematosus
33449	M154000	Lupus erythematosus chronicus
2667	M154100	Discoid lupus erythematosus
40797	M154200	Lupus erythematosus migrans
65391	M154300	Lupus erythematosus nodularis
46148	M154400	Lupus erythematosus profundus
44984	M154500	Lupus erythematosus tumidus
63955	M154600	Lupus erythematosus unguium mutilans
25390	M154700	Subacute cutaneous lupus erythematosus
7522	M154z00	Lupus erythematosus NOS
100692	Myu7800	[X]Other local lupus erythematosus
4909	N....11	Connective tissue diseases
7871	N000.00	Systemic lupus erythematosus
20007	N000000	Disseminated lupus erythematosus
57675	N000100	Libman-Sacks disease
29519	N000300	Systemic lupus erythematosus with organ or sys involv
11920	N000400	Systemic lupus erythematosus with pericarditis
101433	N000600	Cerebral lupus
42719	N000z00	Systemic lupus erythematosus NOS
3670	N001.00	Scleroderma
28417	N001.12	Systemic sclerosis
44141	N001000	Progressive systemic sclerosis
17675	N001100	CREST syndrome
2360	N002.00	Sicca (Sjogren's) syndrome
4908	N003.00	Dermatomyositis
28316	N003X00	Dermatopolymyositis, unspecified
15511	N004.00	Polymyositis
12177	N006.00	Antiphospholipid syndrome
20271	N00y.00	Other specified diffuse collagen diseases
27603	N04..00	Rheumatoid arthritis and other inflammatory polyarthropathy
20615	N04..11	Inflammatory polyarthropathy
844	N040.00	Rheumatoid arthritis
7454	N065.11	Polyarthropathy NEC
52860	Nyu4.00	[X]Systemic connective tissue disorders
58706	Nyu4300	[X]Other forms of systemic lupus erythematosus
71763	Nyu4500	[X]Other forms of systemic sclerosis
68965	Nyu4700	[X]Other systemic diseases of connective tissue
92421	Nyu4C00	[X]Systemic disorders/connective tissue in other diseases CE
95437	Nyu4E00	[X]Dermatopolymyositis, unspecified

Appendix 6 – Clinical Practice Research Datalink (CPRD)

Research Protocol

ISAC APPLICATION FORM
PROTOCOLS FOR RESEARCH USING THE CLINICAL PRACTICE RESEARCH DATALINK (CPRD)

ISAC use only: Protocol Number Date submitted		**IMPORTANT** **If you have any queries, please contact ISAC Secretariat:** ISAC@cprd.com

1. Study Title
   Undiagnosed Lupus-Like Autoimmunity and unexplained stillbirth

2. Principal Investigator (full name, job title, organisation & e-mail address for correspondence regarding this protocol)
   Dr Clare Tower, Honorary Clinical Senior Lecturer / Consultant in Obstetrics and Feto-Maternal Medicine, University of Manchester. Email: Clare.Tower@manchester.ac.uk

3. Affiliation (full address)
   Maternal and Fetal Health Research Centre, St Mary's Hospitals, Central Manchester NHS Foundation Trust, Oxford Road,Manchester, M19 3WL

4. Protocol's Author (if different from the principal investigator)
   Dr Hannah Kither, Clinical Research Fellow in Maternal and Fetal Health, University of Manchester. Email: Hannah.Kither@manchester.ac.uk

5. List of all investigators/collaborators (*please list the names, affiliations and e-mail addresses* of all collaborators*, other than the principal investigator)
   Prof. Darren Ashcroft, Professor of Pharmacoepidemiology, School of Pharmacy & Pharmaceutical Sciences, University of Manchester. Email: darren.ashcroft@manchester.ac.uk

   Dr Ian Crocker, Senior Lecturer in Maternal and Fetal Health, University of Manchester Email: Ian.Crocker@manchester.ac.uk

   Professor Ian Bruce, Professor of Rheumatology and Honorary Consultant in Rheumatology. University of Manchester. Email: Ian.Bruce@manchester.ac.uk

   Dr Hannah Kither, Clinical Research Fellow in Maternal and Fetal Health, University of Manchester. Email: Hannah.Kither@manchester.ac.uk

   Dr Rosa Parisi, Research Associate, Manchester Pharmacy School, University of Manchester. Email: Rosa.Parisi@manchester.ac.uk

   Dr Mark Lunt, Senior Statistician, University of Manchester. Email: Mark.Lunt@manchester.ac.uk

   Please note that your ISAC application form and protocol **must be copied to all e-mail addresses listed above at the time of submission of your application to the ISAC mailbox. Failure to do so will result in delays in the processing of your application.*

6. Type of Institution (please tick one box below)
   Academia ✓ Research Service Provider Pharmaceutical Industry
   NHS Government Departments Others

7. Financial Sponsor of study
Pharmaceutical Industry *(please specify)* ☐ Academia*(please specify)* ☐

Government / NHS *(please specify)* ☐ None ☐

Other *(please specify)* ✓ **Charity - SPARKS**

8. Data source *(please tick one box below)*
Sponsor has on-line access ✓ Purchase of ad hoc dataset

Commissioned study

Other *(please specify)*

Co-applicant Prof. Ashcroft has an institution-wide agreement with the CPRD, which enables him to access the data directly online via the University of Manchester School of Pharmacy & Pharmaceutical Sciences

9. Has this protocol been peer reviewed by another Committee?
Yes* ✓ No

** In our protocol we have stated the name of the reviewing Committee and have provided an outline of the review process and outcome.*

10. Type of Study *(please tick all the relevant boxes which apply)*
Adverse Drug Reaction/Drug Safety | Drug Use | **Disease Epidemiology** ✓

Drug Effectiveness | Pharmacoeconomic | Other

11. This study is intended for:
Publication in peer reviewed journals ✓ Presentation at scientific conference ✓

Presentation at company/institutional meetings ✓ Other

12. Does this protocol also seek access to data held under the CPRD Data Linkage Scheme?
Yes ✓ No ☐

13. If you are seeking access to data held under the CPRD Data Linkage Scheme*, please select the source(s) of linked data being requested.
☐ Hospital Episode Statistics ☐ Cancer Registry Data**

☐ MINAP ☐ ONS Mortality Data

✓ **Index of Multiple Deprivation/ Townsend Score**

☐ Mother Baby Link ☐ Other: (please specify)

** As part of the ISAC review of linkages, the protocol may be shared - in confidence - with a representative of the requested linked data set(s) and summary details may be shared - in confidence - with the Confidentiality Advisory Group of the Health Research Authority.*

***Please note that applicants seeking access to cancer registry data must provide consent for publication of their study title and study institution on the UK Cancer Registry website. Please contact the CPRD Research Team on +44 (20) 3080 6383 or email kc@cprd.com to discuss this requirement further.*

14. If you are seeking access to data held under the CPRD Data Linkage Scheme, have you already discussed your request with a member of the Research team?
Yes ✓ No ☐

**Please contact the CPRD Research Team on +44 (20) 3080 6383 or email kc@cprd.com to discuss your requirements before submitting your application.*

Please list below the name of the person/s at the CPRD with whom you have discussed your request.

Kendal Chidwick – we are aware of limited availability of IMD data (70% of English practices).

15. If you are seeking access to data held under the CPRD Data Linkage Scheme, please provide the following information:

A synopsis of the purpose(s) for which the linkages are required:

Is linkage to a local dataset with <1 million patients being requested?

Yes* ☐ **No** ✓

** If yes, please provide further details:*

16. If you have requested linked data sets, please indicate whether the Principal Investigator or any of the collaborators listed in response to question 5 above, have access to any of the linked datasets in a patient identifiable form, or associated with a patient index.
    Yes* ☐ **No** ✓

** If yes, please provide further details:*

17. Does this protocol involve requesting any additional information from GPs?
    Yes* **No** ✓

    ** Please indicate what will be required:*

Completion of questionnaires by the GP[Ψ] Yes ☐ No ☐

Provision of anonymised records (e.g. hospital discharge summaries) Yes ☐ No ☐

Other (please describe)

[Ψ] Any questionnaire for completion by GPs or other health care professional must be approved by ISAC before circulation for completion.

18. Does this protocol describe a purely observational study using CPRD data (this may include the review of anonymised free text)?
    Yes* ✓ No** ☐

** Yes: If you will be using data obtained from the CPRD Group, this study does not require separate ethics approval from an NHS Research Ethics Committee.*

*** No: You may need to seek separate ethics approval from an NHS Research Ethics Committee for this study. The ISAC will provide advice on whether this may be needed.*

19. Does this study involve linking to patient *identifiable* data from other sources?
    Yes ☐ **No** ✓

20. Does this study require contact with patients in order for them to complete a questionnaire?
    Yes ☐ **No** ✓

N.B. Any questionnaire for completion by patients must be approved by ISAC before circulation for completion.

21. Does this study require contact with patients in order to collect a sample?
    Yes* ☐ **No** ✓

** Please state what will be collected*

22. Experience/expertise available

Please complete the following questions to indicate the experience/expertise available within the team of researchers actively involved in the proposed research, including analysis of data and interpretation of results

Previous GPRD/CPRD Studies	Publications using GPRD/CPRD data
None	

1-3		
> 3	✓	✓

	Yes	No
Is statistical expertise available within the research team?	✓	
If yes, please outline level of experience	Advanced: applicant DA, RP, ML	
Is experience of handling large data sets (>1 million records) available within the research team?	✓	
If yes, please outline level of experience	Advanced: applicant DA, RP, ML	
Is UK primary care experience available within the research team?	✓	☐
If yes, please outline level of experience	*will consult with local GPs*	

23. References relating to your study
Please list up to 3 references (most relevant) relating to your proposed study.

Ulff-Moller CJ, et al. Reproductive factors and risk of systemic lupus erythematosus: nationwide cohort study in Denmark. Journal of Rheumatology. 36(9), 1903-9.(2009).

Dhar JP, Essenmacher LM, Ager JW, Sokol RJ. Pregnancy outcomes before and after a diagnosis of systemic lupus erythematosus. *Am. J. Obstet. Gynecol.* 2005;193(4):1444–55.

Jørgensen KT, Nielsen NM, Pedersen BV, Jacobsen S, Frisch M. Hyperemesis, gestational hypertensive disorders, pregnancy losses and risk of autoimmune diseases in a Danish population-based cohort. *J Autoimmun.* 2012;38(2-3):J120–8

PROTOCOL CONTENT CHECKLIST

In order to help ensure that protocols submitted for review contain adequate information for protocol evaluation, ISAC have produced instructions on the content of protocols for research using CPRD data. These instructions are available on the CPRD website (www.cprd.com/ISAC). All protocols using CPRD data which are submitted for review by ISAC must contain information on the areas detailed in the instructions. IF you do not feel that a specific area required by ISAC is relevant for your protocol, you will need to justify this decision to ISAC.

Applicants must complete the checklist below to confirm that the protocol being submitted includes all the areas required by ISAC, or to provide justification where a required area is not considered to be relevant for a specific protocol. Protocols will not be circulated to ISAC for review until the checklist has been completed by the applicant.

Please note, your protocol will be returned to you if you do not complete this checklist, or if you answer 'no' and fail to include justification for the omission of any required area.

	Included in protocol?		
Required area	Yes	No	If no, reason for omission
Lay Summary (max.200 words)	✓	☐	
Background	✓	☐	
Objective, specific aims and rationale	✓	☐	

Study Type *Descriptive* *Hypothesis Generating* *Hypothesis Testing*	✓ ✓ ☐ ✓	☐ ☐ ✓ ☐	
Study Design	✓	☐	
Sample size/power calculation *(Please provide justification of sample size in the protocol)*	✓	☐	
Study population *(including estimate of expected number of relevant patients in the CPRD)*	✓	☐	
Selection of comparison group(s) or controls	✓	☐	
Exposures, outcomes and covariates *Exposures are clearly described* *Outcomes are clearly described*	✓ ✓ ✓	☐ ☐ ☐	
Use of linked data *(if applicable)*	✓	☐	
Data/ Statistical Analysis Plan *There is plan for addressing confounding* *There is a plan for addressing missing data*	✓ ✓ ✓	☐ ☐ ☐	
Patient/ user group involvement †	✓	☐	
Limitations of the study design, data sources and analytic methods	✓	☐	
Plans for disseminating and communicating study results	✓	☐	

† ***It is expected that many studies will benefit from the involvement of patient or user groups in their planning and refinement, and/or in the interpretation of the results and plans for further work. This is particularly, but not exclusively true of studies with interests in the impact on quality of life. Please indicate whether or not you intend to engage patients in any of the ways mentioned above.***

Voluntary registration of ISAC approved studies:
Epidemiological studies are increasingly being included in registries of research around the world, including those primarily set up for clinical trials. To increase awareness amongst researchers of ongoing research, ISAC encourages voluntary registration of epidemiological research conducted using MHRA databases. This will not replace information on ISAC approved protocols that may be published in its summary minutes or annual report. It is for the applicant to determine the most appropriate registry for their study. Please inform the ISAC secretariat that you have registered a protocol and provide the location.

[PLEASE INSERT THE STUDY PROTOCOL DOCUMENT HERE]

1. Lay Summary:

Pregnant women with the auto-immune disease Systemic Lupus Erythematosus (SLE) are at increased risk of miscarriage or stillbirth. It is possible that for some women these pregnancy losses occur before their SLE has been diagnosed. This research will investigate if this is the case. If this is known, it might help us better understand the causes of these pregnancy problems, and also allow us to improve women's health by diagnosing autoimmune disease early, before it has chance to cause serious ill health. This study will look to examine whether there is a link between complications of pregnancy and the later development of SLE or related disorders. The research will be conducted entirely using the Clinical Practice Research Datalink (CPRD). The applicant team will meet regularly to consider the emerging findings and their relevance. The co-applicant team covers clinical and research knowledge in obstetrics, autoimmune disease, epidemiology and biostatistics, as well as expertise in conducting research with the CPRD and other large clinical datasets.

2. Background:

In the UK, approximately 1 in 200 pregnancies end in stillbirth defined as a fetal death after 24 weeks gestation and 4 times this number end in late miscarriage, defined as pregnancy loss after 12 weeks gestation. At least 50% of these losses remain classified as "unexplained", meaning that no cause has been found. In these situations the inability to define a specific cause can be distressing for both families and clinicians involved.

A number of contributing factors for death *in utero* are described in the literature, including aberrant placental function, fetal growth restriction, knots in the umbilical cord, uterine rupture and infection. However, it is likely that many causes are as yet undiscovered and that some of these fetal deaths could be avoided.

In defining a potential cause of *in utero* fetal death, either through miscarriage or stillbirth, a relevant starting point should be the risk factors associated with these devastating outcomes. The highest risk for pregnancy loss is in subjects with pre-existing co-morbidities including diabetes, hypertension and thrombophilia. Of these medical disorders systemic lupus erythematosus (SLE), a multisystem autoimmune disorder is associated with the highest specific risk. Within this patient group an estimated 20% of pregnancies will end in pregnancy loss (miscarriage/stillbirth), 21% will succumb to preeclampsia or fetal growth restriction, 3% will end in neonatal death and 39% will deliver prematurely[1].

Therefore, in the study of fetal loss and its underlying causes, SLE and related autoimmune disorders may provide the most relevant patient group in which to investigate potential novel pathways in the aetiology and potential prediction of early/late fetal/neonatal mortality. The pathogenesis of SLE is a multifactorial interaction of genetic and environmental factors. Of relevance from an obstetric viewpoint is that 90% of those with SLE are women and of these 80% first develop SLE during their childbearing years[2].

The central immuno-pathological feature of patients with SLE is the production of autoantibodies, i.e. antibodies directed against an individual's own cells or tissues. While often subject to significant delays in its diagnosis, SLE can be confirmed by the recognition of anti-nuclear antibodies (ANA test) and/or antibodies to extractable nuclear antigen (anti-ENA) including anti-Smith (Sm) anti-double stranded DNA (anti-dsDNA), anti-U1 RNP, anti-Ro (SS-A) and anti-La (SS-B) antibodies as well as hypo-complementaemia. SLE is also associated, but not defined by, antiphospholipid antibodies (APS) which are already implicated in adverse pregnancy outcomes. It is also well recognised that many women in the community have incomplete forms of SLE which do not fulfil classic or newer criteria (which is the presence of 4 or more of the 11 criteria listed in appendix 5)[3,4] and are often described as having undifferentiated connective tissue disease (UCTD), lupus-like disease

(LLD). Such patients have some clinical and serological features to suggest SLE, but do not completely satisfy usual diagnostic criteria sets[5].

It is the experience of us and other groups that many patients with adverse pregnancy outcomes associated with autoimmunity fall into this UCTD/LLD group and it is known that UCTD patients also display endothelial dysfunction similar to that seen in SLE[6]. Whether immunological changes

associated with lupus-like autoimmunity may also be associated with *in utero* fetal loss has not, to our knowledge, been formally investigated. Therefore, this proposal forms the basis of this project. It is recognised that active SLE is associated with a poor pregnancy outcome, contributing to a 4-6-fold increase in perinatal mortality and a 2-3-fold decrease in full-term birth[7].

In addition, SLE flares prior to conception and in the prenatal period are also implicated in fetal loss[7]. Serological changes associated with active disease include elevated anti-dsDNA titres and hypocomplementamia and histologically by immune complex deposition in relevant target organs e.g. kidney and skin. It is unclear whether this can also occur at a subclinical level. Interestingly,a study of a nationwide cohort of 1.4 million Danish women between 1955 and 1989, noted a relationship between idiopathic 'unexplained' pregnancy loss and the subsequent development of SLE, most notably in the first 5 years after pregnancy[8]. Taken together the adjusted risk to women of developing clinically accepted SLE after one or more pregnancy losses was >14%, 7 times higher than those of uncompromised outcomes. Therefore early 'pre-clinical' SLE may indeed be a risk factor for women with a history of miscarriage or stillbirth. As such, it seems likely that fetal outcomes may be indicative of a predisease state and that subclinical immunological and physiological changes in women destined to develop SLE, may provide significant clues to the development of hitherto unexplained pregnancy loss. The hypothesis is that women who have had an adverse pregnancy outcome (as defined by delivery before 34 weeks, abruption, pre-eclampsia, or stillbirth) will have an increased rate of development of SLE or LLD compared to their healthy pregnancy counterparts.

3. Rationale, Objective & Specific Aims:

Rationale: Despite advances in healthcare the UK stillbirth rate remains grossly unchanged. Research suggests a role in for undiagnosed SLE, LLD and UCTD disease in the pathogenesis of stillbirth. Our findings aim to examine this theory in the UK population.
Objective: To gain insight into the relationship between stillbirth and adverse pregnancy outcome with subsequent development of SLE, LLD and UCTD disease
Specific Aims: We will tackle several research questions

1. What is the incidence of development of SLE, LLD and UCTD in women who have had a stillbirth
2. Is this incidence greater than the incidence of SLE, LLD and UCTD disease in women who have not had a stillbirth?
3. Does the relative increase in incidence change with time following a stillbirth
4. Is there a relationship between adverse pregnancy outcome (pre-eclampsia, pre-term birth, fetal growth restriction) and subsequent development of SLE, LLD and UCTD disease?

4. Study Type and Design:

This is a retrospective cohort based study with the intention of establishing whether there is a link between adverse pregnancy outcome and subsequent development of autoimmune disease.

We aim to identify women with adverse pregnancy outcomes and examine if they are at an increased risk of developing autoimmune disease (specifically SLE, LLD and UCTD) compared to women with previous healthy pregnancies. Data analysis will be based on using poisson regression on data of women identified as having suffered an adverse outcome (appendix 3 – adverse outcomes READ codes) to measure the incidence rate of SLE, LLD or UCTD. (appendix 4 – READ codes for SLE, LLD and UCTD) and time to events analysis to measure the hazard ratio for women developing SLE, LLD or UCTD following an adverse pregnancy outcome compared to matched controls who had a livebirth without an adverse outcome (appendix 2 – pregnancy codes).

UCTD diagnosis will be based on coding for the disease as outlined in appendix 4 due to many of the features from the American College of Rheumatologists (ACR) criteria unlikely to be coded for by practitioners (appendix 5 – ACR criteria for SLE).

Due to an established prolonged time period between antibody positivity and development of clinical SLE we will also do secondary analysis in women who have positive immunology

(appendix 4 – immunology codes) in accordance with those listed in the ACR criteria for SLE (appendix 5).

These controls will also be analysed to establish the background risk of development of SLE, LLD and UCTD. We expect to observe a higher rate of development of SLE, LLD and UCTD in women who have suffered an adverse outcome. We will look for changes in the hazard ratio over time since delivery to aim to identify any potential interventions to reduce risk in future pregnancies.

Women with an adverse outcome will be matched to 5 control cases from the same GP practice with the same age range (within 5 years).

Entry criteria into the study will be age of ≥18 at time of entry.

Patients will be censored on earliest date of diagnosis of SLE, LLD and UCTD, end of follow-up, death or leaving the practice. Women in the "normal" pregnancy outcome cohort will be removed from that cohort if they also develop an adverse outcome in subsequent pregnancies. Women with SLE, LLD and UCTD prior to an adverse pregnancy event will be excluded.

Cofounders such as hypertension, smoking status and BMI will be recorded.

5. Sample Size / Power Calculation:

Our cohort will consist of an estimated 24,000 stillbirths and N>135,000 composite adverse pregnancy outcomes (antepartum haemorrhage, abruption, pre-eclampsia, fetal growth restriction and stillbirth) and 120,000 controls (5 per stillbirth), based on a livebirth rate of 400,000 for the study timeframe (based on feasibility counts in CPRD Gold for dates 2000-2010 taken May 2014).
Assuming a background risk of developing SLE of 0.05% this would give the study a statistical power of 80% to detect a doubling of this rate.

6. Expected Study Population Estimate:

Our open cohort study will consist of all patients registered at a UK CPRD practice with a first recorded episode of livebirth or adverse pregnancy outcome during 1st January 2000 to 31st December 2010, with follow up to 31st December 2013 for women with an adverse outcome in 2010 (three year follow up minimum).
This cohort is estimated to consist of N>56,000 patients who meet our inclusion criteria. In Appendix 2, 3 and 4 we have provided detail regarding the derivation of this coding list for identification of livebirth, adverse pregnancy outcome and SLE, LLD and UCTD identification respectively.

7. Selection of Comparison Group:

This study will be conducted within a CPRD-based cohort of people who have had an adverse pregnancy outcome. We will also identify pregnancies without adverse outcome to act as a control group and identify their rate of development of SLE, LLD and UCTD disease.

8. Exposures, Outcomes and Covariates:

Primary outcomes: Development of antibodies consistent with SLE, LLD and UCTD (appendix 4) or a formal diagnosis of SLE or lupus like disease (appendix 4).

Main exposures: Adverse pregnancy outcome as defined by late miscarriage, stillbirth, prematurity, growth restriction, abruption or hypertensive disease of pregnancy and subsequent development of SLE or lupus like disease.

Covariates: Assess effect modification and confounding by age, hypertension, smoking and BMI.

9. Use of Linked Data:

IMD data will be used as part of sensitivity analysis for a nested cohort of women

10. Statistical Analysis Plan:

The first stage of the analysis will be to describe the distributions of the potential confounders within the adverse outcome and control groups, and test for any differences between the groups. The mean incidence rate in each group will be determined using Poisson regression (or a generalization of this if necessary, e.g. if there is overdispersion). The follow up period will be divided into 6 month intervals, and a separate incidence rate calculated for each interval, to see if rate is changing over time. Finally, Cox regression will be used to determine the hazard ratio for the adverse outcome group compared to the controls. This analysis will control for all of the potential confounders, as measured at baseline. Again, a separate hazard ratio will be calculated for each 6 month interval, to examine changes in hazard ratio over time.

All analyses will be conducted using Stata v13 software (StataCorp, 2013).

11. Limitations the Data Sources, Study Design and Analytical Methods:

Data sources - missing data: Due to the nature of data being collected it may be difficult to identify women with missing data as these will appear as women who do not develop autoimmune mediated disease: ie misclassification rather than as missing data. . We will use sensitivity analyses to determine the impact on our findings of plausible levels of misclassification.
Due to the varied nature of record of patient-level IMD in the CPRD only approximately 70% coverage will be expected: analysis involving IMD will be restricted to this subgroup, but the groups of subjects with complete and incomplete data will be compared on all other measured variables to ensure that the subgroup with complete data is representative. Similarly data for BMI and smoking may be missing and as such may only allow subgroup analysis.
Study design & analytical methods: We will take a robust approach in dealing with any areas of uncertainty in coding of the explanatory or outcome variables, by conducting exhaustive sensitivity analyses.

12. Prior Peer Review and Funders' Panel Assessment:

During 2012 my colleagues submitted a bid for this research proposal to SPARKS. In 2013 we were notified that a research grant of £148,257 is to be awarded to fund the study, at 100% Full Economic Cost over the 24 month period from January 2014.

13. Patient and Public Involvement (PPI):

To ensure that our PPI strategy is not one directional we will engage in two-way communication with relevant interest groups (as outlined below) from the inception of the project through to dissemination of the findings. Beyond this study, we will seek PPI input to identify, focus and prioritise further novel research questions on this topic.

14. Plans for Disseminating and Communicating the Study Findings:

The applicants and institution are fully committed to patient/public involvement. The University of Manchester was the first to become a HEFCE Beacon for Public engagement and has an active and productive media office. All major findings will be reported/disseminated through this facility.

Outcomes (positive or negative) will be presented at international conferences in related clinical fields, rheumatology and obstetrics. Confirmed results will be published in the most appropriate, widely read and cited journals.

The Trust and University Websites will be used to disseminate major findings, along with newsletters and media from interested parties, support groups and charities both regionally and nationally, building on the applicant's current roles and connections.

In terms of direct public engagement, our pilot studies of lupus in pregnancy have already featured in Lancashire and Cheshire Lupus UK patient newsletter and at the annual patients' feedback meeting at the Kellgren Centre for Rheumatology and the Musculoskeletal Research User Group. The Maternal and Fetal Health Research Centre User Group includes service users from the postnatal group at the 'Baby Café', doulas (birth companions), members of the National Childbirth Trust and midwives. Elements of this project, specifically its preliminary findings on lupus patients, have been presented and discussed at this forum and positive feedback and useful comments were received and incorporated. These groups will be fully updated throughout these studies and their suggestions enacted where necessary.

The applicants have authored a number of clinical guidelines on lupus and stillbirth, including recently developed regional guidelines for the management of Lupus in Pregnancy to coincide with the newly established LiPS (Lupus in Pregnancy Scanning) clinic. Further examples include; the EULAR NPSLE guidelines for the management of SLE with neuropsychiatric manifestations, cardiovascular risk guidelines in rheumatology, RCOG green-top guidelines for the management of reduced fetal movements, and contributions to the RCP Map of Medicine.

Appendix 7 – T cell analysis optimisation

As described previously (section 2.7, page 49) Th17 cell assessments in PBMCs were performed using a commercial kit (R+D Systems, Abingdon, UK), either directly on fresh isolates or after stimulation, following optimisation of the process as outlined below. Unless otherwise stated, all centrifuge cycles were performed for 5 minutes at 5^0c at 250g.

Optimisation of Th17 cell analysis

Isolated PBMCs ($1x10^6$ cells/well) were added to media (RPMI60, 10% heat inactivated FBS, 2mmol Glutamin, 200u/ml Penicillin, 100 µl/ml streptomycin (G1146 Sigma)). This was left overnight (16 hours). Four differing stimulation protocols were trialled using 5µl Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich, Dorset, UK) (50ng/ml), 5µl Ionomycin calcium salt (Sigma-Aldrich, Dorset, UK) (200ng/ml), 5µl interleukin 23 (IL-23) (R+D Systems, Abingdon, UK) and 5µl Lipopolyssaccharide (LPS) (Sigma-Aldrich, Dorset, UK) (1mg/ml). Stop phases included the use of 5µl Monensin Sodium (5mmol), 5µl PMA (50ng/ml) and 5µl Ionomycin calcium salt (200ng/ml). The same patient samples were run through each protocol to determine optimum Th17 yield alongside ease of experimental procedure.

1) Media only
2) Media (16 hours), then PMA, Ionomycin calcium salt, Monensil 4 hours
3) Media with addition of PMA, Ionomycin calcium salt, Il-23, LPS (16 hours), followed by addition of Monensil for 2 hours
4) Media (16 hours) with addition of PMA, Ionomycin calcium salt, Il-23, LPS, then PMA, Ionomycin calcium salt for 2 hours, followed by addition of Monensil for 2 hours

Table 1 - Results of Th17 Optimisation Protocols

Protocol Number	Percentage Th17 cells
1	5.88%
2	5.44%
3	11.34%
4	9.73%

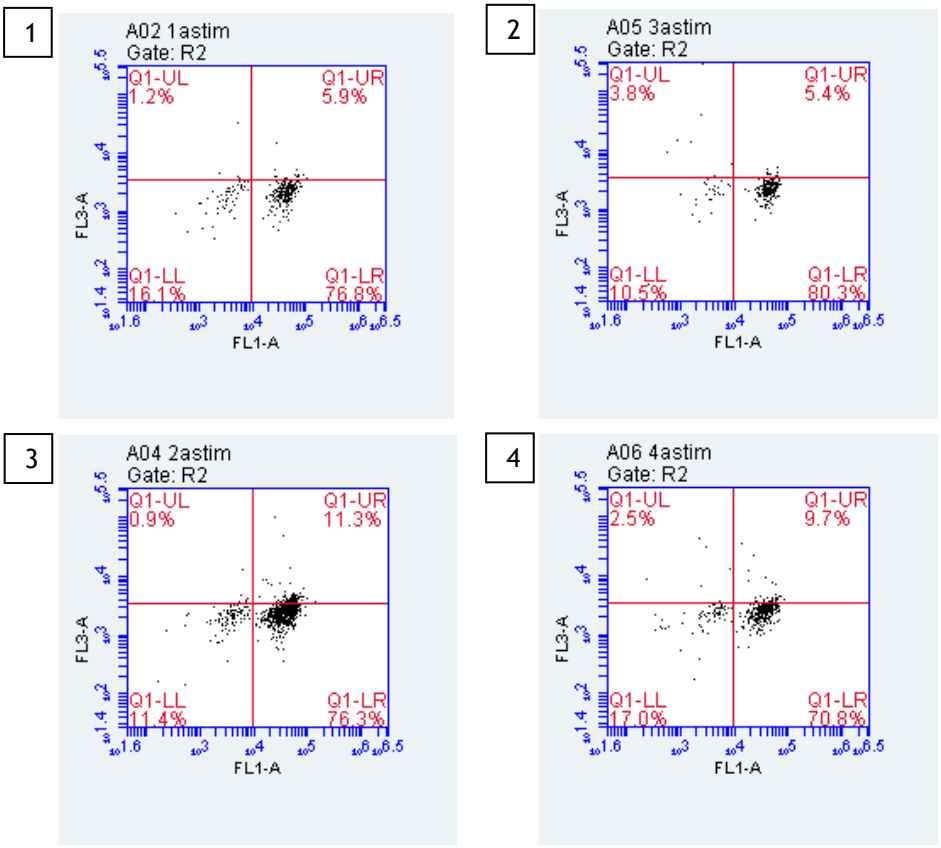

Fig 1 - Flow Cytometry Analysis of Th17 Cell Optimisation

As shown in the table protocol 3 had the greatest Th17 yield and so was selected for use. The full protocol is outlined below alongside staining.

Final Th17 Protocol

Isolated PBMCs ($1x10^6$ cells/well) were placed in a 12 well culture plate (Scientific laboratory Supplies Ltd, Nottingham, UK). 2 wells per patient - control and test. 490µl media (RPMI60, 10% heat inactivated FBS, 2mmol Glutamin, 200u/ml Penicillin, 100 µl/ml streptomycin (G1146 Sigma)) was then added alongside 5µl PMA (50ng/ml) and 5µl Ionomycin calcium salt. Samples were then incubated at 37^0c for 16 hours before addition of 5µl Monensin Sodium (5 mmol). A further 2 hour incubation was undertaken followed by two wash phases with 1ml PBS.

Cells were then stained - 0.5ml Fixation/Permeabilization Buffer was added and cells incubated in the dark for 30 minutes at 2-8^0c before centrifuging. The supernatant was then removed and 100µl Permeabilization/Wash buffer added. 10µl of each antibody, or corresponding isotype control, were added prior to 16 hours of incubation at 2-8^0c (in the dark). An additional 1ml Permeabilization / Wash buffer was then added prior to centrifugation. Following this, the supernatant was removed and cell pellets resuspended in 500µl PBS prior to FACS analysis (section 2.7.4).