

Factor V Leiden and adverse pregnancy outcome

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BMed FRACP

A thesis submitted for the degree of Doctor of Philosophy

School of Medicine and Public Health

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Tracy Dudding

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Abstract

Intrauterine fetal death, fetal growth restriction (FGR) and pre-eclampsia are major causes of fetal and maternal morbidity and mortality; and placental insufficiency is frequently proposed as the most important underlying mechanism. Given the importance of establishing and maintaining an adequate placental circulation, hereditary thrombophilias are postulated as a possible cause of placental insufficiency. Despite initial reports supporting an association between factor V Leiden (fVL) and adverse pregnancy outcomes, a number of other studies yielded conflicting results. A systematic review and meta-analysis of the literature up to January 2003 was undertaken to address the question of whether the common maternal fVL genotype is associated with an increased risk of adverse pregnancy outcomes (pre-eclampsia, fetal growth restriction and fetal loss). Subsequent meta-analyses were also evaluated. To address the shortfalls observed in the large number of small and possibility underpowered case-control studies, a decision was made to undertake a large nested case-control study within the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort. The aim of this study was to evaluate the association between: 1) maternal fVL and intrauterine fetal death, fetal growth restriction and pre-eclampsia; 2) fetal fVL and intrauterine fetal death, fetal growth restriction; 3) maternal prothrombin gene variant (PGV) and intrauterine fetal death, fetal growth restriction and pre-eclampsia and 4) fetal PGV and intrauterine fetal death, fetal growth restriction and pre-eclampsia. Data from other published cohort studies was combined by meta-analysis to increase the power of detecting an association. Overall, the results of the study within the large ALSPAC cohort show no statistically significant association between maternal/fetal fVL or PGV, either alone or in combination with birth weight <10th centile. Furthermore, the FGR meta-analysis which pooled the results of this cohort study and other cohort studies found no evidence of an effect of maternal fVL on FGR. Given the size of the pooled sample, there was 80% power to detect an OR of 1.09, indicating that if an effect of fVL on FGR was missed by this meta-analysis, it would be quite small. The results of this study within the large ALSPAC cohort show no statistically significant association between maternal or fetal fVL or PGV, either

alone or in combination with pre-eclampsia. However, increasing the power by combining this study with other cohort studies by meta-analysis revealed a positive association between maternal fVL and pre-eclampsia with an OR of 1.49 (95% CI 1.13-1.96 $p=0.003$). A narrative review was subsequently published examining the translation from statistical association to change in clinical practice with respect to fVL and adverse pregnancy outcomes. The thesis concludes with a discussion of management in different clinical scenarios relating to fVL and adverse pregnancy outcomes, and the identification of future priority research areas.

Overview

Chapter One

Intrauterine fetal death, fetal growth restriction (FGR) and pre-eclampsia are major causes of fetal and maternal morbidity and mortality. Chapter One reviews the disease burden, underlying mechanisms, etiology, risk factors, optimal definition and diagnosis of each of these adverse pregnancy outcomes. Although each adverse pregnancy outcome has a complex etiology, placental 'insufficiency' is frequently proposed as the most important underlying mechanism.

The chapter includes a description of normal placental development as a benchmark to show the importance of adequate placental size, placentation and placental circulation. Given the importance of establishing and maintaining an adequate placental circulation, hereditary thrombophilias are postulated as a possible cause of placental insufficiency.

Chapter One concludes with a discussion of hereditary thrombophilias, with particular reference to factor V Leiden (fVL) and the conflicting results of a large number of studies that have investigated a possible association between fVL and adverse pregnancy outcomes.

Chapter Two

Despite initial reports supporting an association between factor V Leiden (fVL) and adverse pregnancy outcomes, a number of other studies yielded conflicting results. Publication I is a systematic review and meta-analysis of the literature up to January 2003 addressing the question of whether the common maternal fVL genotype is associated with an increased risk of adverse pregnancy outcomes (pre-eclampsia, fetal growth restriction and fetal loss). Publication II is addendum to Publication I.

The chapter progresses to a critical review of subsequently published meta-analyses (up to January 2007), evaluating possible associations between maternal fVL and

adverse pregnancy outcomes. During this process, a similar association between maternal prothrombin gene variant G20210A (PGV) and adverse pregnancy outcomes became evident. In light of this, it was apparent that including PGV in this review was important.

Chapter Two also reviews the literature with respect to possible associations between: 1) fetal fVL and adverse pregnancy outcomes; and 2) fetal PGV and adverse pregnancy outcomes. It concludes with the study hypotheses to be tested.

Chapter Three

Chapter Three is Publication III which describes the methods, results and conclusions of a nested case-control study within the Avon Longitudinal Study of Parents and Children (ALSPAC). In this study, 6755 mother/infant pairs were genotyped to determine whether maternal or fetal fVL or PGV, either alone or in combination is associated with FGR or pre-eclampsia. The results of this cohort study are also added to previous cohort studies using meta-analysis.

Chapter Four

This chapter is Publication IV, a narrative review examining the translation from statistical association to change in clinical practice with respect to fVL and adverse pregnancy outcomes.

Chapter Five

Chapter Five integrates results relating to fVL and analysis of the concurrent research to discuss possible management in the different clinical scenarios relating to pregnancy outcomes. The thesis concludes with the identification of future priority research areas.

CHAPTER 1

ADVERSE PREGNANCY OUTCOME

1.1 Introduction

Intrauterine fetal death, fetal growth restriction (FGR) and pre-eclampsia are major causes of fetal and maternal morbidity and mortality. Although each of these adverse pregnancy outcomes has a complex aetiology influenced by an interaction between maternal, fetal and placental factors; placental 'insufficiency' is frequently proposed as the most important underlying mechanism.

Chapter One reviews the disease burden, underlying mechanisms, aetiology, risk factors, optimal definition and diagnosis of each of these adverse pregnancy outcomes. The chapter concludes with a discussion of normal placental development and possible mechanisms of placental insufficiency including hereditary thrombophilias.

1.2 Intrauterine fetal death

1.2.1 The burden of late intrauterine fetal death

Stillbirth, defined as late fetal loss (after 20 weeks gestation) or fetal loss with a fetal birthweight greater than 400g, occurs in one in 200 pregnancies, and accounts for 50% of all perinatal deaths. Death is defined as no heartbeat or spontaneous respiration at the time of delivery. In 85% of all stillbirths, death occurs prior to labour (1, 2). However, despite extensive evaluation, the cause of second and third trimester intrauterine fetal death remains unexplained in many cases. Using the ReCoDe (relevant condition at death) classification system, which incorporates customised fetal growth charts, 15% of stillbirth cases are classified as unexplained, and the most common relevant condition at death was fetal growth restriction (43%)(3). The Stockholm classification system which identifies primary and associated diagnoses reports an 18% rate of unexplained stillbirth (4).

Giving birth to a stillborn child is psychologically traumatic. A stillbirth is often unexpected, and the emotional changes associated with this experience are enormous. Perinatal loss of a child has appreciable psychiatric long-term morbidity in 20-30% of women (5, 6).

1.2.2 Mechanisms of late intrauterine fetal death

Bendon (2001) describes three mechanisms of death which manifest in the stillborn fetus. Each mechanism causes 'the interruption of gas exchange between fetus and mother' and ultimately results in fetal death.

They are:

- 1) *Fetal hydrops* (anasarca). Defined as the accumulation of fluid in the entire body of the fetus, fetal hydrops is separated into immune (eg, Rh isoimmunization) and non-immune types. The accumulation of edema results from: 1) alterations in the Starling balance between osmotic pressure and hydrostatic pressure; 2) lymphatic obstruction; or 3) increased capillary permeability. Different pathologies, such as structural heart disease, tachyarrhythmia, anaemia or infection, may lead to hydrops through changes in one or more of these homeostatic mechanisms.
- 2) *Asphyxia*. This is defined as a deficiency of oxygen in the blood accompanied by an increase in carbon dioxide in the blood and tissues. Placental abruption, defined as the separation of part or the entire placenta from the uterus prior to delivery of the infant, is one cause of asphyxia. Placental infarction secondary to fetal thrombotic vasculopathy is also a cause of asphyxia. In the absence of placental abruption and maternal asphyxia, lesions of the umbilical cord may explain autopsy findings consistent with asphyxia.
- 3) *Shock*. Defined as a disruption of the circulation, shock is likely to be either low-volume cardiac shock due to cardiac failure or haemorrhage; or septic shock due to infection. Acidemia due to chronic utero-placental ischemia can also cause peripheral vasodilation, decreased cardiac contractility and shock (7).

1.2.3 Aetiology of late intrauterine fetal death

The different aetiologies of late intrauterine fetal death eventually cause death via one of the mechanisms described in 1.2.2. The following refers to the etiology of stillbirth in developed countries.

1.2.3.1 Umbilical cord accidents

Umbilical cord accidents are estimated to account for up to 15% of stillbirths, but it can be difficult to know if the cause of death is directly caused by the blood flow disruption in the umbilical cord. The various types of cord accidents cause fetal death by different mechanisms:

- 1) Prolapsed cord, which causes cessation of blood flow to the fetus;
- 2) Velamentous insertion, vasa previa, vasa rupture and fetal blood loss, which can cause death by umbilical cord haemorrhage and exsanguination; and
- 3) Cord entanglement, torsion, true knots and loops, which may cause chronic or acute disruption of umbilical blood flow (8).

1.2.3.2 Placental causes of stillbirth

Placental abruption complicates one percent of pregnancies, but accounts for up to 20% of stillbirths. Fetal growth restriction (FGR) due to placental dysfunction is the second most common cause of stillbirth(9). The mechanisms and aetiology of placental insufficiency are summarised in Section 1.8.

1.2.3.3 Infection

Infection can cause fetal death by a number of possible mechanisms: 1) the mother may have a severe infectious illness; 2) the infection may be present in the placenta and interfere with blood supply; or 3) the fetus may become infected causing damage to vital organs. Recognized infectious causes of intrauterine fetal death include: 1) spirochetes such as *Treponema pallidum*; 2) protoza; 3) viruses including parvovirus, coxsackie A and B, cytomegalovirus and human immunodeficiency virus; 4) bacteria including *Escherichia coli*, group B streptococci, *klebsiella*, *enterococcus*, *Ureaplasma urealyticum*, *Listeria monocytogenes*, *Mycoplasma hominus*, *Bacteroidaceae*, and *Chlamydia trachomatis*. The role of infection as a cause of fetal death is difficult to interpret for a number of reasons. Firstly, the isolation of an infectious agent from the placenta or fetal tissue does not prove that infection is the cause of death because the infection may be incidental or have occurred after death. An isolated positive maternal serology, although suggestive, does not prove causation. Taking these limitations

into account, the estimated proportion of stillbirths that are due to infection is between nine and 15 percent (10, 11).

One study, using PCR techniques on placental and/or fetal tissue from stillbirths to identify viral nucleic acid for parvovirus, cytomegalovirus (CMV) and enterovirus, suggests that PCR may improve diagnostic accuracy for infections; nevertheless, the issue of how best to diagnose infection as a cause of fetal death remains unresolved (12). A large study, including 428 stillbirths, reported that an absence of fetal inflammatory response to chorioamnionitis was associated with unexplained antepartum death (13).

1.2.3.4 Genetic conditions

There are a number of rare autosomal recessive metabolic conditions that can cause intrauterine fetal death; and the presence of non-immune hydrops, dysmorphic features or suggestive autopsy findings may give possible clues to a metabolic aetiology (14). X-linked hydrocephalus and Rett syndrome are examples of X-linked genetic conditions that may present as intrauterine fetal death. Chromosomal abnormalities are estimated to account for six to 19 percent of stillbirths, with a higher percentage in macerated fetuses or those with congenital abnormalities (15). The most common chromosomal abnormalities are trisomies (one more than the normal number of chromosomes within a cell) and 45X O, whereby a fetus has one X rather than two sex chromosomes (16).

Confined placental mosaicism (CPM) is defined as a chromosomal abnormality confined to the placenta. This chromosomal abnormality, which occurs in one to two percent of placentas, may cause stillbirth by interfering with the function of the placenta. During early embryogenesis, the zygote (union of egg and sperm) undergoes rapid cell division resulting in a ball of cells (up to 64 cell phase) called the morula.

Two possible mechanisms involving the morula can result in confined placental mosaicism. They are:

- 1) When a zygote is trisomic, an early chromosomal rescue in one cell of the morula will result in two cell lines. If the disomic cell line becomes the inner cell mass (the precursor to the fetus), the fetus has been rescued from a lethal trisomy; this trisomic rescue produces CPM which is meiotic in origin.
- 2) When a disomic zygote undergoes mitotic non-disjunction very early in embryogenesis, the morula contains both disomic and trisomic cell lines. If the inner cell mass is disomic, but the

extra-embryonic trophoblast (which becomes the placenta) is trisomic, the placental trisomy will be confined to the placenta and produce CPM which is mitotic in origin.

Unless CPM has been diagnosed at the time of prenatal testing, it is possible to miss areas of mosaicism if a number of placental samples are not collected. At this stage, the number of unexplained fetal deaths that can be attributed to CPM remains unknown (17, 18). It is important to consider possible genetic conditions when investigating the aetiology of stillbirth because amniotic fluid or specific tissue often needs to be collected in order to confirm a suspected clinical diagnosis.

1.2.3.5 Brain injury

Under-diagnosed brain injury has been raised as a possible cause of stillbirth. Neuropathology including periventricular leukomalacia, gliosis, hemorrhage, cerebral infarcts, pontosubicular necrosis (PSN), and spinal cord or brainstem necrosis is not uncommon in stillborns, and it is hypothesised that hypoxia/ischemia or infection/cytokines may be the underlying pathophysiology (19).

1.2.4 Risk factors for late intrauterine fetal death

A risk factor for stillbirth is defined as a 'maternal characteristic associated with stillbirth, but without a known pathway leading to the death' (1).

1.2.4.1 Maternal medical disease

Maternal hypertension, diabetes, obesity, systemic lupus erythematosus (SLE), chronic renal disease, thyroid disease and cholestasis of pregnancy are estimated to account for 10% of late intrauterine fetal death (20, 21).

A reported RR of 4.4 (95% CI 2.2-8.8) for stillbirth associated with chronic hypertension with superimposed proteinuria (22) supports the conclusions of previous observational and case-control studies (23, 24). However, studies assessing the risk of stillbirth associated with pre-existing hypertension without proteinuria have conflicting results (22, 25, 26). Placental abruption, placental insufficiency and fetal-maternal haemorrhage are believed to be the

pathogenic processes responsible for the increased risk of stillbirth associated with hypertension (20).

The perinatal mortality reported for diabetic women is approximately twice that reported in the general population. The stillbirth rate among women with pre-pregnancy diabetes is 10 in 10,000; but with improved surveillance and diabetic control the risk is closer to five in 10,000. Gestational diabetes increases the risk of perinatal complications such as shoulder dystocia, birth injuries and hypoglycaemia, but is not associated with an increased risk of perinatal mortality (27-29). The mechanism by which maternal diabetes increases the risk of fetal death remains unclear. The fetus is often macrosomic, and there may be associated maternal hypertension, placental vascular disease or poor glycemic control (30).

Systemic lupus erythematosus continues to be associated with significant fetal mortality, with a stillbirth rate of approximately seven percent when SLE was active at the time of conception. Nephritis and the positivity of antiphospholipid antibodies are independent predictors of adverse fetal outcome, and lupus nephritis is associated with the highest risk (30-32).

Women with moderate-to-severe renal failure have a stillbirth rate of 30 to 100 per 1000 births, and features associated with poor outcome include associated hypertension and anemia. Dialysis during pregnancy is associated with 75-80% fetal mortality (30).

Although rare, untreated maternal hyperthyroidism is associated with a stillbirth rate of 100 in 1000, and hypothyroidism is associated with a two-fold increased risk of stillbirth (33, 34).

Cholestasis, occurring in 0.1% of pregnancies, has a stillbirth rate of 25 to 30 per 1000 births. This condition is associated with abnormal sulfation of steroid compounds, which affects the metabolism of progesterone and bile acids in the fetal/placental compartment. Although this interferes with the transport of bile acids across the placenta, the exact cause of fetal death is unknown. Management includes close maternal-fetal surveillance and ursodeoxycholic acid (UDCA), which alters the composition of the bile acid pool in maternal blood (35).

1.2.4.2 Previous stillbirth or growth-retarded infant

For women who have had a stillbirth, the risk of a subsequent stillbirth increases by a factor of two- to 10-fold (36, 37). Birth weight less than the 10th centile is a recognized risk factor for intrauterine fetal death; however, studies often used standard population growth charts which fail to differentiate between fetal growth restriction (FGR) and genetic smallness. Using the

customised growth charts, which take into account maternal height, weight, parity, gender and race, 52% of unexplained stillborn fetuses ≥ 22 weeks gestation were reported below the 10th percentile for optimal fetal growth; and the odds ratio (OR) of fetal death, associated with suboptimal growth, was 7.0 (95% CI of 3.3-15.1). In the presence of concurrent maternal obesity, the risk of fetal death associated with FGR increased to an odds ratio of 71 (95% CI 14-350, $P < 0.001$) (38).

1.2.4.3 Socioeconomic status and race

Low socioeconomic status and black race are frequently reported as risk factors for stillbirth, but the exact mechanism responsible for these associations remains unclear. African-American women in the United States have a two-fold increased risk of stillbirth compared to Caucasian women, even in the presence of prenatal care (8, 39). The issue of whether periodontal disease is associated with an increased risk of adverse pregnancy outcome remains controversial (40).

1.2.4.4 Maternal age

Advanced maternal age has been associated with an adverse pregnancy outcome in a number of studies (41-43). Jacobsson and colleagues (2004) report OR of 3.8 (95% CI 2.2-6.4) for intrauterine fetal death in women aged 45 years or older and 2.1 (95% CI 1.8-2.4) in women aged 40 to 44 compared to a control group of women aged 20 to 29 years (41).

1.2.4.5 Maternal weight

The literature suggests a two-to-four-fold increased risk of stillbirth among obese ($\text{BMI} \geq 30$) nulliparous women; however, weight gain during early or late pregnancy does not appear to increase the risk. Whether the obesity, the associated diabetes or hypertension is responsible for this increased risk remains unknown (44-46).

1.2.4.6 Multiple pregnancies

Although multiparity was reported to be associated with an increased risk of stillbirth, it was unclear whether or not this risk was independent of advanced maternal age (47, 48). Recently

a large population-based study controlling for maternal age and other potential confounders reported that grand multiparity (5-9) was only associated with a small increased risk of stillbirth (OR 1.05 CI 1.02-1.07); however, the risk continued to increase with parity. The OR was 1.97 and 2.31 for 10 to 14 and 15 or more previous live births, respectively. Eighteen or more live births were associated with a 16-fold risk of stillbirth (CI 8.77-29.82) (49).

1.2.4.7 Post dates

There have been a number of studies confirming that prolonged pregnancy is a risk factor for unexplained stillbirth (50, 51). A large study confirmed that, when calculated per 1000 ongoing pregnancies, the rate of stillbirth increased six-fold from 0.35 per 1000 ongoing pregnancies at 37 weeks to 2.12 per 1000 ongoing pregnancies at 43 weeks of gestation (52). A similar result was replicated by Shankar and colleagues (2002) with the risk of unexplained stillbirth per 1000 ongoing pregnancies rising to 1.6 at 40 to 41 weeks (53).

1.2.4.8 Cigarette smoking

Smoking is clearly associated with an increased risk of stillbirth, with a number of studies confirming a dose-response relationship (54, 55). Raymond et al (1994) showed that, in smokers, the risk of stillbirth was eliminated when women with FGR, placental abruption, and placenta previa were excluded from the analysis. They concluded that the association between stillbirth and smoking can be explained entirely by the associated growth restriction and placental pathology (55). A cohort study of 25,000 singleton pregnancies confirmed that tobacco smoke in utero was associated with a two-fold increased risk of stillbirth, and concluded that 25% of all stillbirths could be avoided if all pregnant women stopped smoking by the 16th week of gestation (56).

1.2.4.9 Drugs and alcohol

There are a large number of studies evaluating the fetal effects of cocaine with conflicting results; however, a meta-analysis supports a positive association with a six-fold (95% CI 1.18-31.3) increased risk of stillbirth associated with FGR and placental abruption in cocaine-only users compared to non-drug users(57). A more recent meta-analysis of cocaine users also

showed that polydrug use, including cocaine, was associated with higher risks of placental abruption and premature rupture of membranes (58).

Apart from a small but statistically detectable decrease in birth weight, there is no evidence that cannabis use during pregnancy is associated with an increased risk of adverse pregnancy outcomes (59).

In a large, well-designed prospective Danish cohort study, the risk of stillbirth was 1.37 per 1000 births in women who drink less than one standard alcoholic drink per week. This rate increases up to 8.8 per 1000 in women who consume \geq to five drinks per week. The increased stillbirth rate is believed to relate to alcohol increasing the risk of FGR, fetal malformation and preterm delivery (60).

The consumption of eight or more cups of coffee per day during pregnancy is associated with a three-fold (95% 1.5-5.9) increased risk of stillbirth compared to women who did not drink coffee. This risk was still increased after adjusting for smoking and alcohol use (61).

1.2.4.10 Haemoglobin concentration

There have been a number of studies reporting a possible association between high and low haemoglobin concentration and adverse pregnancy outcomes (62-64). In a well-designed, population-based case-control study comparing the haemoglobin concentration of 702 women with stillbirths to control women with live births, a maternal haemoglobin concentration of 146g/l or higher was associated with a 1.8-fold increased risk of stillbirth. Restricting the analysis to small-for-gestational age (SGA), antepartum, non-malformed stillbirths, the OR increased to 4.2. One possible explanation is that a higher haemoglobin level indicates a low plasma volume. No association was found between stillbirth and a haemoglobin concentration below 110g/L (63).

1.2.4.11 Serum maternal proteins

Within a multicentre cohort of 7934 women, Smith et al (2004) studied the risk of stillbirth in relation to maternal serum levels of placental proteins during the first 10 weeks of pregnancy. PAPP-A was strongly associated with stillbirth defined as placental abruption, or unexplained

stillbirth associated with FGR (OR 46.0 [95%CI 11.9-178]; $P < 0.001$). Adjustment for all the known risk factors for stillbirth did not affect the association. Although this is a very strong association, they report the positive predictive value for placental causes of stillbirth was only 1.8%. Nevertheless, it may be a helpful guide to predict women who may benefit from more intensive surveillance with Doppler ultrasound during pregnancy. The association is biologically plausible, as PAPP-A is a protease for insulin-like growth factor binding proteins 4 and 5, and low levels of PAPP-A are likely to result in lower levels of free insulin-like growth factor. The authors conclude that the risk of stillbirth due to placental insufficiency may be determined by placental dysfunction in the first 10 weeks (2).

1.2.4.12 Maternal birth weight

Although not studied in humans, intergenerational data about reproductive consequences of rhesus monkeys showed that mothers born SGA had a 3.4-fold risk (1.47-7.86) of delivering a stillbirth infant (65).

1.3 Fetal growth restriction

1.3.1 The burden of fetal growth restriction

1.3.1.1 Increased infant morbidity and mortality

The burden of illness associated with fetal growth restriction (FGR) is considerable. The risk of death in the first year is five- to 10-fold in growth-restricted compared to normal birth weight infants and becomes more significant as the birth weight falls from below the 10th centile to the first centile (66, 67). There is also a strong association between FGR and antepartum stillbirth because a chronic incremental uteroplacental insufficiency can eventually result in fetal death due to perinatal asphyxia (68). Initially the fetus attempts to compensate for acute hypoxia by preferential perfusion of vital organs including the adrenal glands, brain and heart (69), which can result in asymmetrical growth restriction. Although the fetus adapts to a hypoxic, hypoglycaemic environment by a number of mechanisms aiming to conserve energy,

prolonged perinatal hypoxia places the fetus at increased risk of metabolic acidosis and intrapartum distress (70, 71).

The majority of stillbirths are reported as unexplained. However, using customised growth charts, FGR was identified as an important risk factor in 52% of sudden, unexplained stillbirths (38).

Growth-retarded fetuses that survive the perinatal period have an increased incidence of neurological sequelae. A birth weight between the 3rd-10th centile or below the third centile is associated with an increased risk of newborn encephalopathy, with odds ratios of 4.37 and 38.2, respectively (72). In addition to an increased risk of hypoxic encephalopathy, resulting in impaired cognitive function or cerebral palsy, growth-restricted fetuses have an increased incidence of congestive cardiac failure, acute tubular necrosis, pulmonary transition difficulties, meconium aspiration, necrotizing enterocolitis, intracranial haemorrhage, polycythemia, impaired cellular immunity and metabolic disturbances (68, 70, 73, 74).

Reports confirm an association between FGR and risk of premature labour (75-77). Bukowki and colleagues report that approximately one quarter of premature fetuses, delivered at or before 34 weeks gestation, had a customised birth weight less than the 5th centile. Overall, 40% of the premature infants did not reach their 25th centile of growth potential (78).

The overall prognosis of a growth-restricted infant varies depending on the severity of the growth restriction and the underlying cause. Earlier and longer toxic exposures result in a poor long-term prognosis. Symmetrical FGR (affecting all growth parameters to the same degree) due to genetic or chromosomal disorders, is usually associated with a poor prognosis; whereas the symmetrical constitutionally (i.e. genetically) SGA infant may not have any abnormalities (74).

1.3.1.2 Implications for disease in adulthood

Not only is FGR associated with increased infant morbidity and mortality, there is mounting epidemiological evidence that it places the fetus at increased risk of chronic diseases in adulthood (79-81). The fetal origins hypothesis states that 'fetal under nutrition in middle to late gestation, leading to disproportionate fetal growth, programs later chronic hypertension, diabetes, stroke and coronary artery disease as an adult' (80). Individuals with a low ponderal index (birth weight X100/(crown heel) tend to develop the combination of insulin resistance,

hypertension, non-insulin dependent diabetes and lipid disorders known as syndrome X. These associations occur at all levels of social class, smoking, obesity or alcohol consumption; however, the adult lifestyle factors can add to the effect of fetal under-nutrition (82). The fetal response to poor nutrition is the redistribution of blood and nutrients to vital organs (such as the brain and lungs) at the expense of other visceral organs (such as the pancreas); and animal studies confirm that fetal under-nutrition results in a 40% reduction in pancreatic beta cell mass (83). Subsequently, the hypothesis that under-nutrition would result in a predisposition to glucose intolerance was supported by a study of 468 men aged 64 years in Hertfordshire UK, which showed that the individuals with the worst glucose tolerance were those who were born small but were currently obese (84).

A second study of individuals, who were in utero during the Dutch Hunger Winter of 1944 to 1945, compared to the year after the famine, also concluded that prenatal exposure to famine is linked to decreased glucose tolerance in obese adults (85). Studies of men and women born in Hertfordshire between 1911 and 1930 also showed that the death rate from coronary heart disease was linked to growth in utero, with mortality from coronary artery disease decreasing progressively between those who weighed less than 2500g at birth and those who were 4310g. These associations were seen in babies born SGA rather than those born prematurely (86). The results of these studies have been replicated in the United States and Sweden (80). Studies of medical records in Sheffield, UK, showed a link between disproportionate size at birth and disordered cholesterol metabolism and blood coagulation.

One interpretation is that the reduced abdominal circumference in relation to head circumference reflected impaired liver growth to support brain growth, consequently, reprogramming liver metabolism (87). An association between low birth weight and raised blood pressure in adulthood has been found in 21 studies. Possible mechanisms for this association include loss of elasticity in vessel walls or the effects of glucocorticoid hormones as a result of under-nutrition (80).

1.3.2 Definition of small-for-gestational age versus fetal growth restriction

There is considerable debate as to the definitions of SGA and FGR. For clinical purposes, SGA is often defined as a weight less than the 10th percentile of the population based standards

because these infants have an increased risk of adverse pregnancy outcome (68). However, the international SGA advisory board consensus (88) uses the term SGA to describe a 'neonate whose birth weight or birth crown-heel length is at least two standard deviations below the mean ($<2SD$) for the infant's gestational age based on the data derived from a reference population'. This is approximately the third percentile for gestational age, and this definition of SGA is most likely to include the majority of infants with disordered fetal growth. Neonates with either low birth weight (SGAW) or length (SGAL) or both (SGAWL) for gestational age should be considered SGA (88).

The term 'fetal growth restriction' has been introduced into the literature, and is defined as the situation whereby an infant has not reached their genetically determined growth potential (89). Fetal growth restriction suggests diminished growth velocity documented by ultrasound growth assessments; however, serial ultrasounds are not routine clinical practice (88). Therefore, despite these concepts, the new terminology remains confusing, and clinicians often use the terms SGA and FGR interchangeably. Using these terms interchangeably is problematic because some SGA infants may be constitutionally small and have none of the clinical features of the growth-restricted infant. No cause can be established in 30 to 40% of SGA infants, and it is possible that this idiopathic SGA group may represent constitutionally small infants as a result of genetic factors (90).

1.3.3 Diagnosis of fetal growth restriction

1.3.3.1 Standard population growth charts

Despite a number of limitations, standard population growth charts are commonly used. There are different cut-offs for the 10th centile for charts derived from California birth data compared to the US national data; consequently, it is unclear whether population-based growth charts can be generalized to all Caucasian populations (67). Another shortcoming of the population-based charts is that the normal values for prematurely born neonates are obtained from prematurely born neonates who have a higher incidence of growth restriction. Apart from gestational age, factors such as parental height and weight, parity and ethnicity are not taken into consideration; hence, population charts do not account for the genetic

growth potential of the neonate (78). As with any type of growth chart, the accuracy of birth measurements and gestational dating is important.

1.3.3.2 Symmetry versus asymmetry

In a fetus with symmetric FGR, all the biometric measurements are reduced to the same degree. In asymmetric FGR, the abdominal circumference is reduced compared to the other growth indices (74, 91), and the head growth usually remains normal or may drop during late pregnancy (74, 92). Symmetrical versus asymmetrical FGR is thought to relate to the primary underlying process which is causing the growth restriction. Symmetric FGR is typically seen with extrinsic conditions during early pregnancy or intrinsic genetic or chromosomal conditions that decreased the growth potential of the fetal. Asymmetrical FGR is typically associated with uteroplacental insufficiency. Animal studies suggest that growth asymmetry results from fetal adaption to nutritional deprivation with redistribution of cardiac output in favour of brain development. Although asymmetry is typical, severe and prolonged placental insufficiency may result in symmetrical growth restriction with a poor prognosis (93). Therefore, symmetric versus asymmetric FGR cannot be used to predict the underlying aetiology of FGR with any certainty. However, it is worthwhile comparing the biometric measurements because a low weight-to-length ratio is associated with an increased risk of perinatal morbidity, even in fetuses with a weight over the 10th centile (91).

1.3.3.3 Anthropometric measurements

Anthropometric measurements, such as the ponderal index, are regarded as useful tools for assessing the nutritional status of a newborn. The advantage of using a measure that includes both weight and length is that it is likely to give a better idea of adiposity and, therefore, nutritional status.

The ponderal index (PI) identifies infants whose soft tissue mass is low for their skeletal development. Although these infants may not be classified as SGA, the PI suggests that they have not reached their growth potential and are, therefore, growth restricted (74). A number of studies have questioned the usefulness of the ponderal index (94-96). A cross-sectional study using 53,934 term singleton live born infants found that birth weight alone is unable to

perfectly predict indicators of FGR such as skin thickness, the ratio of abdominal circumference to biparietal diameter or organ asymmetry; however, it was a better predictor than the PI (96). Other studies, however, support the use of the ponderal index, reporting that infants with FGR with a proportionate PI tend to remain lighter and shorter; whereas, the FGR infants with a low ponderal index experience catch up growth within the first few months (97, 98). The low birth-weight infants with a proportionate ponderal index may represent the constitutionally (genetically) SGA infants.

1.3.3.4 Customised growth charts

It is estimated that between 28% and 75% of SGA infants are genetically small infants and not at increased risk of adverse pregnancy outcome (99, 100). Customised growth charts, calculating an optional term weight, have been developed by using coefficients of maternal height, pre-pregnancy weight, parity, ethnicity, fetal sex and gestational age at delivery (based on dating ultrasound). The percentile growth curves are then calculated from the variance of the term birth weights (101). Data on paternal height, paternal birth weight or maternal birth weight are not included in the analysis.

In a study of more than 300,000 pregnancies, customised standards, compared to population-based charts, provided an improved capacity to predict adverse outcomes such as stillbirth, neonatal death and Apgar scores below four at five minutes. In two out of three cases, there was agreement between the standard and customised chart. When the two methods did not agree, there was a strong association between the customised definition of SGA and adverse pregnancy outcome. Fetuses defined as growth retarded by the customised chart had the highest risk of adverse pregnancy outcome; the ORs were 6.1 (5.0-7.5), 4.1 (2.4-4.8) and 2.2 (1.9-2.7) for stillbirth, neonatal death and Apgar scores < four at five minutes respectively. Babies considered small by the population standard, but not by the customised standard, did not have a greater risk of adverse pregnancy outcome or low Apgar scores compared to average-for-gestational age babies (102). Customised growth charts are also superior at identifying SGA infants at increased risk of long-term neurological sequelae (78).

The usefulness of customised growth charts remains controversial. In a cohort of 274 low-risk pregnancies, customised fetal weight $\leq 5^{\text{th}}$ centile were predictors of anthropometric features

of FGR with likelihood ratios of 4.9 (95% CI 2.7-6.3), 6.8 (95% CI 4.5-10.6) and 6.3 (95% CI 3.7-14) for skin-fold thickness <10th centile, ponderal index <25th centile and mid-arm circumference to occipito-frontal circumference <-1 SD, respectively (103, 104).

However, in a smaller study of 51 unexplained stillbirths, customised weight standards were not superior to standard growth charts at predicting growth restricted stillborn infants based on brain/liver ratios (105). A recent study showed that the use of customised fundal height charts significantly increased the detection rate of small for gestational age (SGA) fetuses in low risk nulliparous Australian women ($P < 0.001$; OR 3.10; 95% CI 1.73-5.57)(106).

1.3.3.4 Catch-up growth

Although unproven, catch-up growth is thought to represent a child's return to their genetic growth trajectory after a period of FGR. Theoretical reasons why a fetus may not reach their genetic growth potential include:

- 1) Physical uterine restraint such as multiple gestations, uterine abnormality or the effect of first parity;
- 2) Placental dysfunction; and
- 3) Reduced oxygen and nutrient supply from the mother.

Ong et al (2000) studied predictors of catch-up growth in a cohort of 848 full-term babies. A gain in SD score of greater than 0.67 SD for weight between birth and age two years was taken to indicate significant catch-up growth. A change of 0.67 SD was used, as this is the width between each of the centiles on the standard growth chart. 30.7% of the infants showed significant catch-up growth, with weight increases greater than 0.67 SD scores. These children tended to be shorter and thinner at birth, and have taller fathers. Catch-up weight gain was also associated with low maternal birth weight, primiparous pregnancies and a higher risk of obesity at five years of age (107).

Catch-up growth is closely related to maternal factors, such as smoking and weight gain during pregnancy, which are also independent risk factors for fetal growth restriction. The

presence of catch-up growth is also more common after the first pregnancy, which possibly represents the effect of in-utero growth restraint (108).

Recent evidence shows that insulin resistance, at eight years of age, is also associated with the presence of catch-up growth (108).

Harding and McCowan (2003) evaluated postnatal growth patterns in SGA babies up until 18 months of age. SGA babies with late-onset or less severe growth restriction were more likely to show catch-up growth to >10th centile by six months of age. Late (after six months) or failed catch-up was associated with early gestation at diagnosis of SGA, short birth length and increased placental weight/ birth weight ratio. Birth length was the best predictor of length and head circumference at 18 months. The association between failure of catch-up growth and increased placental weight/birth weight ratio suggests that the placenta size is not the factor limiting fetal growth, and that this group may represent the intrinsic or genetically small fetuses rather than fetal growth restriction (109).

1.3.3.5 Summary

Assessment of fetal well-being and fetal vascular changes measured by Doppler study can also be assessed antenatally to help differentiate the healthy SGA infant from those with FGR. Unfortunately, this information is not always available in large cohort studies. Overall, the literature provides evidence that the customised growth chart, developed by Gardosi et al (1992), helps to identify 1) genetically small healthy babies and 2) growth-restricted infants who are not classified as SGA based on the population charts (102, 110). Although SGA and fetal growth restriction are not synonymous, customised growth charts are likely to be superior to population charts at identifying true fetal growth restriction. Although not commonly used to diagnose fetal growth restriction, the presence of catch-up growth may be a useful parameter to measure, especially in a large cohort study where post-natal growth has also been measured. Being able to accurately diagnose the true phenotype of fetal growth restriction will increase the power to be able to detect a true association in genetic association studies.

1.3.4 Factors contributing to fetal growth restriction

1.3.4.1 Introduction

Fetal growth and health is dependent on: 1) the genetic growth potential modulated by the health of the fetus and 2) adequate supply of nutrients to the fetus from the mother via the placenta. Fetal growth restriction (FGR) is not a disease, but rather the result of multiple factors influencing each other. Possible factors contributing to FGR are traditionally divided into: 1) extrinsic environmental factors; 2) maternal factors; 3) intrinsic fetal factors; or 4) placental factors (68). The literature now suggests that paternal factors may also be important. From a clinical perspective, fetal abnormality (chromosomal/anatomical) and inadequate uteroplacental circulation establishment and maintenance are the main causes of FGR. In a normally formed fetus, placental insufficiency causes approximately 60% of cases of intrauterine growth restriction (73).

1.3.4.2 Extrinsic environmental factors

Reported environmental factors associated with FGR include high altitude, variable nutrition, low folate and/or low dairy diet, pollution, hyperthermia and irradiation. Experimental animal studies, evaluating protein/caloric deficiency and selected nutrient deficiency and micronutrient deficiency, confirm the effect of maternal under-nutrition as a possible mechanism for FGR (111). However, the effect of maternal nutrition on fetal development in industrialized countries has been controversial. Earlier observational studies of British women suggest that maternal diet influenced infant and placental size; however, protein and energy supplementation produced inconsistent results (112).

1.3.4.3 Maternal factors

Maternal factors associated with FGR can be divided into: 1) medical conditions; 2) poor obstetric history; and 3) other conditions. A range of acute and chronic maternal medical conditions are associated with an increased risk of FGR. Maternal disease associated with FGR includes hypertension, anaemia, cyanotic heart disease and renal disease. Reduced uteroplacental perfusion due to maternal vascular disease accounts for approximately 25-30%

of all FGR infants (74). Although maternal blood pressure is intrinsically good for uteroplacental perfusion, severe early onset pregnancy-induced hypertension is associated with a 15-20 fold risk of FGR; and renal disease and mild pregnancy induced hypertension are each associated with a five-fold increased risk of FGR (113). Maternal conditions causing hypoxia, such as asthma, cystic fibrosis and cyanotic heart disease may impair the normal oxygen supply to the fetus. Chronic disease such as inflammatory bowel disease may result in maternal under-nutrition. Maternal substance abuse can cause FGR via a direct effect on the fetus or due to an association with poor nutrition, antenatal care or socioeconomic factors (114). Smoking is a major risk factor for FGR with 15 to 30% of women reported to smoke during pregnancy (115, 116). A study cohort of 170,254 pregnancies confirmed that the birth weight of neonates of mothers who smoke was lower compared to non-smoking mothers across all gestational ages. They found a reduction in birth weight of -111g, -175g and -236 g for women who smoked one to five; six to 10; and > 10 cigarettes per day (115). In developed countries, smoking is estimated to account for 40% for growth-restricted newborns (117). Heavy maternal alcohol consumption (defined ≥ 14 glasses a week) is a well recognised risk factor for FGR (118, 119); however, moderate alcohol consumption (≤ 14 glasses a week) does not appear to be a risk factor (119, 120). The OR for an association between low BMI during pregnancy or failure to gain > 0.2 kgs per week during pregnancy is reported between 1.5 and 2.5 (121, 122). Maternal height less than 157.5-158cm is reported to have a relative risk of FGR of 1.27 (117); however, these fetuses may represent normal genetically small babies.

Obstetric factors can also influence the risk of FGR. A woman's first baby tends to be smaller (RR FGR 1.23), which is believed to be the result of superficial placentation. A prior history of a SGA baby is associated with a 2.75 RR of SGA baby in a subsequent pregnancy. Prior stillbirth appears to be associated with an increased risk of FGR in a subsequent pregnancy, but it is difficult to know whether a previous stillbirth is an independent risk factor, as it may be a proxy for a previous child with FGR (117).

1.3.4.4 Paternal factors

The importance of paternal factors, particularly paternal height as a predictor of birth weight and birth length, is emerging. Initially, it was reported that standardised birth weights were

greater when a baby was born to a tall father (123, 124). The effect of a tall father is greater if the mother is also tall and less if the mother is shorter, which is consistent with the concept of maternal constraint (125). Subsequently, the effect of paternal height on fetal size has been shown to be greater than the effect of fetal sex. In women of average height, a baby will be 183g lighter if the father's height is ≤ 2 SD compared with a tall father (126). Analysing data from a French population-based maternity registry of 5,989 couples, the risk of offspring being born SGA was 4.7 times greater for mothers and 3.5 times greater for fathers who were born SGA and 16.3 times greater if both parents were born SGA (127). Paternal obesity is also an independent risk factor for SGA (128). Despite the importance of paternal height, this variable is not routinely used in the customised birth weight charts because 1) in historical cohorts, paternal data was not routinely collected (101) and 2) the issue of false paternity may make the data inaccurate.

1.3.4.5 Intrinsic fetal factors

1.3.4.5.1 Congenital malformations and chromosomal abnormalities

Fetal causes such as congenital malformations and chromosome disorders are responsible for approximately 20% of FGR (67). In a cross-sectional study of 458 fetuses, referred for further assessment of growth restriction at 17-39 weeks gestation, 19% had an abnormal karyotype. The incidence of chromosomal abnormalities was significantly higher in fetuses with FGR detected between 18-25 weeks compared to later gestations. Triploidy was present in 58% of those diagnosed with FGR < 26 weeks gestation, whereas trisomy 18 was the most common (46%) in later gestations. The presence of fetal malformations, in addition to FGR, was associated with a 40% incidence of chromosomal abnormality compared to isolated growth restriction (2%) (129). Although chromosomal abnormalities are often associated with early-onset symmetric impairment in growth of all parts of the body, this is not always the case. Triploidy and chromosomal abnormalities where FGR is diagnosed after 30 weeks are usually asymmetrically growth restricted (129).

Uniparental disomy occurs when two copies of a chromosome are inherited from a single parent. Studies in both man and mouse have shown that specific genes or collections of genes

are imprinted (gene expression switched off by epigenetic mechanisms) depending on the parent of origin. If the imprinted genes on a particular chromosome are involved in fetal growth, uniparental disomy for that chromosome can affect the dosage of specific gene expression and result in specific growth disorders. In a cohort of 35 babies with idiopathic growth restriction, maternal uniparental disomy for chromosome 16 was found in 5%, and structural chromosomal abnormalities were found in 11% (130).

Inborn errors of metabolism, genetic syndromes and Rhesus disease are rarer causes of FGR.

1.3.4.5.2 Fetal infection

For most cases of chronic villitis, no infectious cause can be found, and infections such as rubella, toxoplasmosis, cytomegalovirus (CMV), varicella-Zoster, malaria, syphilis, herpes, listeria, tuberculosis, chlamydia, and Mycoplasma are estimated to account for 5-10% of cases of FGR. CMV before 20 weeks is the most frequent viral etiology of FGR in developed countries (74, 131, 132).

1.3.4.6 Placental factors

An abnormality of adequate establishment and maintenance of the uteroplacental circulation is the single most common cause of FGR. Placental factors which can reduce nutrient supply from the mother to the fetus include: 1) limited placental perfusion; 2) reduced placental membrane area; or 3) altered permeability. Placental insufficiency has been established as a recognised factor contributing to the etiology of both intrauterine fetal death and intrauterine growth restriction, and placental function also plays a central role in the pathogenesis of the adverse pregnancy outcome pre-eclampsia (section 1.7:*Placental insufficiency*). Fetal thrombotic vasculopathy as a cause of limited placental perfusion is discussed in section 1.8.

1.4 Pre-eclampsia

1.4.1 Definition

“Preeclampsia is a multi-system disorder unique to human pregnancy characterised by hypertension and involvement of one or more other organ systems and/or the fetus”(133). Based on the Society of Obstetric medicine of Australia and New Zealand (SOMANZ) guidelines (133), a diagnosis of preeclampsia can be made when hypertension arises after 20 weeks gestation and is accompanied by one or more of the following:

Renal involvement:

- Significant proteinuria – dipstick proteinuria subsequently confirmed by spot urine protein/creatinine ratio $\geq 30\text{mg/mmol}$. In view of the close correlation between spot urine protein/creatinine ratio and 24 hour urine excretion, the latter is rarely required.
- Serum or plasma creatinine $> 90\text{ }\mu\text{mol/L}$
- Oliguria

Hematological involvement

- Thrombocytopenia
- Hemolysis
- Disseminated intravascular coagulation

Liver involvement

- Raised serum transaminases
- Severe epigastric or right upper quadrant pain.

Neurological involvement

- Convulsions (eclampsia)
- Hyperreflexia with sustained clonus
- Severe headache
- Persistent visual disturbances (photopsia, scotomata, cortical blindness, retinal vasospasm)
- Stroke

Pulmonary edema

Fetal growth restriction

Placental abruption

Severe pre-eclampsia is defined as blood pressure above 160/110mmHg and proteinuria greater than 5g/24hours; however, the differentiation between mild and severe pre-eclampsia is still a matter of debate(134).

Severe pre-eclampsia may progress to the syndrome of hemolysis, elevated liver enzymes, and low platelets (HELLP) or eclampsia. Eclampsia, due to the involvement of the cerebral vessels, is defined as 'the life-threatening convulsive phase of pre-eclampsia which tends to occur after mid-pregnancy, at delivery or within 48 hours post-partum' (135).

Another form of classification divides pre-eclampsia in terms of early onset (before 34 weeks) and late-onset (34 weeks or more), with the early onset form most likely to be associated with fetal growth restriction and abnormal uterine artery Doppler consistent with abnormal placentation (136). The concept of maternal verses placental pre-eclampsia is discussed in section 1.4.5 (Aetiology and pathophysiology of pre-eclampsia).

1.4.2 Burden of illness of pre-eclampsia

The incidence of pre-eclampsia is estimated to range from two to six percent in healthy, nulliparous women, with the incidence of severe pre-eclampsia being one in 200 (0.5%); and although maternal mortality in developed countries has decreased, pre-eclampsia remains a major factor contributing to FGR, pre-term birth and perinatal mortality. Pre-eclampsia accounts for 15% of preterm deliveries, which are associated with an increased risk of mortality and long-term neurological sequelae (135). There is compelling evidence for an association between FGR and cardiovascular disease in adulthood (79). Pre-eclampsia is a risk factor for maternal cardiovascular disease later in life, with the highest risk in the subgroup of women with early onset or recurrent pre-eclampsia (137, 138).

1.4.3 Risk factors for pre-eclampsia

There are a number of maternal, paternal, pre-conception and pregnancy related risk factors associated with pre-eclampsia which are summarised in Table 1. Pre-eclampsia is more common in first pregnancies and advanced maternal age. Several studies suggest that pre-eclampsia has a familial predisposition, but there is no evidence for a single dominant gene. The fact that pre-eclampsia is more common in mothers, daughters and sisters with pre-eclampsia suggests that maternally inherited genes play an important role; however, the high degree of discordance in risk of pre-eclampsia between monozygous twins confirms that other factors in addition to maternal genes influence the risk (139, 140). The role of paternal genes was raised by a study reporting that men who were the product of a pregnancy complicated by pre-eclampsia were significantly more likely, compared to controls, to have a child who was the product of a pregnancy complicated by pre-eclampsia (141).

Table 1. Risk factors for pre-eclampsia

Risk factor		Effect size
Patient related factors	Maternal age ≥ 40	RR 1.96 (1.3-2.8) (142)
	Nulliparity	RR 2.9 (95% CI 1.2-6.6) (142)
	Interval between pregnancies	RR 1.12 (1.11 -1.13) For each year increase in interval
	Short duration of sexual relationship	(< or =6 months adjOR 1.88, 95% CI 1.05-3.36) (< or =3 months adjOR 2.32, 95% CI 1.03-5.25) (143)
	Previous history of pre-eclampsia	RR 7.19 (5.85-8.83) (142)
	Family history of pre-eclampsia	RR 2.9 (1.7-4.9) (142) OR 2.0 (1.3 to 3.0) (144)
	Raised blood pressure at booking	

	Diastolic ≥ 80 mmHg	RR 1.38 (1-1.8) (142)
	Systolic ≥ 130 mm Hg	RR 2.37 (1.78-3.15)
	Pre-existing hypertension (Diastolic blood pressure before 20 weeks ≥ 110 mmHg)	RR 5.2 (1.5-17.2) (142)
	Underlying renal disease	5.3% v 1.8% (142)
	Raised body mass index before pregnancy and insulin resistance	RR 2.47 (1.6-3.67) (142)
	Increase of 5 in Body Mass Index	1.3 (1.1 to 1.4) (144)
	Pre-existing diabetes	RR 3.6 (2.5-4.9) (142)
	Thrombophilias	See literature review
	Antiphospholipid antibodies	RR 9.7 (4.3-21.7) (142)
	Autoimmune disorders	RR 6.9 (1.1-42.3) (142)
	Maternal urinary infection	6.7% v 2.6% (142)
	Family history of coronary heart disease¶	OR 1.9 (1.2 to 2.8) (144)
Partner related factors	Primipaternity	
	Donor insemination	
	Oocyte donation	
	Partner who fathered a pre- eclamptic pregnancy in another women	

Pregnancy associated factors	Multiple pregnancies	RR 2.9 (2-4.2) (142)
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Hydrops fetalis

Chromosome anomalies
(triploidy and trisomy 13)

Hydatidiform moles

List adapted from (145, 146)

1.4.4 Pathological features of pre-eclampsia

The earliest pathological changes in pre-eclampsia are found in the utero-placenta unit. These include poor spiral artery remodelling with the failure of the extravillous trophoblast (EVT) cells to express a vascular endothelial phenotype accompanied by poor decidual invasion (147). The subsequent maternal multi-system pathology is characterized by endothelial swelling, oedema, microinfarction, microhaemorrhage and decreased perfusion to virtually all organs. The main target organs are the brain, liver, kidneys, adrenals, heart and lungs. The characteristic kidney changes, including glomerular capillary endothelial and mesangial

swelling sufficient to occlude the capillary lumen and inclusions in the glomerular basement membrane, are not seen with other causes of hypertension (148).

1.4.5 Aetiology and pathophysiology of pre-eclampsia

1.4.5.1 Introduction

Pre-eclampsia results from an imbalance between factors produced by the placenta and the maternal adaptation to these factors. Redman et al 2009 distinguishes placental pre-eclampsia from maternal pre-eclampsia, but notes that most cases have a combination of both these classes of pre-eclampsia. "Placental pre-eclampsia is the outcome of poor placentation in early pregnancy (weeks 8-18)"(149). Abnormal placental development early in pregnancy is believed to result in placental underperfusion and ischemia. This leads to the release of antiangiogenic factors into the mother's circulation, which leads to arteriolar vasospasm and endothelial damage. This results in increased peripheral vascular resistance, reduced tissue perfusion and systemic hypertension. Pregnancy is usually associated with an increased refractoriness to pressor agents such as angiotension and vasopression; however, the vasculature of women with pre-eclampsia has an increased sensitivity to such pressor agents (150, 151). This process is further exacerbated by: 1) activation of the coagulation cascade with the formation of microthrombi; and 2) loss of fluid into the intravascular space resulting in reduced plasma volume. Renal pathophysiologies vary in different degrees. Glomerular endothelial dysfunction results in mild to severe proteinuria and reduced glomerular filtration rate (148).

1.4.5.2 Placental ischemia

In the decreased placental perfusion model, the pathogenesis is initiated by 'decreased placental perfusion interacting with maternal constitutional factors to result in oxidative stress, endothelial activation, and a multisystemic maternal disease' (152). It is widely accepted that pre-eclampsia is a disorder relating to the placenta rather than the fetus or the uterus. Hydatidiform mole, which is associated with very little fetal tissue, is associated with an increased risk of pre-eclampsia. Pre-eclampsia can occur without uterine distension in ectopic pregnancies, and pre-eclampsia is cured by the delivery of the placenta (135). Studies confirm

that the endovascular invasion of fetal trophoblasts and spiral artery remodelling occur either superficially or not at all in pre-eclampsia. Defective trophoblast invasion is thought to be due to poor differentiation of trophoblasts and a failure to change their adhesion molecular expression from those characteristic of an epithelial cell to those characteristic of an endothelial cell (153, 154). This failure of normal spiral artery remodelling leads to abnormal placentation and significantly reduced perfusion to the placenta. Poor implantation is thought to be due to immunological (Section 1.4.6.3) and genetic (Section 1.4.6.4) factors or a combination of both.

The process of normal placentation is usually complete by 20 to 22 weeks. Therefore, despite clinical features of pre-eclampsia presenting in late pregnancy, the underlying pathological changes seen in the placenta are present in the first half of the pregnancy (155). Other conditions such as thrombophilias or maternal microvascular disease may also reduce placental blood supply and increase the risk of pre-eclampsia (152). The pathological changes seen in the placenta due to reduced perfusion are also seen in association with FGR, suggesting that other genetic/environmental factors, in addition to reduced placental perfusion, are involved in the pathogenesis of pre-eclampsia (152).

1.4.5.3 Immune dysfunction

The placenta has the same genotype as the fetus, and the normal process of placentation requires maternal-fetal immune tolerance. Immune intolerance is believed to interfere with the normal process of implantation and, consequently becomes a causal factor in the development of pre-eclampsia. There is evidence that macrophages, residing in excess in the placental bed of preeclamptic women, are able to limit extravillous trophoblast invasion of spiral arterial segments through apoptosis mediated by tumor necrosis factor-alpha (TNF- α) secretion (156, 157).

In support of the couple-specific immune tolerance theory are reports that the risk of pre-eclampsia is higher in women who: 1) conceive by artificial insemination (158, 159); 2) had shorter periods of co-habitation prior to pregnancy (160, 161); and 3) use barrier contraception (161). Einarsson and colleagues (2003) reported a 17-fold increased risk of pre-eclampsia in women who cohabited for less than four months or used barrier methods for contraception

compared with women with more than 12 months of cohabitation before conception (162). Kho et al 2009 reported that short duration of sexual relationship was more common in women with preeclampsia compared with uncomplicated pregnancies (≤ 3 months adjOR 2.32 and ≤ 6 months adj OR 1.88) (143). Seminal-vesicle-derived transforming growth factor β (TGF β 1) is a critical stimulating agent in the post-coital inflammatory response which may induce immune tolerance to seminal antigens (163). Although there are numerous studies supporting the concept of partner-specific mucosal tolerance, there are large epidemiological studies which challenge the importance of couple specific immune maladaptation in the etiology of pre-eclampsia (164). Ness and colleagues report ORs for the association between pre-eclampsia and barrier conception and length of sexual experience (mean eight months) of 1.0 (95% CI 0.6-1.6) and 1.6 (95% CI 0.5-4.3) respectively, concluding lack of support for the immune maladaptation theory of pre-eclampsia (165); however, this cohort of 2211 women (85 with pre-eclampsia) may have been underpowered to detect a true difference.

Immune intolerance is also hypothesised to have a genetic basis, whereby interaction between uterine natural killer (uNK) cells and extravillous trophoblastic cells (EVT) control placental implantation (see 1.6.5). Studying polymorphisms in the killer immunoglobulin receptors (KIR) on maternal natural killer cells, Hiby et al (2004) reported that mothers with a KIR-AA genotype in combination with a fetal HLA-C2 genotype were at greatly increased risk of pre-eclampsia (166); however, a number of studies show inconsistent results, and the immune theory for pre-eclampsia remains controversial (167).

Recent studies report that women with pre-eclampsia possess angiotensin II (AngII) receptor (AT1) agonistic autoantibodies that activate AT1 receptors promoting physiological changes characteristic of pre-eclampsia through induction of tumor necrosis factor alpha (TNF-alpha) (168).

1.4.5.4 Genetics of pre-eclampsia

The fact that the placenta has the same genotype as the fetus suggests that genes inherited from the father as well as the mother may play an important role in the regulation of normal placentation and the development of pre-eclampsia (139).

This was supported by an analysis of pre-eclampsia in 701,488 pregnancies of 244,564 siblings from a Swedish birth registry, which found that the heritability conferred by maternal genes was 35% (95% CI 0.33-0.36). The heritability due to fetal genes was 20% (95% CI 0.11-0.24), with equal contribution of maternal and paternal genetic effects(169). This makes conventional linkage studies more difficult. Genome-wide screening has detected a number of loci suggestive of linkage including chromosome 2 (2p13, 2p12, 2p25, 2p22, 2q22) and chromosome 9 (9p13) which supports the concept that the aetiology of pre-eclampsia is multifactorial rather than due to a major dominant susceptibility gene (170-173). A number of population association candidate gene studies have been undertaken, often with conflicting results and no clear conclusions. Examples include factor V Leiden, prothrombin gene, lipoprotein lipase, MTHFR; AGT (encoding angiotensinogen); NOS3 (encoding endothelial nitric oxide synthase), FAS and FAS ligand (174-181).

1.4.5.5 Oxidative stress hypothesis

The oxidative stress hypothesis of pre-eclampsia proposes 'that hypoxia at the fetal-maternal interface results in the generation of free radicals that may lead to oxidative stress dependent upon the maternal constitution' (135). It is the maternal susceptibility to this oxidative stress that is thought to be the link between reduced placental perfusion (section 1.4.4.2) and the maternal syndrome of pre-eclampsia.

Oxidative stress 'is the presence of active oxygen species in excess of the available antioxidant buffering capacity' (182). The reactive oxygen species are highly toxic and cause oxidative damage to DNA, proteins, and lipids interfering with their structure and function. An imbalance in the body's normal mechanisms to buffer the reactive oxygen species can be caused by reduced antioxidants, or an excessive production of reactive oxygen species (182).

1.4.5.5.1 Tissue ischemia as a cause of oxidative stress

Tissue ischemia followed by reperfusion is a common pathological cause of excess production of reactive oxygen radicals. Changes in uterine and placental blood flow during pregnancy may be influenced by exercise, posture and uterine contractions (182). These normal variations may be further compromised by pathological causes of reduced placental perfusion. Oxidative stress is the current hypothesis explaining the endothelial changes seen in atherosclerosis

(183). The observation that atherosclerosis and pre-eclampsia have similar risk factors and share a similar dyslipidemia profile also raises the possibility that a common genetic susceptibility is involved (148). Markers of oxidative stress have been demonstrated in the blood, maternal tissue, decidua and placenta in pre-eclampsia (135). The generation of superoxide anion radicals from xanthine oxidase has been implicated in post ischemia-reperfusion tissue injury. The placental activity of xanthine oxidase was found to be increased in the placenta of pre-eclamptic women. In addition, the activity of the anti-oxidant superoxide dismutase was reduced in the same cells, and a major product of oxidative stress, peroxynitrite (ONOO⁻), was increased in the placenta (184).

1.4.5.5.2 Maternal susceptibility to oxidative stress

Maternal factors which may increase susceptibility to oxidative stress include decreased antioxidants, sensitised endothelium; and lipoproteins sensitive to oxidation (152). In a two-stage model, reactive oxygen species generated as a consequence of decreased placental perfusion evoke endothelial cell activation which results in the maternal vascular malfunction characteristic of pre-eclampsia (185). An altered endothelial dysfunction is suggested by the glomerular and pathophysiological changes seen in pre-eclampsia. Plasma levels of circulating markers of endothelial dysfunction are increased in women with pre-eclampsia, and vessels removed from women with pre-eclampsia have impaired endothelial vasodilator functions (148). Several possible mechanisms, by which free radicals formed in the intervillous space result in systemic endothelial activation, have been proposed. These include: 1) neutrophils and monocytes activated by oxidative stress in the placenta could generate free radicals on contact with systemic endothelium; 2) the formation of stable products of lipid peroxidation such as malondialdehyde in the placenta may injure systemic endothelium; 3) oxidized fragments of syncytiotrophoblasts (the part of the placenta that actively invades the uterine wall forming the outermost fetal component of the placenta) entering the systemic circulation; and 4) release of placental cytokines with the potential to cause oxidative stress (152). Placental oxidative stress occurs in all pregnancies, but it is hypothesised that superimposed additional placental and maternal factors increase placental oxidative stress resulting in an increased risk of developing pre-eclampsia.

1.4.5.6 Angiogenic factors in pre-eclampsia

The normal process of placentation requires a balance between the proangiogenic and antiangiogenic factors produced by the placenta. An imbalance of the proangiogenic and antiangiogenic factors released from the developing placenta appear to play an important role in the development of pre-eclampsia (186). Proangiogenic factors include VEGF and PlGF, while antiangiogenic factors include soluble fms-like tyrosine kinase 1 receptor (sFlt-1/soluble VEGF receptor type1) and soluble endoglin (sEng) (187, 188). sFlt binds to the receptor-binding domains of VEGF and PlGF, blocking their ability to bind to their endothelial receptors, which inhibits their proangiogenic activity. Increased placental secretion of sFlt-1 and, consequently decreased levels of circulating PlGF and VEGF, are seen in women with pre-eclampsia; and this imbalance of antiangiogenic factors contributes to endothelial dysfunction in pre-eclampsia (189). Evidence that cytotrophoblasts are able to increase sFlt-1 production under reduced oxygen supports the hypothesis that increased sflt production is triggered by factors released in response to placental ischemia (190, 191). It remains unclear whether the increased sFlt-1 production is a cause of the placental abnormalities or a response to placental ischemia. Although the mechanism remains unclear, evidence suggests that soluble endoglin (sEng) secreted by the placenta also plays a role in the pathogenesis of pre-eclampsia (186).

Intrauterine fetal death, intrauterine growth restriction and pre-eclampsia all have placental insufficiency as a common link. The following sections detail the normal development of the fetomaternal circulation, genetic factors involved in normal placentation and the aetiology of placental insufficiency.

1.5 Feto-maternal circulation

1.5.1 Normal blood supply to the uterus

The uterus receives its blood supply from the uterine and ovarian arteries. The uterine arteries give off numerous arcuate arteries to supply the myometrium (middle muscular layer) and

basal layer of the endometrium (lining of the uterus). The arcuate arteries branch to form the coiled spiral arteries that supply the functional layer of the endometrial (192).

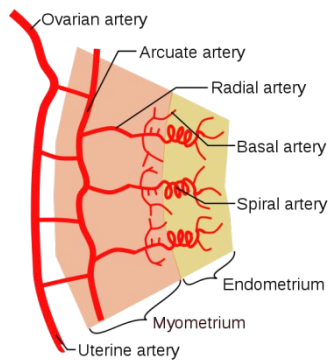


Figure 1.
Arterial vasculature of the non-pregnant uterus
J Clin Pathol 1976;29:9-17 copyright has been
released in the public domain.

1.5.2 Implantation and development of the placenta

Cells that give rise to the placental unit arise very early in development. Following fertilisation, the zygote divides into two cells. The cells continue to divide to form the morula (12 cells), which enters the uterus day three post-fertilisation. Shortly after entering the uterus, a fluid-filled space appears in the morula forming the blastocyst. The fluid separates the cells into an inner cell mass (which gives rise to the embryo) and an outer trophoblast (which gives rise to the placenta). The differentiated trophoblasts are specialised epithelial cells that physically connect the embryo and the uterus (193, 194). Allocation of cells to the trophoblast lineage is dictated by cell position, and trophoblasts that overlie and remain proximal to the inner cell mass continue to divide. At the time of attachment of the blastocyst to the endometrium, the blastocyst is composed of approximately 100 to 250 cells. Shortly before it invades the endometrium, the trophoblast differentiates into: 1) and outer syncytiotrophoblast; and 2) the inner cytotrophoblast.

The invading trophoblast then burrows into the endothelium and the blastocyst becomes embedded within the endothelium (195).

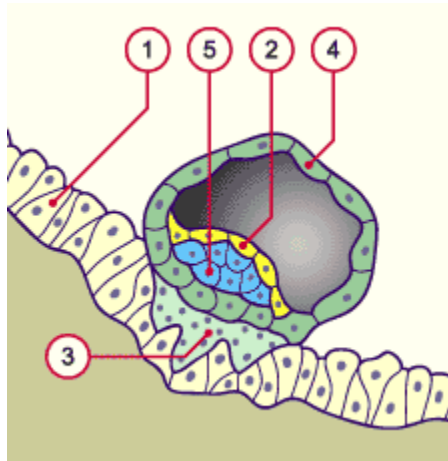


Figure 2. Implantation 6th-7th day

1. Epithelium of the uterine endometrium
2. Hypoblast
3. Syncytiotrophoblast (ST)
4. Cytotrophoblast (CT)

<http://www.embryology.ch/anglais/fplacenta/fecond03.html>

The phenomenon of implantation is also associated with the transformation of the uterus. Initially the uterine blood vessels become more permeable. Subsequently, the uterus undergoes a decidual response whereby the uterine epithelium is lost and stromal (decidual) cells undergo epithelioid transformation and proliferation to form a thick uterine wall. There is recruitment of inflammatory cells into the decidual tissue that show immunological properties such as reduced alloreactivity (194).

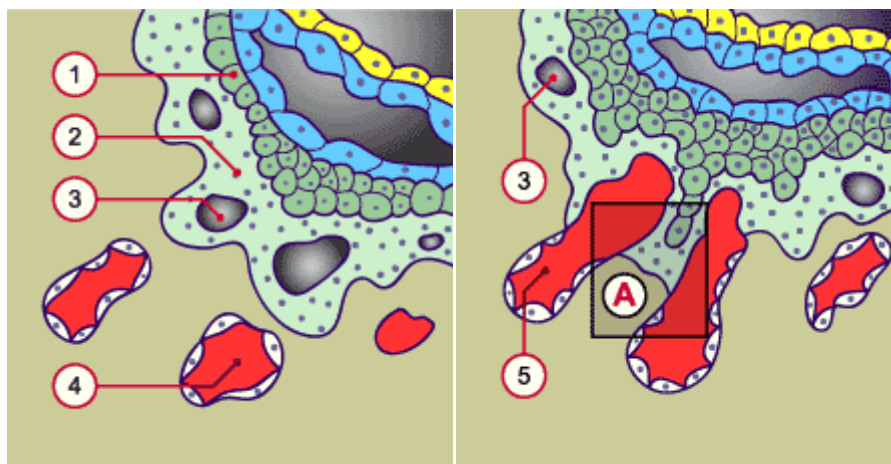


Figure 3. 9th-10th day

1. Cytotrophoblast
2. Syncytiotrophoblast
3. Spaces between syncytiotrophoblast (Lacunae)
4. Maternal vessel
5. Maternal vessel, eroded by the syncytiotrophoblast which form the maternal sinusoids through communication with Lacune

An Enlarged section in figure 4

<http://www.embryology.ch/anglais/fplacenta/villosite02.html>

As early as 7.5 days after fertilisation, the inner cell mass (destined to be the embryo) differentiates into a bilaminar embryonic disc. Simultaneously, spaces called lacunae appear within the syncytiotrophoblast. By approximately nine days post-endometrial implantation, the syncytiotrophoblast erodes the maternal endometrial capillaries and the lacunae fill with maternal blood forming sinusoids (these sinusoids will eventually communicate with each other to form the intervillous space). With deeper burrowing into the endometrium, strands of trophoblast branch and form primitive villi transversing the lacunae (195). While these primitive villi contain only trophoblast, they are called primary villi.

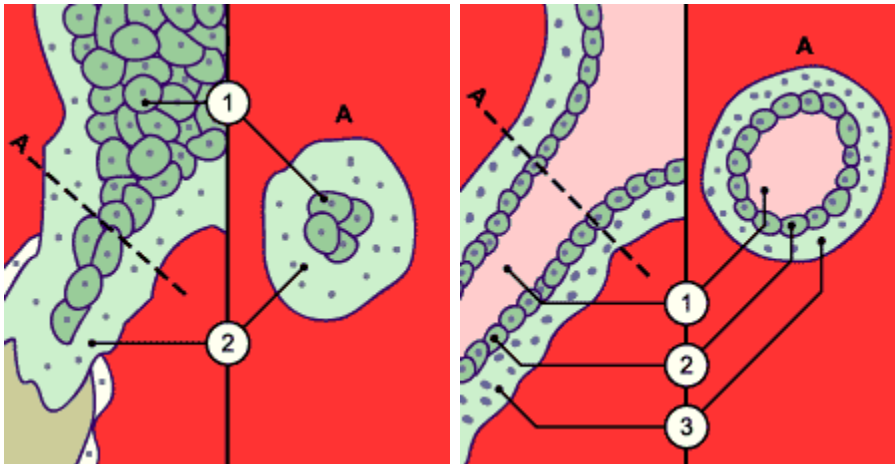


Figure 4. 11th-13th day

1. Cytotrophoblast
2. Syncytiotrophoblast

<http://www.embryology.ch/anglais/fplacenta/villosite02.html>

Figure 5. Day 16

1. Extra-embryonic mesoblast
2. Cytotrophoblast
3. Syncytiotrophoblast

<http://www.embryology.ch/anglais/fplacenta/villosite02.html>

Primary villi are invaded centrally by mesenchyme to form secondary villi. By week five to six, via a process termed vasculogenesis, fetoplacental capillaries form within the villi and transform them into tertiary villi. There continues to be further development of these tertiary (vascularised) villi into subclasses termed mesenchymal villi, intermediate villi, stem villi, mature intermediate villi and terminal villi. The number of terminal villi increases exponentially (196), reaching a surface area of 13m² at term. The capillaries within the terminal villi contain 80mls of blood, which is 25% of the fetoplacental blood volume (196).

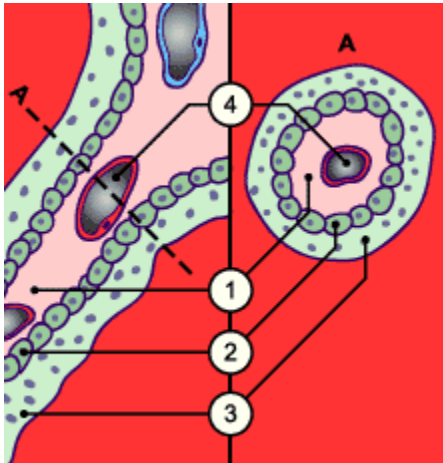


Figure 6. day 21

- 1.Extra-embryonic mesoblast
- 2.Cytotrophoblast
- 3.Syncytiotrophoblast
- 4.Fetal capillaries

<http://www.embryology.ch/anglais/fplacenta/villosite02.html>

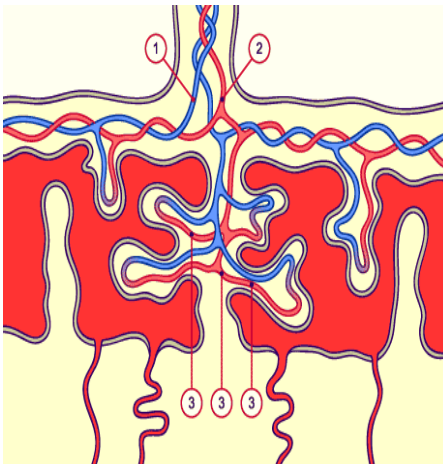


Figure 7. Fetal maternal circulation

- 1.Umbilical artery
- 2.Umbilical Vein
- 3.Fetal capillaries

Due to their low oxygen content, the fetal umbilical arteries are shown in blue and the oxygen-rich veins in red.

<http://www.embryology.ch/anglais/fplacenta/circulplac01.html>

The lacunae continue to enlarge and ultimately fuse to form the intervillous space. The fetal and maternal circulations are only separated by a single thin membrane of syncytiotrophoblast, which optimises the physiological exchange of gases, nutrients and waste between the mother and her fetus (193). The endometrial spiral arteries supply oxygenated blood into the lacunae. The maternal blood temporarily leaves the maternal circulation when it enters the intervillous space. Within the intervillous space, the maternal blood is like a lake of blood that surrounds and bathes the large arterio-capillary-venous system within the chorionic villi. The deoxygenated blood from the intervillous space returns back to the maternal circulation via the endometrial veins (193).

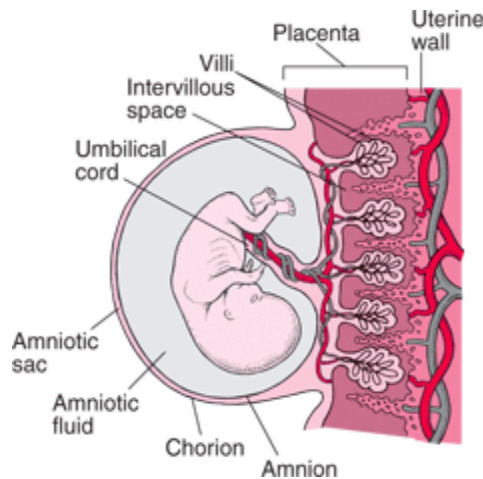


Figure 8. Placenta and embryo at 8weeks
<http://www.merckmanuals>

1.5.3 Fetal blood supply to the placenta

The fetal cardiovascular system reaches a primitive functional state by the end of the third week. Blood flows from the primitive fetal cardiovascular system through the umbilical cord via two umbilical arteries. The umbilical arteries branch to form truncal arteries, which each supply a single placental lobe. The fetal circulation passes through the large arterio-capillary-venous system of chorionic villi, and fetal oxygenated blood then returns to the fetus via the umbilical vein (193).

1.5.4 The process of uterine spiral arteries remodelling

The uterine spiral arteries provide the maternal blood supply to the endometrium of the uterus. During the early stages of uteroplacental development, the elastic and the muscular walls of the spiral arteries undergo a process of 'vascular remodelling'. At this stage, they are transformed from vaso-reactive vessels to non-compliant vessels of low resistance. This physiological remodelling, which allows the spiral arteries to conduct the 10-fold increase in blood flow during the pregnancy, is the result of an interaction between the extravillous trophoblast (trophoblastic elements outside of the villi) and the deciduas (the part of the endometrium that forms the maternal part of the placenta). Invasion of the spiral artery walls by extravillous trophoblast (EVT) is associated with degenerative changes within all layers of the arterial wall, which causes the vascular smooth muscle to become unrecognisable (197). One important consequence of the disappearance of musculo-elastic layers of spiral arteries is the formation of gaps that allow increased low pressure flow in the intervillous space (198) . These 'physiologic changes' in the spiral arteries commence as early as the fourth week of

gestation and are complete by week eight (197, 198). The invasion of the spiral arteries by the EVT cells also protects the early circulatory system from high pressure by allowing plug formation. These plugs of cytotrophoblast, interposed between the spiral artery flow and the intervillous space prevent maternal blood from penetrating the intervillous space freely and quickly (197).

During spiral artery remodelling, the invasive cytotrophoblasts downregulate epithelial-like receptors and replace them with endothelial adhesion molecules to allow the cytotrophoblasts to invade and differentiate (199).

The flow of blood in and out of the intervillous space is produced by changes in maternal blood pressure. At the height of the blood pressure, blood spurts towards the chorionic plate (the part of the placenta that gives rise to the villi) followed by lateral spread. The next increase in arterial pressure pushes the previous blood towards exits in the basal chorionic plate to be drained by endometrial veins. The 150ml of maternal blood in the intervillous space is replaced with oxygenated blood three to four times each minute (200).

1.6 Genetic factors involved in normal placentation

Trophoblastic invasion and normal placental implantation is a complex event with molecules involved in a number of processes including trophoblastic differentiation, extracellular matrix degeneration, angiogenesis and avoidance of immune surveillance (114). Therefore, genes encoding these molecules are important genes with respect to normal development and maintenance of the feto-placental circulation.

1.6.1 Proteins involved in the formation of normal placenta

There are a number of molecules involved in the complex process of EVT cell proliferation and villous cytotrophoblastic proliferation and function. For example, during normal placentation, the binding of the proangiogenic molecules VEGF and PGF to the Fms-like tyrosine kinase 1 (Flt1) receptors located on the cytotrophoblast stimulates the production of nitric oxide (NO) in the presence of sufficient tissue L-arginine. The vasodilatory and angiogenic properties of nitric oxide and down pathway regulation of matrix-degrading proteases play a vital role in the normal cytotrophoblast endovascular invasion during placentation. The antiangiogenic

soluble form of Fms-like tyrosine kinase 1 (sFlt1) normally competes with VEGF at the FLT1 receptors (188, 201).

Factors involved in EVT cell migration/invasiveness include the insulin-like growth factor (IGF) II produced by the placenta; IGF binding protein (IGFBP)-1 produced by the decidua; trophoblast derived uPA; endothelin; hepatic growth factor and SGHPL4. Factors inhibiting EVT cell proliferation, migration and invasion include TGFbeta and decorin, both produced by the decidua (147).

In addition, there is a balance between vasoconstrictor and vasodilator substances that helps maintain the unique low resistance to blood flow in the placenta. Vasoconstrictor substances include Thromboxane A2, angiotensin II and endothelin- derived relaxing factor and atrial natriuretic peptide (ANP) (147, 202).

1.6.2 The importance of imprinted genes

This is a complex and continually evolving area of genetics. Some genes can act on both the fetus (increasing for example, cell proliferation) and the placenta (influencing placental size and function and thus food supply); whereas some genes specifically control fetal growth and others exclusively influence placental growth and development (203, 204). Transgenic and gene knock-out mice studies have allowed the study of genes which are important for normal growth and, of interest, is the role of imprinted genes in the control of fetal and placental growth. Genomic imprinting is the phenomenon by which one of the two alleles of a subset of genes is preferentially expressed according to its parent of origin. Although imprinted genes only make up 1% of genes in the mammalian genome, a number of imprinted growth and growth inhibitory genes are involved in fetal and placental growth. Deletion of the paternally expressed *Igf2*, *peg1/Mest*, *Peg3* or *Ins/Ins2* genes result in fetal growth restriction; whereas deletion of the maternally expressed *Igf2r* or *H19* genes or over-expression of the *Igf2* gene results in fetal overgrowth (204). According to the conflict hypothesis, paternally expressed genes acting on the placenta are predicted to extract more resources from the mother to enhance fetal growth; whereas, maternally expressed genes are predicted to restrain fetal growth to conserve resources in the interest of the lifetime reproductive fitness of the mother. In light of this concept, normal fetal growth is the balanced result of two opposite genetic forces based on the monoallelic expression of the imprinted genes involved (203). In

particular, mouse studies have highlighted the importance of the insulin-like growth factor system. A newborn IGF-I-null-mutant weighs 60% of the wild type mouse despite a normal sized placenta (111). Constancia et al (2002,) showed that deletion from the *Igf2* gene of a transcript specifically expressed in the placenta leads to reduced growth of the placenta, followed several days later by fetal growth restriction. This study provides evidence for imprinted gene action within the placenta which directly controls the placental supply of nutrients and fetal size (204) .

1.6.3 Immunological factors involved in normal placentation

The placenta has the same genotype as the fetus, and the normal process of placentation requires mechanisms which protect the fetal semi-allograft from immune rejection by the mother. A leading area of research is why a semi-allogeneic fetus, which has inherited half its histocompatibility antigens from its father, is not rejected by the mother.

The major histocompatibility complex (MHC) is subdivided into three classes of molecules: MHC 1a, MHC 1b and MHC class II. The villous trophoblast, which is exposed to maternal blood, lacks expression of the class 1 and class II MHC molecules, which means they are not recognised as foreign by the mother's immune system (205). Within the decidua (uterine lining during pregnancy), there is a population of uterine specific natural killer (uNK) cells. Compared to classical circulating natural Killer T cells, uNK cells produce more cytokine molecules (involved in cell communication) compared to cytolytic molecules (causing cells to burst) (206). MHC complex molecules of the trophoblast cells interact with inhibitory receptors of the uNK cells and inhibit the cytolytic response of the uNK cells. The uNK cells also produce a number of molecules which are involved in angiogenesis and vascular stability and, therefore, spiral artery remodelling and normal implantation (205, 207). HLA-C (human leucocyte antigen-C) is part of the MHC 1a. Paternally-derived HLA-C and maternally-derived killer cell immunoglobulin-like receptors (KIRs) on uNK cells can each occur in several forms i.e. are polymorphic. Therefore, every pregnancy has a different HLA-C/KIR combination. Pre-eclampsia is more common in mothers lacking most or all activating KIR (inhibitory AA genotype) compared to women with the stimulator BB genotype, and this effect is more marked when the fetus is homozygous for HLA-C2. Therefore, placentation is better and pre-eclampsia less prevalent when the fetal trophoblast strongly stimulates the

maternal uterine killer cells. However, the fact that not all pregnancies with this combination develop pre-eclampsia and only a minority of pregnancies with pre-eclampsia has this combination suggests that other factors are involved (166, 201, 206).

Studies have also shown that patients with pre-eclampsia have increased antibodies to the angiotension AT-1 receptor. A mouse model supporting the hypothesis shows that autoregulation of this receptor by autoantibodies increases Angiotension II which increases hypertension and vascular damage (208).

1.7 Aetiology of placental insufficiency

Placental factors which can reduce nutrient supply from the mother to the fetus include: 1) limited placental perfusion; 2) reduced placental membrane area; and 3) altered permeability. Therefore, any pathological process which significantly interferes with normal implantation and the establishment and maintenance of the uteroplacental circulation flow will result in placental insufficiency (74).

1.7.1 Placental size

There is evidence that the size of the placenta may influence fetal size. Heinomen et al (2001) showed that placental weights were smaller in SGA infants compared to appropriate-for-gestational age (AGA) infants. The placental weight: birth weight ratio is also lower in the SGA compared to an AGA group of the same birth weight suggesting that the size of the placenta determines the size of the fetus (209). Earlier studies of experimental reduction in placental size in sheep showed smaller fetuses, which is also consistent with the concept that placental size dictates fetal size (210). As described in Sections 1.5 and 1.6, normal placental growth is regulated by complex metabolic growth factors. Apart from a disruption of the normal growth mechanisms, reduced placental size can also be due to focal lesions, placental infarcts and confined placental mosaicism (18, 211).

1.7.2 Abnormal implantation

Although the exact pathway of normal implantation remains unclear, it is proposed that an imbalance between the complex array of migration/invasion–promoting molecules and migration/invasion–controlling molecules (described in section 1.6) may lead to inadequate implantation and, consequently, reduced placental perfusion.

1.7.3 Abnormal fetoplacental blood flow

Greiss (212) described the uteroplacental circulation as having three characteristics: 1) dynamic vasodilatory capacity enabling 100- to 200-fold increase in uterine blood flow; 2) the ability to divert large amounts of total blood flow from the uterus to the placenta; and 3) minimal vascular resistance. Therefore, the placental blood flow is usually more than sufficient to meet the demands of the growing fetus if the development and function of the placenta are adequate. Non-invasive Doppler ultrasound can access the fetoplacental circulation by measuring vascular resistance. Possible mechanisms responsible for increased vascular resistance and a compromised fetoplacental circulation include: 1) inadequate development of the placenta; 2) obliteration of stem villous arteries by an embolic process; and 3) a disorder of vasomotor regulation (114, 202).

1.7.4 Conclusion

There are a large number of genes involved in the transcription of molecules necessary for the complex process of normal placentation. It is likely that the aetiology of placental insufficiency is multifactorial due to combined genetic factors and environmental factors, and that any gene that is involved in trophoblastic invasion, vascular remodelling, immune tolerance, normal coagulation or vasomotor regulation could be a potential candidate gene for causing placental insufficiency. Because of the importance of normal development and maintenance of the fetoplacental circulation, inherited thrombophilias have been raised as possible cause of placental insufficiency.

1.8 Inherited thrombophilias as a potential cause of placental insufficiency

1.8.1 Normal haemostasis

Haemostasis is the human body's normal response to injury and bleeding. Tissue factor, a cell membrane bound glycoprotein, is the primary initiator of haemostasis. As a consequence of vascular disruption, tissue factor complexes with plasma derived factor VII. This initiates a complex cascade of events involving platelets and blood clotting that ultimately leads to the formation of thrombin. In the final steps of the coagulation pathway, thrombin cleaves fibrinogen to form an insoluble fibrin monomer. Fibrin monomers self-polymerize and are cross-linked covalently by factor XIII to form a stable hemostatic plug. Thrombin also mediates platelet activation and aggregation forming a platelet clot (213, 214).

To prevent fatal thrombotic disease, a group of serine protease inhibitors (SERPINs) rapidly inhibits the excess thrombin activity. The fibrinolytic system controls the extent of the formation of the fibrin clot (215).

1.8.2 Thrombophilias

Thrombosis is defined as the formation of a blood clot (thrombus) within a blood vessel. Thrombophilia is defined as a condition that increases the risk of thrombotic disease, and can be classified as congenital (genetic) or acquired. Thrombophilias should not be thought of as diseases, but rather as risk factors predisposing to thrombosis. The acquired thrombophilias include lupus anticoagulant and anticardiolipin antibodies (216).

The most common inherited thrombophilias include autosomal dominant deficiencies of antithrombin III, protein C, and protein S, as well as activated protein C resistance due to the factor V Leiden (fVL) mutation; a function enhancing mutation in the prothrombin gene (G20210A) and hyperhomocystinemia.

Rare familial thrombophilias include dysfibrinogenemia and hyperfibrinogenemia (215, 216). A genetic predisposition to clotting is particularly important during pregnancy because pregnancy independently increases the risk of thromboembolism six-fold (217).

The inherited thrombophilias may tip the coagulation pathway balance in favour of thrombosis by: 1) partial deficiency of an anticoagulant protein (antithrombin, protein C, or protein S); 2) dysfunction of an anticoagulant factor (activated protein C resistance); or 3) gain of coagulation factor (prothrombin gene mutation (218)).

1.8.3 Factor V Leiden

The factor V Leiden (fVL) mutation is the most common form of inherited thrombophilia (219). A point mutation in the factor V gene at nucleotide position 1691, resulting in an arginine to glutamine substitution, reduces the sensitivity of the factor V protein to inactivation by activated protein C (activated protein C resistance) resulting in a pro-coagulant state and an increased risk of thrombosis (220). The trait is inherited in an autosomal dominant manner with the risk of thrombosis increased seven times in heterozygotes and 80 times in homozygotes (221). Studies have shown that the distribution of the factor V Leiden mutation varies in different populations, being present in about five percent of Caucasians and virtually absent in Africans and Asians (222).

1.8.3.1 Factor V Leiden and adverse pregnancy outcome

Despite early reports supporting an association between fVL and adverse pregnancy outcomes (223-230), a number of other studies have yielded conflicting results (231-235). Possible sources of heterogeneity include different sources for recruitment of cases and controls, different severity of disease outcome between studies, and failure to exclude fetuses with a known cause of intrauterine death or fetal growth restriction in the case group. It is possible that thrombophilias only exert their effect as part of a two hit model, for example, the combination of immune maladaptation and inherited thrombophilia (236), or the combination of thrombophilia and environmental factors, such as smoking.

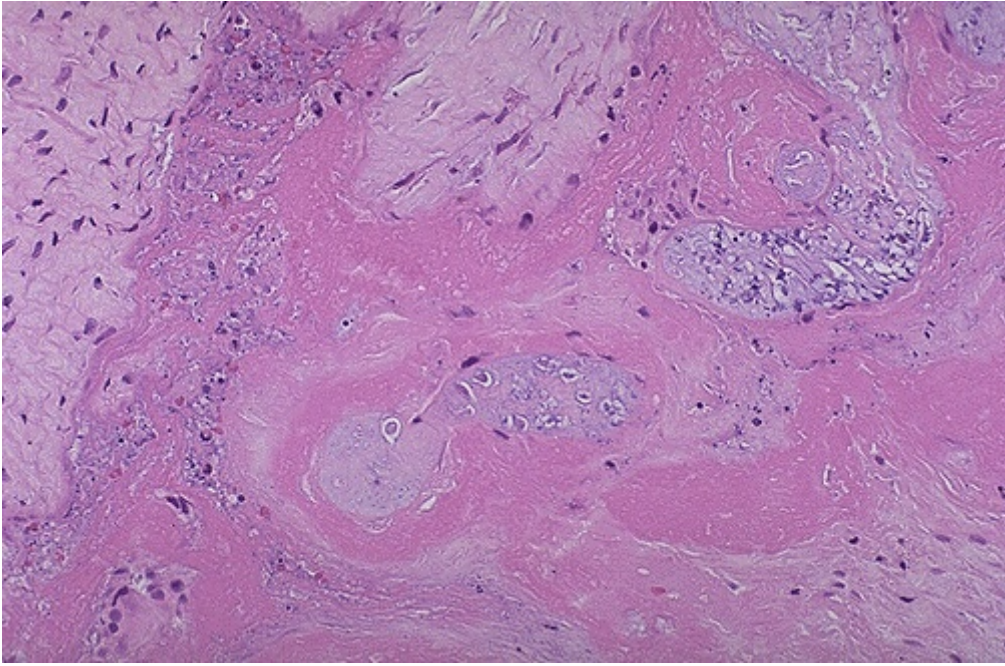


Image 1. Pale necrotic chorionic villi secondary to placental infarction due to fetal thrombotic vasculopathy.

1.9 Summary

Intrauterine fetal death, fetal growth restriction and pre-eclampsia continue to be major causes of fetal and maternal morbidity and mortality. Although a number of causes and risk factors have been identified for each of these adverse pregnancy outcomes, the underlying mechanism/s responsible for placental insufficiency remains poorly understood. Given the importance of establishing and maintaining an adequate placental circulation, hereditary thrombophilias have been postulated as a possible cause of placental insufficiency. Factor V Leiden is the most common form of hereditary thrombophilia, being present in five percent of the Caucasian population. A large number of studies have investigated a possible association between fVL and adverse pregnancy outcomes with conflicting results.

The focus of Chapter Two is a systematic review of the literature and meta-analysis aimed at clarifying the association between fVL and adverse pregnancy outcomes.

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CHAPTER 2

REVIEW OF THE EFFECT OF MATERNAL AND FETAL FACTOR V LEIDEN AND PROTHROMBIN GENE VARIANT ON ADVERSE PREGNANCY OUTCOMES

2.1 Introduction

Prior to Publication I, a number of small case-control studies had shown an association between factor V Leiden (fVL) and recurrent fetal loss, late fetal death, pre-eclampsia and fetal growth restriction (FGR); however, other studies had not shown a clear association. Possible sources of heterogeneity included different sources for recruitment of cases and controls. This systematic review of the literature and meta-analysis was performed to address the question of whether the maternal fVL genotype is associated with an increased risk of adverse pregnancy outcomes.

Although the meta-analysis of case-control studies suggested that fVL is associated with a 1.8-fold higher risk of a first-trimester fetal loss, this estimate was viewed cautiously due to clinical heterogeneity and the lack of a dose response curve: those with a stronger history of previous first-trimester losses actually had a lower risk associated with fVL. This made the validity of this relationship doubtful, and in combination with the negative result of the only cohort study, it was interpreted as there being at best a very weak relationship, but probably no relationship, between fVL and first-trimester fetal loss. A possible source of heterogeneity between studies is the inclusion of patients who had experienced a first trimester miscarriage prior to the establishment of the intervillous space as these miscarriages would be unrelated to thrombophilia.

In contrast, the meta-analysis indicated a strong relationship between second- or third-trimester fetal loss and fVL, with the odds ratio (OR) increasing as the number of previous fetal losses increased, and the further into the pregnancy the losses occurred. For second- or third-trimester fetal loss, there was a consistent and graded increase in risk: the OR was 2.4 (95%CI 1.1-5.2) for isolated (non-recurrent) third-trimester fetal loss, rising to 10.7 (95%CI 4.0-28.5) for those with two or more second-or third-trimester fetal losses.

The meta-analysis is the first to evaluate how fVL influences pre-eclampsia and fetal growth restriction (FGR) (birth weight <10th centile). Studies evaluating a possible association between fVL and pre-eclampsia were heterogenous and were divided according to study design. Meta-analysis of cohort studies, reflecting an unselected group of fVL positive

women, did not show a statistically significant association between fVL and pre-eclampsia with a pooled OR of 1.1 (95% CI 0.4-2.9, 3032 pooled women $p=0.5$). Case-control studies were heterogeneous, and therefore divided *post hoc* according to severity of pre-eclampsia. Cases diagnosed with severe pre-eclampsia were homogeneous with a combined odds ratio of 3.0 (95%CI 2.0-4.7).

Studies assessing the risk of FGR (defined as a birth weight < 10th centile) were heterogeneous ($P=0.01$) and the three articles not excluding known causes of FGR were dropped from the analysis. The combined OR for the remaining five studies were homogeneous ($P=0.3$) with a combined OR of 4.7 (95%CI 2.3-9.5). Subsequent to the publication of this meta-analysis, we had feedback and discussion with Professor Claire Infante-Rivard, the content of which is summarised in 'Ammendum to: The association between adverse pregnancy outcomes and maternal factor V Leiden genotype. A meta-analysis', which is included as Publication III. The fact that the three excluded case-control studies, which used a relatively unselected population with a total of 2116 participants, were homogeneous with a pooled OR of 1.07 (95%CI 0.67-1.75), highlighted the need for further research in this area. Another possible source of heterogeneity, which was not explored in this meta-analysis, is the proportion of cases with severe early onset intrauterine growth restriction.

Overall, the results of the meta-analysis highlighted a trend towards a greater association with fVL as the severity and number of previous adverse pregnancy outcomes increased.

Chapter Two progresses to a critical review of subsequently published meta-analyses (up to January 2007) evaluating possible associations between maternal fVL and adverse pregnancy outcomes. During this process, a similar association between maternal prothrombin gene variant G20210A (PGV) and adverse pregnancy outcomes became evident. In light of this, it was apparent that including PGV in this review was important.

The same literature was also reviewed with respect to possible associations between: 1) fetal fVL and adverse pregnancy outcomes; and 2) fetal PGV and adverse pregnancy outcomes.

Chapter Two concludes with the study hypotheses to be tested.

2.2 Maternal factor V Leiden, maternal prothrombin gene variant G20210A and adverse pregnancy outcome

2.2.1 Definition of a meta-analysis

Meta-analysis can be defined as ‘the statistical analysis of a large collection of results from individual studies for the purpose of integrating the findings’ (1). It is essential to undertake a meta-analysis using a systematic approach and explicitly report the methods of meta-analysis so the reader can accurately assess the validity of the combined results (2).

2.2.2 Maternal fVL Meta-analysis: Publication 1 and Publication II

Blood Coagulation, Fibrinolysis and Cellular Haemostasis

The association between adverse pregnancy outcomes and maternal factor V Leiden genotype: a meta-analysis

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Summary

The conclusions of studies to date which evaluate a possible association between factor V Leiden and adverse pregnancy outcome have been conflicting. This study was undertaken to further investigate this association. Our objective was to evaluate the association between adverse pregnancy outcomes and maternal factor V Leiden genotype by meta-analysis. Inclusion criteria were: (a) cohort or case control design; (b) outcomes clearly defined as one of the following: first or second/ third trimester miscarriage or intrauterine death, preeclampsia, fetal growth retardation, or placental abruption; (c) both the case and control mothers tested for the factor V Leiden mutation; (d) sufficient data for calculation of an odds ratio. Both fixed and random effect models were used to pool results and heterogeneity and publication bias were checked. For first trimester fetal loss, the pooled odds ratio was heterogeneous ($p=0.06$) and no dose-response curve could be found. For sec-

ond/third trimester fetal loss, there was a consistent and graded increase in risk: the odds ratio was 2.4 (95% CI 1.1-5.2) for isolated (non-recurrent) third trimester fetal loss, rising to 10.7 (95% CI 4.0-28.5) for those with 2 or more second/third trimester fetal losses. Factor V Leiden is associated with a 2.9 fold (95% CI 2.0-4.3) increased risk of severe preeclampsia, and a 4.8 fold (95% CI 2.4-9.4) increased risk of fetal growth retardation. These results support factor V Leiden testing for women with recurrent fetal loss in the second/third trimester. Women with only 1 event may also warrant testing if the fetal loss occurred in the third trimester. Conversely, in those women known to have the factor V Leiden mutation, monitoring for adverse pregnancy outcomes is warranted; whether this means increased vigilance or anti-coagulant prophylaxis is still contentious.

Keywords

Factor V Leiden, fetal death, intrauterine growth retardation, preeclampsia, pregnancy, meta-analysis

Thromb Haemost 2004; 91: 700-11

Introduction

Approximately 1%-5% of pregnant women have a serious pregnancy outcome such as preeclampsia, abruptio placentae, intrauterine death, or severe fetal growth retardation (1). Recurrent fetal loss is also a significant public health problem with two or more losses affecting up to 5% of women (2). Known etiologies of recurrent fetal loss include chromosomal abnormalities, ana-

tomic alterations of the uterus, autoimmune, and endocrinological abnormalities. However, a significant fraction of poor pregnancy outcomes remain unexplained by these factors and much research has focused on identifying further risk factors. Given that a successful pregnancy outcome is highly dependent on the establishment and maintenance of an adequate placental circulation (3), it is possible that abnormalities of placental vasculature, leading to inadequate fetomaternal circulation, may be

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responsible for at least some poor pregnancy outcomes. This has led to an interest in the thrombophilias as risk factors for fetal loss. The factor V Leiden mutation is the most common form of inherited thrombophilia (4). A point mutation in the factor V gene at nucleotide position 1691, resulting in an arginine to glutamine substitution, reduces the sensitivity of the factor V protein to inactivation by activated protein C (activated protein C resistance) resulting in a pro-coagulant state and an increased risk of thrombosis (5). The trait is inherited in an autosomal dominant manner with the risk of thrombosis increased seven times in heterozygotes and 80 times in homozygotes (6). Studies have shown that the distribution of the factor V Leiden mutation varies in different populations, being present in about 5% of Caucasian individuals (Europeans, Jews, Arabs and Indians) and virtually absent in Africans and Asians (7). A number of studies have shown an association between factor V Leiden and risk of recurrent fetal loss, intrauterine fetal death, preeclampsia and intrauterine growth retardation; however other studies have not shown a clear association (8-13). Possible sources of heterogeneity include different sources for recruitment of cases and controls and failure to exclude fetuses with a known cause of intrauterine death in the case group. We undertook a systematic review and meta-analysis of the literature to address the question of whether the maternal factor V Leiden genotype is associated with an increased risk of adverse pregnancy outcome.

Methods

Literature search and study selection

We performed a MEDLINE and EMBASE search (up to January 2003) using the headings: factor V Leiden (textword), and pregnancy, OR spontaneous abortion, OR fetal death OR miscarriage OR stillbirth OR preeclampsia OR fetal growth retardation (MeSH headings) OR placental abruption (textword). The search was limited to human studies published in English. The references of the identified articles were also reviewed. Inclusion criteria were: cohort or case control design; outcomes clearly defined as one of the following: first, combined second/ third trimester miscarriage or intrauterine death, pre-eclampsia, intrauterine fetal growth retardation, or placental abruption; both the case and control mothers tested for the factor V Leiden mutation; sufficient data to enable the calculation of an odds ratio.

Data extraction and analysis

Data were extracted independently by the authors on (a) the general characteristics of the study (title, author, place), (b) research question, methodology (study design, recruitment and characteristics of cases and controls, outcome definition), (c) potential confounders (whether or not other causes of fetal death had been excluded) and (d) outcome data. We analysed mater-

nal factor V Leiden results in association with 5 separate outcomes: a) first trimester fetal loss b) second or third trimester fetal loss c) preeclampsia d) intrauterine growth retardation e) placental abruption. Testing for heterogeneity was performed using the Breslow-Day method; p value threshold for heterogeneity was set at $p < 0.1$. When data were heterogeneous, subgroup analysis was used to explore the reasons. When data were homogeneous, they were pooled using a fixed effects model (Mantel-Haenszel χ^2 statistic) as well as a random effects model (DerSimonian-Laird). Since fixed effects models did not substantially change the interpretation of results, we report only the random effects model. 95% confidence intervals were calculated by the Robins, Breslow, Greenland method. Publication bias was checked using the Egger test. All calculations and Forest plots were performed using Stats Direct software (v2.2.3, 16/10/02, <http://www.statsdirect.com/>). The odds ratios we derived were for both heterozygous and homozygous factor V Leiden individuals pooled together.

Results

Figure 1 summarises the process of identifying and choosing studies. We identified 76 case-control or cohort studies relating to maternal factor V Leiden and adverse pregnancy outcome. Based on the above validity criteria, 54 were eligible for inclusion in the meta-analysis. The associations between maternal factor V Leiden and first trimester fetal loss, combined second/third trimester fetal loss, preeclampsia, fetal growth retardation, and placental abruption were evaluated in 15, 18, 26, 8 and 5 studies respectively. Twenty-two studies failed to meet the inclusion criteria because they 1) did not test both cases and controls for factor V Leiden (14, 15, 76); 2) did not break down fetal loss into different trimesters (12, 16-22, 77, 78, 82); and 3) data on number of women with an adverse outcome were unable to be extracted (25, 31, 43, 59-60, 79-81).

First trimester fetal loss

The study characteristics and odds ratios for this group of studies are summarised in Tables 1 and 2. In the one cohort study (28), reflecting a mostly unselected population of factor V Leiden positive women, the presence of factor V Leiden did not significantly increase the risk of first trimester loss, with an odds ratio of 1.1 (95%CI 0.5-2.6, 589 women in cohort). Pooling all case-control studies yielded an overall odds ratio of 1.8 (95%CI 1.2-2.7). Although this was statistically homogeneous ($p=0.11$), we felt that there was clinical heterogeneity, since factor V Leiden is rare in Orientals, and neither of the 2 Oriental studies identified any positive subjects. Removing these 2 studies (8,74) yielded an identical pooled odds ratio and confidence interval, but this was heterogeneous ($p=0.06$). In addition, we were unable to determine a dose-response curve in *post hoc* analyses. When cases had two or more first trimester losses (i.e.

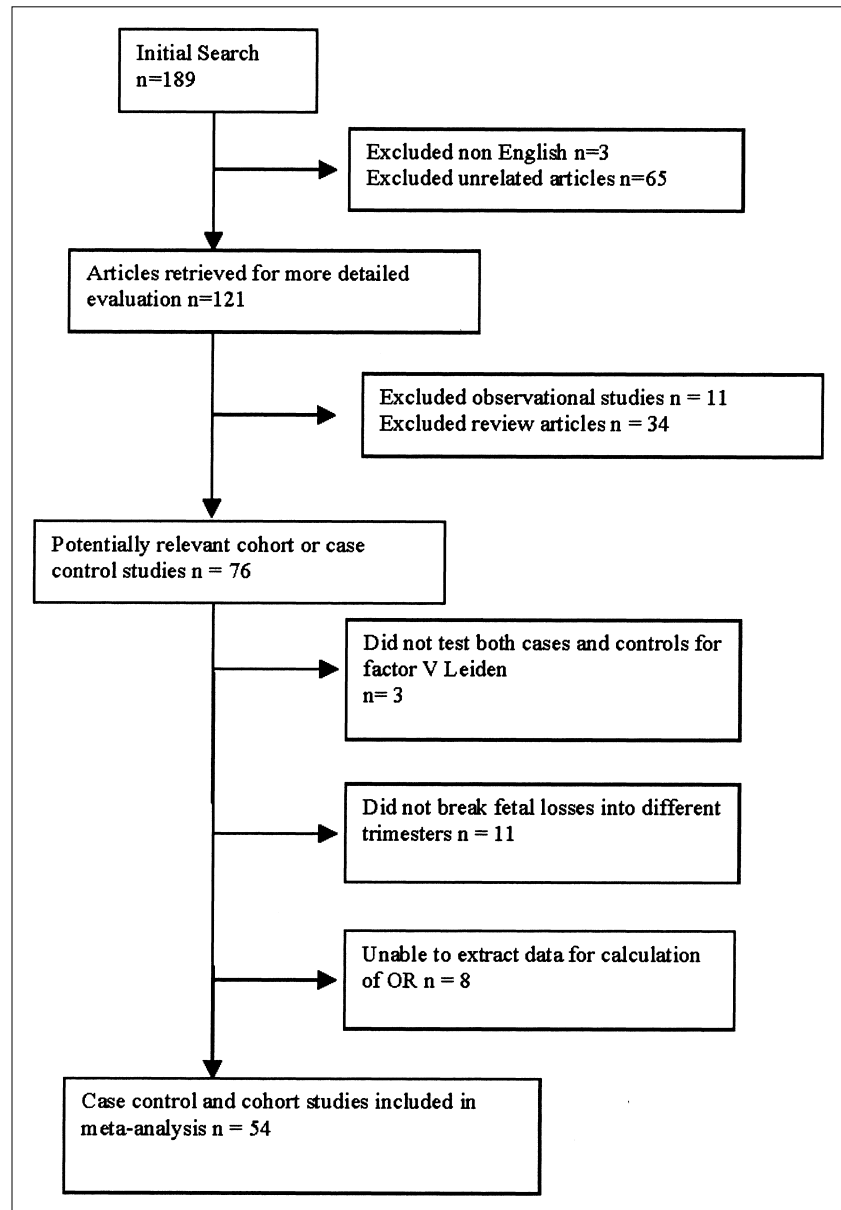


Figure 1: Stages of selection.

a history of one or more previous first trimester losses apart from the index event) (23, 24, 26, 29, 30, 53, 66) the odds ratio was 2.6 (95%CI 1.7-3.8) with no heterogeneity ($p=0.7$, 1415 pooled cases and controls, Fig. 2). When cases had three or more first trimester losses (i.e. a history of two or more previous first trimester losses apart from the index event) (9, 10, 27, 52, 73), the combined odds ratio was 0.9 (95%CI 0.5-1.6) with no heterogeneity ($p=0.3$, 1482 pooled cases and controls). There was no evidence of publication bias for any of these results (Egger test $p>0.4$).

Second/third trimester fetal loss

The study characteristics and odds ratios for this group of studies are summarised in Tables 3 and 4. The pooled odds ratio was

3.6 (95% CI 2.2-5.8). These studies were heterogeneous ($p=0.023$). Therefore, they were divided, *post hoc*, into groups based on study design and number of previous fetal losses. In the cohort studies, reflecting a mostly unselected population of factor V Leiden positive women (28, 34, 35) the overall odds ratio for combined second/third trimester fetal loss was 1.2 (95% CI 0.6-2.5) with no heterogeneity ($p=0.9$, 3418 pooled women). In the case control studies, when focusing on isolated third trimester fetal loss (11, 32, 33, 68, 72, 75) the odds ratio associated with factor V Leiden rose to 2.8 (95% CI 1.3-6.2); there was no heterogeneity ($p=0.4$, 1107 pooled women, Fig. 3). When cases had 2 or more fetal losses, (i.e. history of 1 or more previous losses) one of which was a second/third trimester fetal loss (26, 30, 69) the odds ratio was 3.9 (95% CI 1.9-8.2), with

Table 1: First trimester fetal loss: study characteristics.

Study	Population	Ethnicity	Study design	Control group	Consecutive recruitment	Exclusion of causes of fetal loss
(10) Dizon-Townson 1997	3 or more miscarriages	Caucasian (USA)	case-control	7 or more live births Caucasian	-	yes
(23) Grandone 1997	2 or more unexplained fetal losses	Caucasian (Italy)	case-control	parous women Caucasian	-	yes
(24) Balasch 1997	2 or more first trimester fetal losses	Spanish	case-control	at least one successful pregnancy	yes	yes
(8) Hashimoto 1999	3 or more first trimester miscarriages	Japanese	case-control	matched parous	-	yes
(9) Kuttahl 1999	3 or more first trimester losses	Caucasian (USA)	case-control	at least one normal pregnancy	yes	yes
(26) Pauer 1998	2 or more unexplained first trimester losses	Caucasian (Germany)	case-control	pregnant women with no history of fetal loss	-	-
(27) Rai 2001	3 or more early miscarriages	Caucasian (UK)	case-control	no history of pregnancy complication	yes	yes
(29) Foka 2000	2 or more spontaneous miscarriages	Caucasian (Greece)	case-control	matched controls	yes	yes
(30) Younis 2000	2 or more unexplained first or second trimester losses	Israeli	case-control	at least one successful pregnancy	yes	yes
(52) Fatini 2000	3 or more first trimester fetal losses	Caucasian (Italy)	case-control	history of a normal pregnancy	yes	yes
(53) Reznikoff-Etievant 2001	two or more unexplained early miscarriages before 10 weeks	Caucasian (France)	case-control	healthy female controls	yes	yes
(28) Bare 2000	2 or more miscarriages	Caucasian (Hungary)	cohort	control women	-	-
(66) Finanl 2002	2 or more first trimester losses	Lebanese	Case-control	uncomplicated pregnancy	-	no
(73) Carp 2002	3 or more first trimester pregnancy losses	Israel	Case-control	no history of miscarriage	-	yes
(74) Yamada 2001	recurrent Abortion	Japan	Case-control	no history of miscarriage	-	yes

Table 2: First trimester fetal loss: summary and odds ratio.

Case-control studies	+/- fVL cases	+/+ fVL cases	Total fVL positive cases	+/- fVL controls	+/+ fVL controls	Total fVL positive controls	Odds ratio
(10) Dizon-Towson 1997	0	0	0/22	0	0	0/25	-
(23) Gradone 1997	2	0	2/27	5	0	5/118	1.8
(24) Balasch 1997	1	0	1/55	1	0	1/50	0.9
(8) Hashimoto 1999	0	0	0/52	0	0	0/55	-
(9) Kuttahl 1999	1	0	1/50	2	0	2/50	0.6
(26) Pauer 1998	5	1	6/64	8	0	8/87	1.0
(27) Rai 2001	58	1	59/904	12	0	12/150	0.8
(29) Foka 2000	9	0	9/61	4	0	4/100	4.1
(30) Younis 2000	-	-	6/37	8	0	8/139	3.1
(52) Fatini 2000	-	-	6/59	-	-	2/70	3.8
(53) Reznikoff-Etievant 2001	26	1	27/260	11	0	11/240	2.4
(66) Finan 2002	38	7	45/110	11	0	11/67	3.5
(73) Carp 2002	-	-	2/70	-	-	5/82	0.4
(74) Yamada 2001			0/35			0/109	-
Cohort study			Total outcomes in fVL positive group			Total outcomes in fVL negative group	
(28) Bare et al 2000			8/128			26/461	1.1
+/- = heterozygote for fVL mutation +/+ = homozygote for fVL mutation							

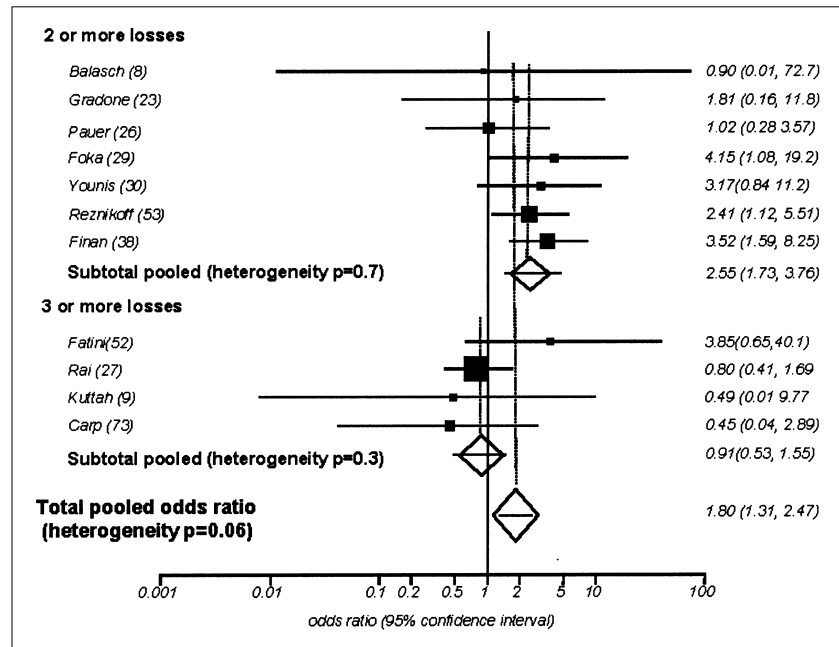


Figure 2: Pooled odds ratios for first trimester fetal loss subdivided by total number of fetal losses (index event plus previous history).

Table 3: Second or third trimester fetal loss: study characteristics.

Study	Population	Ethnicity	Design	Control	Consecutive recruitment	Exclusion of known causes of fetal loss
(10) Dizon-Townson 1997	3 or more miscarriages with at least one second trimester	Caucasian (USA)	case-control	7 or more live births	-	yes
(23) Grandone 1997	2 or more unexplained fetal losses in the second/third trimester	Caucasian (Italy)	case-control	parous controls	-	yes
(26) Pauer 1998	2 or more unexplained fetal losses including at least one second trimester loss	Caucasian (Germany)	case-control	healthy women with no history of pregnancy loss	-	-
(32) Gris 1999	at least one fetal loss after 22 weeks	Caucasian (France)	case-control	matched controls	yes	-
(33) Kupferminc 1999	history of stillbirth	Israeli	case-control	age matched, normal pregnancy.	yes	yes
(36) Mello 1999	fetal loss in the second or third trimester	Caucasian (Italy)	case-control	normal pregnancy	yes	-
(37) Rai 2001	late miscarriage >12 weeks	Caucasian (UK)	case-control	no history of miscarriage	yes	yes
(29) Fokal 2000	2 or more second trimester miscarriage	Caucasian (Greek)	case-control	matched controls	yes	yes
(1) Martinelli 2000	first unexplained late fetal loss (>20 weeks)	Caucasian (Italy)	case-control	women with at least one healthy baby and no history of fetal loss	yes	yes
(30) Younis 2000	2 or more unexplained first or second trimester losses	Israeli	case-control	at least one successful pregnancy & no abortions	yes	yes
(11) Alfirevic 2001	unexplained stillbirth after 23 weeks	Caucasian (UK)	case-control	uncomplicated pregnancies	yes	-
(68) Alonso 2002	at least one fetal loss > 23 weeks	Spanish	case-control	at least one successful pregnancy	yes	yes
(69) Grandone 2002	at least one previous IUFD	Caucasian (Italy)	case-control	healthy parous women	-	yes
(72) Agorastos 2002	fetal loss >24 weeks	Caucasian (Greece)	case-control	healthy parous women	yes	yes
(75) Many 2002	third trimester fetal loss	Israel	case-control	healthy parous women	yes	yes
(35) Lindqvist 1999	fVL positive pregnant women within a population cohort	Caucasian (Sweden)	population based cohort	APCR negative pregnant women within a cohort	-	-
(28) Bare 2000	Intrauterine fetal death	Caucasian (Hungary)	cohort	control women	-	-

+/- = heterozygote for fVL mutation +/- = homozygote for fVL mutation

no heterogeneity ($p=0.4$, 479 pooled women, Fig. 3). When cases had 2 or more second/third trimester fetal losses (23, 29) (i.e. history of one or more previous second/third trimester losses) the odds ratio was 10.7 (95%CI 4.0-28.5) with no heterogeneity ($p=0.9$, 253 pooled women). There was no evidence of publication bias for any of these results ($p>0.4$).

Preeclampsia

The study characteristics and odds ratios for this group are summarised in Tables 5 and 6. The studies were heterogeneous ($P<0.1$) and were divided according to study design. In the cohort studies, reflecting an unselected group of factor V Leiden women, the odds ratio was 1.1 (95% CI 0.4-2.9, 3032 pooled

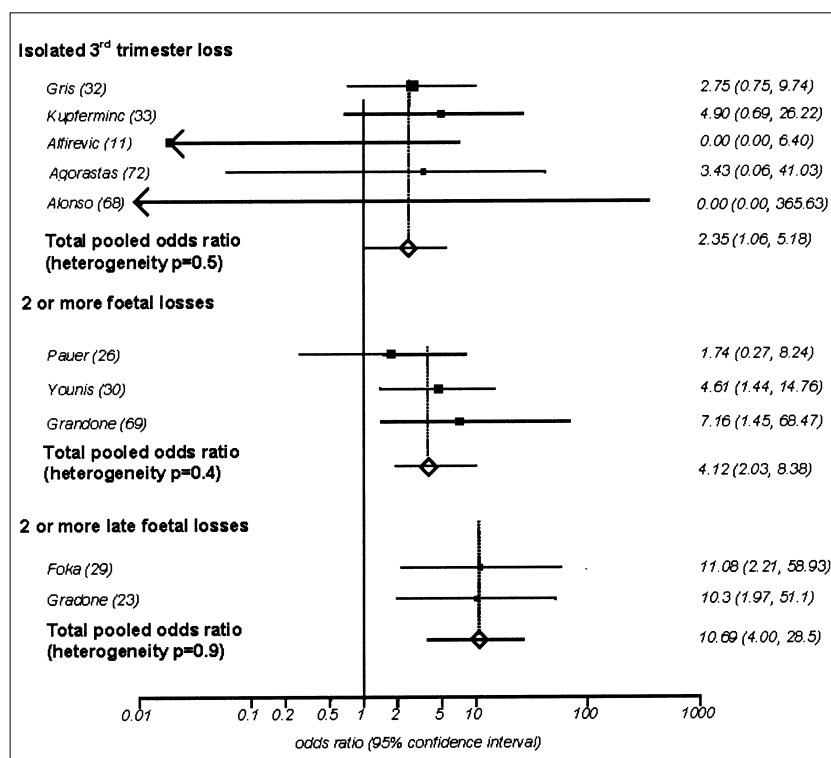


Figure 3: Pooled odds ratios for second/third trimester fetal loss subdivided by severity of fetal losses (index event plus previous history). The risk associated with factor V Leiden appears to increase with increasing number and lateness of previous fetal losses.

Table 4: Second or third trimester fetal loss: summary and odds ratio.

Case-control studies	+/- fVL cases	+/- fVL cases	Total fVL + cases	+/- fVL controls	+/- fVL controls	Total fVL + controls	Odds ratio
(10) Dizon-Townson 1997	-	-	0/18	-	-	0/25	-
(23) Gradone 1997	5	0	5/16	5	0	5/18	10.3
(26) Pauer 1998	3	0	3/20	8	0	8/87	1.7
(32) Gris 1999 1 fetal loss	6	0	6/148	7	0	7/464	2.7
>1 fetal loss	9	0	9/84				7.8
(33) Kuperminic 1999	-	-	3/12	7	0	7/110	4.9
(36) Mello 1999	8	0	8/34	3	0	3/80	7.9
(27) Rai 2001	14	1	15/207	12	0	12/150	0.9
(29) Foka 2000	0	0	6/19	0	0	4/100	11.0
(3) Martinelli 2000	5	0	5/67	6	0	6/232	3.0
(30) Younis 2000	-	-	9/41	8	0	8/139	4.6
(11) Alfirevic 2001	-	-	0/18	-	-	3/44	0.3
(68) Alonso 2002	-	-	0/8	-	-	1/75	2.9
(69) Grandone 2002	10	-	10/84	2	-	2/108	7.2
(72) Agorastos 2002	1	0	1/8	4	0	4/100	3.4
(75) Many 2002	3	0	3/40	3	0	3/80	1.5
Cohort studies			Total outcomes in fVL positive group			Total outcomes in fVL negative group	
(34) Meinardi 1999	-	-	13/228	-	-	6/121	1.2
(35) Lindqvist 1999	-	-	4/270	-	-	25/2210	1.3
(28) Bare 2000	-	-	1/128	-	-	2/461	1.8

women $p=0.5$) (12, 35). Case-control studies were heterogeneous ($P=0.002$) and therefore divided *post hoc* according to severity of preeclampsia. Cases diagnosed on the basis of pro-

teinuria $\geq 5\text{gm}$ in 24 hours and one or more of the features of severe preeclampsia (33, 37, 46, 47, 49, 51, 72) were homogeneous ($p=0.3$) with a combined odds ratio of 3.0 (95% CI

Table 5: Preeclampsia: study characteristics

Study	Population	Ethnicity	Design	Control	Consecutive recruitment
(37) Dizon-Townson 1996	severe preeclampsia *	Caucasian	case-control	normotensive gravid women	-
(38) Grandone 1997	pregnancy induced hypertension	Caucasian (Italy)	case-control	matched parous women	yes
(33) Kupferminc 1999	severe preeclampsia *	Israeli	case-control	1 or more normal pregnancies	yes
(39) Lindoff 1997	preeclampsia	Caucasian (Sweden)	case-control	healthy pregnant women	yes
(13) O'Shaughnessy 1999	preeclampsia	Caucasian (Northern Europe)	case-control	normotensive pregnant women	-
(40) Kobashi 1999	preeclampsia	Japanese	case-control	normotensive pregnant women	-
(41) Nagy 1999	severe preeclampsia	Caucasian	case-control	healthy pregnant and non-pregnant women	Yes
(42) De Groot 1999	preeclampsia	Caucasian (The Netherlands)	case-control	normotensive pregnant women	-
(44) Grandone 1999	gestational hypertension with or without proteinuria	Caucasian (Italy)	case-control	matched normotensive pregnant women	-
(36) Mello 1999	preeclampsia	Caucasian (Italy)	case-control	matched women with a normal pregnancy	Yes
(45) Rigo 2000	preeclampsia	Caucasian (Hungary)	case-control	healthy pregnant controls	Yes
(46) Van Pampus 2000	severe preeclampsia or HELLP *	Caucasian	case-control	healthy women with uncomplicated pregnancies	yes
(47) Kupferminc 2000	severe preeclampsia *	Israeli	case-control	at least 1 healthy pregnancy	yes
(48) Mimuro 2000	preeclampsia	83% Caucasian 17% Asian	case-control	healthy pregnant and non-pregnant women	-
(49) Von-Tempelhoff 2000	severe preeclampsia *	Caucasian (Germany)	case-control	women with a normal pregnancy	-
(50) Livingston 2001	severe preeclampsia	African American	case-control	normotensive pregnancy without a history of PE	yes
(51) Young Ju Kim 2001	severe preeclampsia *	Caucasian (USA)	case-control	at least two term pregnancies unaffected by PE	-
(11) Alfrevic 2001	severe PE/ preeclampsia	90% Caucasian (UK)	case-control	women with uncomplicated pregnancies	yes
(63) Currie 2002	severe pre-eclampsia	Caucasian (Australia)	case-control	normal pregnancy	-
(64) D'Elia 2002	preeclampsia	Caucasian (Italy)	case-control	normal pregnancy	-
(65) Morrison 2002	preeclampsia	Caucasian	nested case-control	-	-
(67) Paternoster 2002	preeclampsia	Caucasian (Italy)	Case-control	normal pregnancy	-
(70) Benedetto 2002	preeclampsia	Caucasian (Italy)	Case-control	normal pregnancy	yes
(72) Agoratos 2002	*Severe preeclampsia	Caucasian (Greece)	case-control	normal pregnancy	yes
(35) Lindqvist 1999	preeclampsia	Caucasian (Sweden)	cohort	-	yes
(12) Murphy 2000	preeclampsia	Caucasian (Ireland)	population based cohort	-	yes

*Severe preeclampsia was diagnosed on the presence of proteinuria of $\geq 5\text{gm}$ in 24 hours and one or more of the following signs and symptoms: a systolic blood pressure of $\geq 160\text{mm Hg}$ or a diastolic pressure of $\geq 110\text{ mm Hg}$, oliguria, elevated liver enzymes, platelet count $<100,000$, cerebral or visual disturbances, epigastric pain or pulmonary oedema.

2.0-4.7) (Fig. 4). There was no evidence of publication bias ($p=0.2$). The source of heterogeneity for the remaining preeclampsia case-control studies could not be ascertained.

Fetal growth retardation

Studies assessing the risk of fetal growth retardation were heterogeneous ($P=0.01$) and articles not excluding other causes of IUGR were dropped from the analysis. The combined odds ratio for studies where IUGR was defined as a value less than the 10th centile (33, 56, 58, 71, 72) were homogeneous $P=0.3$ with a combined odds ratio of 4.7 (95% CI 2.3-9.5) (Fig. 5). There was no publication bias ($p=0.3$).

Placental abruption

Cases with placental abruption, (11, 33, 57, 62, 72) were heterogeneous ($P=0.01$) with a combined odds ratio of 5.4 (95% CI 1.3-22.7) by the random effects model, with no publication bias

($p=0.4$). We were unable to determine the source of the heterogeneity.

Homozygosity

Since homozygous factor V Leiden individuals are so rare, their numbers were small even in this meta-analysis; the exclusion of cases homozygous for factor V Leiden did not alter the conclusions of the meta-analysis.

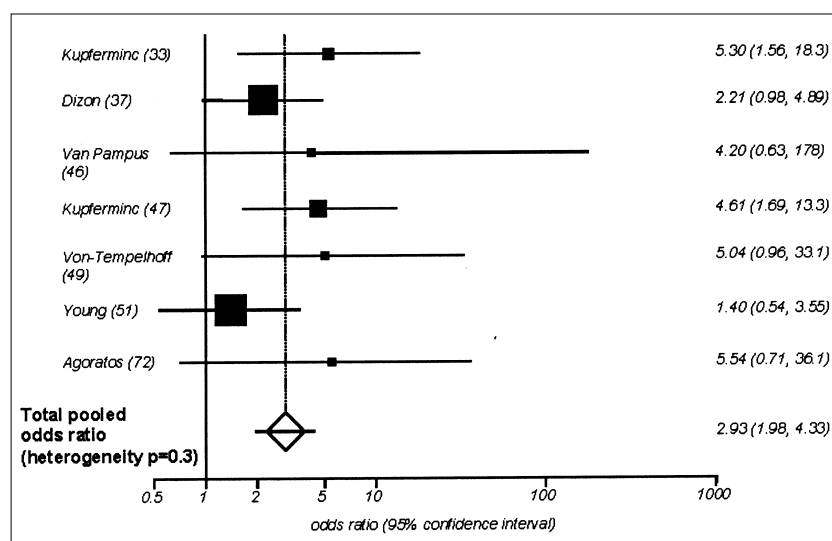
Discussion

Methodological issues

Our meta-analytic method is reasonably rigorous. We searched both EMBASE and MEDLINE and extracted data in duplicate. We were careful to check heterogeneity and only pooled homogenous studies. Nevertheless there are a number of caveats: We did not include abstracts or "grey" literature, i.e.

Table 6: Preeclampsia: summary and odds ratios.

Study	+/- fVL cases	+/-fVL cases	Total fVL cases	+/- fVL controls	+/- fVL controls	Total fVL controls	Odds ratio
(37) Dizon-Townson 1996	14	0	14/158	17	-	17/403	2.2
(38) Grandone 1997	9	1	10/95	3	0	3/128	4.9
(33) Kupferminc 1999	-	-	9/34	7	0	7/110	5.3
(39) Lindoff 1997	9	2	11/50	5	0	5/50	2.5
(13) O'Shaughnessy 1999	15	0	15/283	6	0	6/100	0.9
(40) Kobashi 1999	0	0	0/71	0	0	0/109	-
(41) Nagy 1999	13	0	13/69	8	0	8/129	3.5
(42) De Groot 1999	16	0	16/163	15	0	15/163	1.07
(44) Grandone 1999	-	-	4/70	4	0	4/215	3.2
(46) Van Pampus 1999	16	1	17/284	1	0	1/67	4.2
(36) Mello 1999	11	1	12/46	3	0	3/80	9.0
(45) Rigo 2000	22	0	22/120	3	0	3/101	7.3
(47) Kupferminc 2000	-	-	15/63	-	-	8/126	4.6
(48) Mimuro 2000	-	-	4/50	-	-	1/150	12.9
(49) Von-Tempelhoff 2000	5	1	6/29	2	1	3/61	5.0
(50) Livingston 2001	5	-	5/110	4	-	3/97	1.5
(51) Young Ju Kim 2001	11	-	11/169	12	-	12/253	1.4
(11) Alfirevic et al 2001	-	-	1/63	-	-	3/44	0.2
(63) Currie 2002	4	0	4/48	5	1	6/46	0.6
(64) D'Elia 2002	3	0	3/58	3	0	3/74	1.3
(65) Morrison 2002	17	0	17/394	8	0	8/163	
(67) Paternoster 2002	3	0	3/47	2	0	2/35	1.1
(70) Benedetto 2002	-	-	8/111	-	-	5/111	1.59
(72) Agoratos 2002	3	0	3/16	4	0	4/100	5.5
Cohort studies			Total outcomes in fVL positive group			Total outcomes in fVL negative group	
(35) Lindqvist 1999	-	-	5/270	-	-	34/2210	1.2
(12) Murphy 2000	0	0	0/12	13	0	13/540	1.6


Figure 4: Pooled odds ratio for severe pre-eclampsia.

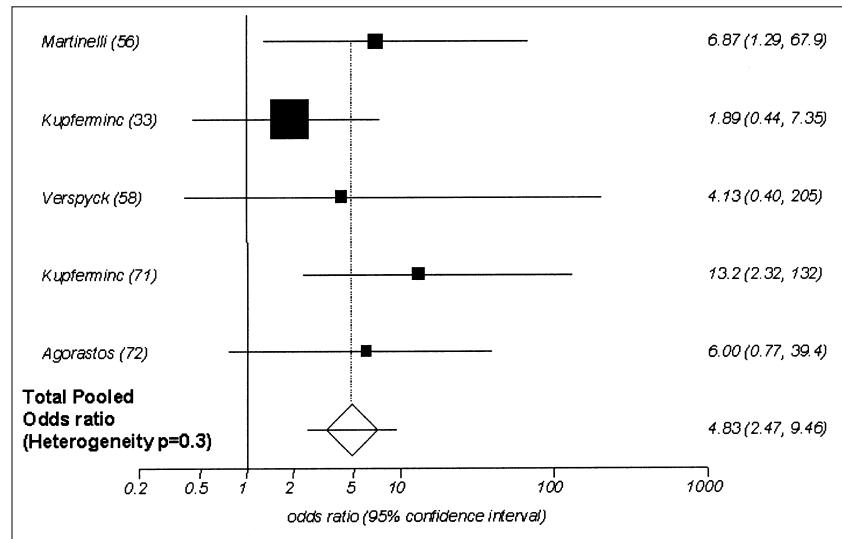


Figure 5: Pooled odds ratio for fetal growth retardation.

unpublished studies, or studies published in monograph form unlikely to be indexed. Previous work (83) suggests that this may overstate effect size by up to 30% presumably by excluding negative studies or those with small effect sizes. Although it is possible that the point estimates in this meta-analysis are slightly inflated, we do not believe that this is a major influence in that none of the results showed evidence of publication bias. Although tests for publication bias are regarded as somewhat weak, we raised the threshold to $p < 0.1$ to compensate for this. We excluded non-English studies. Current work indicates that this is unlikely to bias results (84, 85). We did not do hand searching. Previous work indicates that hand searching appears to pick up an additional 15% of studies missed by both databases; the quality of these studies however is no different and is unlikely to bias results (87).

Interpretation of results

Our results indicate that the risk of fetal loss associated with factor V Leiden depends on the history and timing of previous fetal losses. Although there initially appeared to be an overall 1.8 fold higher risk of a first trimester fetal loss, we view this estimate cautiously due to clinical heterogeneity and the lack of a dose response curve: those with a stronger history of previous first trimester losses actually had a lower risk associated with factor V Leiden. This made us doubt the validity of this relationship, and in combination with the negative results of the only cohort study, our interpretation is that there is likely to be no relationship, or at best a very weak one, between factor V Leiden and first trimester fetal loss. This differs from the only previous meta-analysis on the same topic. Rey, et al. (88) investigated various thrombophilias (factor V Leiden, prothrombin mutation, Protein C, S, and antithrombin deficiencies, and MTHFR) in relation to pregnancy loss. They used a timeframe

of less than 13 weeks (roughly equivalent to our first trimester cutoff) and greater than 19 weeks (roughly equal to our second/third trimester cutoff), and had similar inclusion criteria. They concluded that recurrent first trimester fetal loss was significantly associated with factor V Leiden with an odds ratio of 2.01 (1.13-3.58). We believe that this discrepancy is due to the fact that we identified an additional 6 studies that met the inclusion criteria, and explored the dose response curve, which led us to conclude that the statistical association with first trimester loss was not likely to be biologically significant. Rey et al. used MEDLINE and hand searching, whereas we also used EMBASE. Previous work indicates that up to one third of references may be missed by searching only one database (87), in this case impacting substantially on the conclusions.

Our results indicate a strong relationship between second/third trimester fetal loss and factor V Leiden, with the odds ratios increasing as the number of previous fetal losses increases, and as the timing of those previous losses gets later in pregnancy. We identified 7 extra studies not included by Rey, et al., and excluded 4 other studies that they included. In this instance however, the results were similar; we both found a strong relationship with increasing severity of previous losses.

If we assume that a previous history of adverse outcomes is a surrogate marker for other genetic or environmental risk factors, then there are 2 possible explanations for the graded increase in risk between factor V Leiden and fetal loss. The first possibility is that FVL might interact or potentiate the effect of other genetic or environmental factors and represents a true synergy. However, another interpretation is that multiple genetic/environmental factors each independently contribute to fetal loss, with no synergy. The effect of these other factors may be mistakenly attributed to FVL, simply because this is the only factor being measured, thereby inflating the apparent effect size

of FVL. The study design of a recent article by Rai, et al. (86) aimed to tease out the isolated contribution of factor V Leiden by comparing FVL+ women with recurrent fetal loss to FVL- women with the same history of fetal loss. The live birth rate was significantly lower among the women who carried the factor V Leiden allele confirming that factor V Leiden independently increases the risk of fetal loss.

Our results represent the first meta-analysis of other adverse pregnancy outcomes, i.e. severe preeclampsia, fetal growth retardation (<10th centile), and placental abruption. These are based on fewer studies but indicate a 3-5 fold increase in risk with factor V Leiden.

Clinical conclusions

The factor V Leiden mutation is present in 1 in 20 Caucasian individuals. Asymptomatic female carriers are often identified because of cascade family testing and seek counselling with respect to the risk associated with this mutation. Conversely, women with adverse pregnancy events present the opposite dilemma of whether to initiate genetic testing. We integrate our results and previous results to suggest some possible directions in both these scenarios.

Group 1: factor V Leiden positive women with no previous history of fetal loss

This meta-analysis shows no apparent increased risk of first trimester and combined second/ third trimester fetal loss, although there may be an increased risk of isolated third trimester loss, with an OR=2.4 (95% CI 1.1-5.2). This translates into an absolute risk of 1.2-2.4% compared to a population risk of 0.5-1%. One interpretation of this result is that although this may warrant increased surveillance, it may not be sufficiently high to initiate prophylaxis.

Group 2: factor V Leiden positive women with a previous history of fetal loss

When cases had at least one previous first trimester fetal loss, factor V Leiden was associated with a 2.6 fold (95%CI 1.7-3.8) increase risk of another first trimester fetal loss. Unfortunately, we were unable to clarify this group further as figures for women with just one previous first trimester loss are unavail-

able. When cases had at least one previous second/third trimester fetal loss, factor V Leiden was associated with an up to 10.7 fold (95%CI 4.0-28.5) increased risk of another late fetal loss. The absolute risks definitely warrant increased surveillance of the pregnancy and are likely sufficiently high to initiate prophylaxis, depending on other clinical factors.

In both group 1 and 2 above, the meta-analysis shows a 2.9 fold (95%CI 2.0-4.3) increased risk of severe preeclampsia (defined as proteinuria ≥ 5 gm in 24 hours and one or more of the features of severe preeclampsia), and a 4.8 fold (95%CI 2.4-9.4) increased risk of fetal growth retardation, and potentially a similar risk of placental abruption. Despite the fact that these case-control studies were possibly subject to ascertainment bias, it appears prudent to recommend at least increased vigilance of these pregnancies, especially in the third trimester.

Group 3: genetic testing in women with a first fetal loss

The results would indicate that women with an initial first or second trimester fetal loss do not require screening for the factor V Leiden mutation. However, consideration may be given to testing women with their first third trimester fetal loss.

Group 4: genetic testing in women with recurrent fetal loss

The results support the practice of screening women with a history of recurrent fetal loss. The relative and absolute magnitude of this risk may warrant prophylaxis, in a subsequent pregnancy, depending on other clinical factors. The risk of fetal loss increases with the trimester in which the previous losses occurred, going from 2.6 with "at least" two losses, to 4.1 when one of the two losses was a second/third trimester loss, to 10.7 with at least two second/third trimester losses. With respect to first trimester losses only, it remains unclear whether women should be tested after two or three miscarriages.

It is important to keep in mind that, based on previous evidence with respect to risk of DVT (6), women who are homozygous for factor V Leiden may be at even higher risk than we have estimated here.

We hope that these results will help guide clinicians in decision making around these issues.

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Addendum to: The association between adverse pregnancy outcomes and maternal factor V Leiden genotype. A meta-analysis

Dear Sir,

Subsequent to the publication of our meta-analysis regarding Factor V Leiden and poor pregnancy outcomes (1), we have had some feedback and discussions with Prof Claire Infante-Rivard, and believe that some clarifications and additional analyses are warranted for the outcome of intra-uterine growth retardation. As stated in the paper, we located 8 studies reporting this outcome, but these were heterogeneous. In trying to identify the source of heterogeneity, it appeared that 2 different populations had been recruited:

1. Five studies (2-6) used what appeared to be a relatively selected population, in that these excluded births with congenital abnormalities, chromosomal abnormalities, or infections such as CMV. The pooled odds ratio was reported in this group as 4.7 (95% CI 2.3-9.5), indicating an association with Factor V Leiden, with no heterogeneity and no publication bias. Although this selected population may correspond to what is considered routine screening for IUGR in a genetics or Obs/Gyn clinic, it is worth noting that details of this selection procedure and screening were some-

what scanty, e.g. How many eligible births occurred and how many of these agreed to participate? Was karyotyping performed on everyone, and which infections were screened for and how? These studies also represented a smaller number of participants, 695 in total.

2. Three studies (7-9) used a relatively unscreened population, i.e. all comers except for those with congenital abnormalities or premature births. In the paper, we did not pursue this group any further. However we report here, that the results from these 3 studies were also homogeneous ($p=0.8$ by Breslow-Day), with a pooled odds ratio of 1.07 (95% CI 0.67-1.75) by both fixed and random effects models. These studies include 2116 participants in all, and includes the most rigorous study from a methodological viewpoint (6).

These results raise 2 main possibilities: either Factor V Leiden is not related to IUGR and the 5 pooled studies reflect a degree of selection bias, or Factor V Leiden may be related to IUGR in a selected population where other common causes of IUGR have been already excluded. It is not possible from the results thus far to distinguish between these possibilities and we highlight the need for further research in this area.

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2.2.3 Evaluation of subsequently published meta-analyses on maternal fVL and maternal PGV

In this section, subsequently published meta-analyses (from 2003 until January 2007) exploring possible associations between: 1) maternal fVL and adverse pregnancy outcomes; and 2) PGV and adverse pregnancy outcomes are evaluated using the criteria described by Oxman et al (2), as outlined in Table 1.

Table 1 Meta-Analysis Assessment Criteria

1. Did the review address a focused clinical question?
2. Were the criteria used to select articles for inclusion appropriate?
3. Is it unlikely that important and relevant studies were missed?
4. Was the validity of the included studies appraised?
5. Were assessments of studies reproducible?
6. Were the results similar from study to study?
7. What are the overall results of the review?
8. How precise were the results?

Meta-analysis 1: Rey et al, 2003

1) **Did the review address a focused clinical question?** Rey et al aimed to qualify the magnitude of the association between individual inherited thrombophilias (fVL, MTHFR, PGV, protein C or S deficiency and antithrombin deficiency) and fetal loss.

2) **Were the criteria used to select articles for inclusion appropriate?** The criteria used for inclusion were appropriate. They included case-control, cohort (prospective and retrospective) and cross-sectional studies. The outcome of interest was recurrent (two or more) and non-recurrent fetal loss.

3) **Is it unlikely that important and relevant studies were missed?** The authors searched MEDLINE for articles published between January 1975 and May 2002 with appropriate text and subject headings. They reviewed reference lists of articles to identify any additional

relevant studies. The authors may have missed important articles published in another language; but overall, they attempted to identify all the relevant studies.

4) **Was the validity of the included studies appraised?** Quality scores were assigned to retrieved studies, and studies were graded as weak, moderate or strong according to the assessment grid for observational studies. Criteria used to judge study quality included an adequate description of baseline characteristics of the study population and the control group; description of exposure and outcome; and enough information to allow extraction of data. Only studies rated as moderate or strong were included. Whether or not cases were consecutively recruited was not mentioned.

5) **Were assessments of studies reproducible?** Two authors independently reviewed and rated the retrieved studies and a third reviewer acted as an arbitrator. The agreement between the reviewers was almost 100%.

6 & 7) **Were the results similar from study to study? What are the overall results of the review?** Pooled data on recurrent fetal loss associated with fVL from 18 studies indicated significant heterogeneity ($p=0.018$). However, when recurrent fetal loss before 13 weeks was pooled, the combined odds ratio (OR) was 2.01 (1.13- 3.56) with no statistical heterogeneity ($p= 0.11$). The studies of non-recurrent fetal loss were also heterogeneous ($p=0.09$), but sensitivity analysis indicated a more robust association if non-recurrent fetal loss occurred after 19 weeks gestation, with a combined OR of 3.26 (1.82-5.83); heterogeneity ($p= 0.6$). The pooled data on recurrent fetal loss associated with the PGV from nine case-control studies were homogeneous ($p=0.67$) with a combined OR of 2.05 (1.18-3.54) ($p=0.01$).

8) **How precise were the results?** The findings indicate that maternal fVL is associated with first-trimester recurrent fetal loss and late (after 19 weeks) non-recurrent fetal loss. These estimates appear to be robust.

Meta-analysis: 2 Kosmas et al, 2003 (3)

1) **Did the review address a focused clinical question?** The aim was to evaluate a possible association between the maternal fVL genotype and the risk of hypertension in pregnancy.

2) **Were the criteria used to select articles for inclusion appropriate?** All studies evaluating an association between maternal fVL and hypertension in pregnancy were included regardless of their study design. Inclusion criteria included fVL genotyping on all cases and

controls. Studies with fewer than five hypertensive subjects were excluded. Twenty three potentially eligible reports were identified. Seven studies that did not specify whether or not the hypertension occurred before or during the pregnancy were excluded. Eleven of the 23 included studies reported consecutive recruitment. To prevent duplication of data, they excluded three reports where the same data was used in more than one study. The definition of hypertension was not consistent across all included studies.

3) Is it unlikely that important and relevant studies were missed? It was unlikely the authors missed relevant studies because they searched both MEDLINE and Embase (up to 2002) and used appropriate keywords: 'pre-eclampsia OR', 'eclampsia OR' 'HELLP syndrome OR' 'pregnancy induced hypertension OR' 'gestational hypertension' AND factor V Leiden'. The authors also reviewed the bibliographies of retrieved articles. The literature review was not limited to English language papers.

4) Was the validity of the included studies appraised? Although clear inclusion and exclusion criteria were defined, there was no evidence that the studies were evaluated separately by two independent reviewers, increasing the risk of selection bias.

5) Were assessments of studies reproducible? There was significant heterogeneity when the cases were combined; and as a result, the authors reported random effects ORs. Despite subgroup analysis according to race and definition of pre-eclampsia, heterogeneity remained. The authors note the positive results of earlier studies are incompatible with the negative results of the latter studies with no overlapping of the 95% CI. Although the results of the eight studies reporting data on fVL homozygosity verses wildtype were homogeneous, there were only eight women included in this subgroup meta-analysis.

6 & 7) Were the results similar from study to study? What are the overall results of the review? The results were presented as a random effect OR with 95% CI. In this meta-analysis of 2742 hypertensive women and 2403 controls, maternal fVL increased the odds of hypertensive disease by 2.25-fold (95% CI 1.50-3.38). For women defined to have pre-eclampsia, the OR was 2.52 (95%CI 1.64-3.88).

8) How precise were the results? Although the pooled OR suggests that heterozygosity for maternal fVL is associated with an increased risk of hypertension in pregnancy, the heterogeneity between the studies suggests possible bias. Publication or time-lag bias may

account for the fact that earlier studies were more likely to be positive, while the subsequent and larger studies tended to show no association.

Meta-analysis 3: Kovalevsky et al, 2004

1) **Did the review address a focused clinical question?** The aim of the meta-analysis was to evaluate the relationship between recurrent pregnancy loss (RPL) and the common thrombophilias maternal fVL and maternal PGV.

2) **Were the criteria used to select articles for inclusion appropriate?** The inclusion criteria required that RPL be defined as two or more losses in the first two trimesters and that fVL or PGV was identified. Third trimester fetal losses were not included. Sixteen case-control studies were included while cohort studies were excluded due to design differences.

3) **Is it unlikely that important and relevant studies were missed?** The authors searched English language MEDLINE (1966-2002) using appropriate terms. Pertinent studies were identified from article bibliographies. Articles published in another language may have been missed.

4) **Was the validity of the included studies appraised?** The authors do not mention whether or not the validity of the included studies was appraised.

5) **Were assessments of studies reproducible?** Two authors performed the searches and extracted the information independently. Although the agreement between the authors is not specified, all differences were resolved by consensus.

6 and 7). **Were the results similar from study to study? What are the overall results of the review?** Pooled data on recurrent fetal loss (two or more fetal losses in trimester one or two) with fVL in 16 case-control studies produced a combined OR of 2.0 (1.5-2.7; $p < .001$); however, there was significant heterogeneity ($p = 0.03$). The authors note the exclusion of the Rai et al study eliminated significant between-study heterogeneity; however, it does not appear to be reasonable to exclude one of the largest case-control studies. The pooled data on recurrent fetal loss with PGV produced a combined OR of 2 (1.0-4.0; $p = 0.03$) with no significant between-study heterogeneity ($p = 0.51$).

8) **How precise were the results?** The findings indicate both maternal fVL and maternal PGV double the risk of experiencing two or more miscarriages within the first and second trimester. A large negative study by Rai was the cause of significant study heterogeneity in

the fVL-positive women, which casts some doubt on the positive association within this group.

Meta-analysis: 4 Howley et al, 2005 (4)

1) **Did the review address a focused clinical question?** The authors' objective was to conduct a systematic review of the literature for studies that examined the association between: 1) maternal fVL and FGR; and 2) maternal PGV and FGR and perform a meta-analysis of case-control and cohort studies to determine the pooled estimate of the OR and 95% CI.

2) **Were the criteria used to select articles for inclusion appropriate?** The authors included studies with a case-control or cohort design where the exposure of interest was maternal fVL or maternal PGV, and the outcome was FGR defined on birth weight <10th centile; however, some studies defined FGR as <3rd centile or <5th centile. All except one of the case-control studies excluded known causes of FGR.

3) **Is it unlikely that important and relevant studies were missed?** Relevant studies were unlikely to be missed because the authors searched MEDLINE and Embase up to June 2003. They used medical subject headings and text words with no restriction on language.

4) **Was the validity of the included studies appraised?** The validity of the studies was appraised. Thirty nine manuscripts (21 case-control and 18 cohorts) examined the association between maternal fVL/PGV and FGR. Eleven eligible case-control and five eligible cohort studies were included.

5) **Were assessments of studies reproducible?** Two reviewers independently performed data extraction using standardised data collection forms. Quality assessment was also performed using the Newcastle Ottawa Scale. Whether or not cases and controls were consecutively recruited was not noted.

6) **Were the results similar from study to study?** Statistical heterogeneity was assessed by the Q statistic (heterogeneity chi-squared test). The authors did not provide the p value for the test of heterogeneity; but state that the random-effects model was used to calculate the summary odds ratio of combined case-control studies because of clinical and statistically significant heterogeneity. They conducted post-hoc analyses within subgroups based on

birth weight cuts-offs; however, they do not comment on the results of tests of statistical heterogeneity within these subgroup analyses. With respect to the combination of cohort studies, they mention clinical heterogeneity. There is no mention of the heterogeneity chi-squared test for the meta-analysis of the cohort studies.

7) What are the overall results of the review? The pooled OR for the association between maternal fVL and FGR <10th centile was 1.97 (95% CI 0.84-4.62); and 1.97 (95% CI 0.72-5.40) for maternal PGV and FGR <10th centile. The summary OR was ≈2 fold higher (fVL, 4.7 vs 2.0; PGV, 4.3 vs 2.0) among studies that used a ≤ 5th cut off percentile compared with <10th centile. They note that the case-control studies suggest a possible association between FGR; however, the meta-analysis revealed statistically and clinically significant heterogeneity. Overall, the cohort data does not support an association, but the authors comment on significant methodological limitations such as inadequate power and absence of controls for known confounders.

8) How precise were the results? Although the pooled ORs of case-control studies suggest that heterozygosity for maternal fVL or maternal PGV is associated with an increased risk of FGR, heterogeneity between the studies suggests bias. Conversely, meta-analysis of the cohort studies does not support an association (RR 0.99 range 0.5-1.9), but these results should be interpreted with caution due to heterogeneity between studies and methodological limitations. The included cohort studies did not measure maternal PGV.

Meta-analysis 5: Lin et al, 2005 (5)

1) Did the review address a focused clinical question? Lin et al aimed to quantify the magnitude of the association between two outcomes pre-eclampsia and severe pre-eclampsia - with three genetic forms of maternal thrombophilia - fVL, methylenetetrahydrofolate reductase (MTHFR) and PGV.

2) Were the criteria used to select articles for inclusion appropriate? The authors included studies with a case-control design but excluded the only two prospective cohort studies because they measured relative risk rather than ORs. The exclusion of cohort studies may have overestimated the reported association.

3) **Is it unlikely that important and relevant studies were missed?** The authors' search method was unlikely to miss relevant studies because they searched MEDLINE from 1966 to 2002 and Embase from 1980 to 2002. Appropriate keywords were used and the search was not limited to English language papers.

4) **Was the validity of the included studies appraised?** Of the 349 distinct titles, two separate authors reviewed 47 articles in detail for inclusion and exclusion criteria. The authors included case-control studies evaluating the association between one of three forms of maternal thrombophilia (fVL, MTHFR and PGV) and pre-eclampsia. Cases had to meet the criteria for pre-eclampsia, but it was not specified if cases and controls were consecutively recruited.

5) **Were assessments of studies reproducible?** The inclusion and exclusion criteria and data extraction was done separately by two authors to ensure an accurate evaluation of each of the studies.

6) **Were the results similar from study to study?** When the authors combined all the case-control studies exploring an association between fVL and pre-eclampsia, the studies were heterogeneous ($p=0.04$), and the funnel plot was asymmetrical, suggesting possible publication bias due to fewer smaller studies with negative results. Case-control studies exploring an association between severe pre-eclampsia and maternal fVL were also heterogeneous ($p=0.009$). Therefore, it may not have been appropriate to pool these studies. The pooled studies exploring a possible association between pre-eclampsia and severe pre-eclampsia with the PGV were homogeneous with p values of 0.57 and 0.55 respectively; making it appropriate for them to be pooled.

7) **What are the overall results of the review?** The pooled OR for the association between maternal fVL and all pre-eclampsia and severe pre-eclampsia were 1.81 (95% CI 1.14-2.87) and 2.24 (95%CI 1.28-3.94) respectively. The pooled OR for the association between PGV and all pre-eclampsia and severe pre-eclampsia were 1.37 (95% CI 0.72-2.57) and 1.98 (0.94-4.17) respectively.

8) **How precise were the results?** The pooled ORs suggest that heterozygosity for maternal fVL is associated with an increased risk of pre-eclampsia. However, heterogeneity between the studies casts doubt as to whether the ORs are a reasonable estimate of the true

population OR. The homogeneous pooled articles exploring a possible association between PGV and pre-eclampsia did not show a statistical association.

Meta-analysis 6: Robertson et al, 2006 (6)

1) Did the review address a focused clinical question? The aim of this study was to undertake a systematic review and meta-analysis to evaluate the association between: 1) thrombophilias with adverse pregnancy outcomes; and 2) venous thromboembolism (VTE) with adverse pregnancy outcomes.

2) Were the criteria used to select articles for inclusion appropriate? The inclusion and exclusion criteria were clearly defined. The study population included women who were known to either have one or more forms of thrombophilia, were pregnant or up to six weeks post-partum or had experienced a VTE or an adverse pregnancy outcome (pregnancy loss, pre-eclampsia, fetal growth restriction or placental abruption). The definitions for each of the outcomes of interest were clearly defined.

3) Is it unlikely that important and relevant studies were missed? A literature review was undertaken by two independent reviewers using appropriate key words on major databases including MEDLINE (1996- 2003), Embase (1980-2003) and the Cochrane database of systematic reviews (1998-2003). Only articles published in English were retrieved. Overall, the authors attempted to identify all the relevant studies, but may have missed important articles published in another language.

4) Was the validity of the included studies appraised? The authors assessed the quality of the studies using major criteria including cohort design, appropriate control group, blinded assessment of outcomes, adjustment for confounders and appropriate follow-up. Studies were excluded if: 1) cases were selected on the basis of autoimmune disease; 2) studies had no controls; or 3) controls included current users of hormone contraceptives.

5) Were assessments of studies reproducible? The inclusion and exclusion criteria and data extraction was done separately by two authors to ensure an accurate evaluation of each of the studies.

6 & 7) Were the results similar from study to study? What are the overall results of the review? The authors presented the results as OR with 95% confidence intervals based on the

random effects model for each of the outcomes stratified according to the separate forms of thrombophilia.

- i) The fVL genotype increased the odds of pre-eclampsia by 2.19 fold (95% CI 1.46-3.27), but there was significant heterogeneity between the studies ($p=0.04$). When mild pre-eclampsia was analysed separately, an OR of 2.3 (95% CI 1.27-4.16) was obtained, but heterogeneity remained ($p=0.01$). When restricting the analysis to severe pre-eclampsia, an OR of 2.04 (95%CI 1.23-3.36) was obtained and evidence of heterogeneity was removed ($p=0.13$). Homogeneous studies ($p=0.58$) exploring an association between PGV and pre-eclampsia had a combined OR of 2.52 (1.52-4.23).
- ii) The studies exploring the association between maternal fVL and recurrent fetal loss were heterogeneous ($p=0.001$), but all studies exploring an association between fVL and non-recurrent fetal loss (OR 4.12; 95%CI 1.93-8.8) and late fetal loss (OR 2.06; 95%CI 1.10-3.86) were homogeneous. Pooled studies exploring an association between PGV and recurrent first-trimester fetal loss (OR 2.7; 95%CI 1.37-5.34); non-recurrent second trimester fetal loss (OR 8.6; 95%CI 2.18-33.95) and late fetal loss (OR 2.66; 95%CI 1.28-5.53) were homogeneous.
- iii) The meta-analysis of studies exploring a possible association between 1) maternal fVL and birth weight <10% and 2) maternal PGV and birth weight <10th centile showed no significant association, but pooled studies were heterogeneous.

8) How precise were the results? The results of an association between fVL and severe pre-eclampsia may be generalisable but, unfortunately, the authors do not provide their definition of severe pre-eclampsia.

The studies exploring an association between maternal fVL and non-recurrent fetal loss or late fetal loss are generalisable. The studies exploring an association between maternal PGV and recurrent first-trimester fetal loss, non-recurrent second-trimester loss or late fetal loss are generalisable

2.2.4 Summary of published meta-analyses on maternal fVL/PGV and adverse pregnancy outcome (up until 2007)

2.2.5.1 Intrauterine fetal death

The results of four meta-analyses evaluating a possible association between: 1) maternal fVL and intrauterine fetal death; and 2) PGV and intrauterine fetal death are summarised in Table 2. Overall, there were six different outcome subgroups, making it difficult to compare the results of different meta-analyses. Meta-analyses of homogeneous case-control studies suggest that fVL is associated with around a 1.7-fold increased risk of single first-trimester fetal loss (6-8); however, the one cohort study within this meta-analysis does not support an association (OR 1.1; 95%CI 0.5-2.6) (8). The meta-analyses of homogeneous case control studies suggest that fVL is associated with around a three-fold increased risk of single loss after 19 weeks (7, 8); however, the cohort studies within the meta-analysis casts doubt on this association (OR1.2; 95% CI 0.6-2.5). Within case-control studies, the increase in OR to 10.7 (95% CI 4-28.5) associated with the outcome of two or more second/third-trimester fetal losses is consistent with a true association with maternal fVL. While meta-analyses of case-control studies support a possible association between maternal PGV and recurrent first-trimester fetal loss and single or recurrent second-trimester fetal loss, no cohort studies were available (6, 7, 9). Therefore, although evidence from case-control studies supports an association between recurrent fetal loss with fVL and PGV, a number of small cohort studies cast doubt on an association between fVL and non-recurrent fetal loss.

Table 2. Summary of meta-analyses exploring an association between 1) fVL and fetal loss and 2) PGV and fetal loss.

Trimester	Number of fetal losses	Gene variant	Study design included in meta-analysis	Results	Rey 2003 (7)	Dudding 2004 (8)	Kovalevsky 2004 (9)	Robertson 2006 (6)
Any trimester	Single fetal loss	fVL	Case-control	OR	1.73			
				95%CI	1.18-2.54			
				heterogeneity	$p=0.086$			
			Cohort	OR				
				95%CI				
				heterogeneity				
First trimester	Single fetal loss	fVL	Case- control	OR		1.8		1.68
				95% CI		1.2-2.7		1.09-2.58
				heterogeneity		$p=0.11$		$p=0.08$
			Cohort	OR		1.1		
				95% CI		0.5-2.6		
				heterogeneity		One study		
		PGV	Case-control	OR				2.49

				95% CI	1.24-5.0			
				heterogeneity	<i>P</i> =0.19			
	>1 fetal loss	fVL	Case-control	OR	2.01	2.6	1.6	1.91
				95% CI	1.13-3.58	1.7-3.8	1.2-2.2	1.01-3.6
				heterogeneity	<i>p</i> =0.11	<i>p</i> =0.7	-	<i>p</i> =0.001
		PGV	Case-control	OR	2.32		3.4	2.70
				95% CI	1.12-4.79		1.5-8	1.37-5.34
				heterogeneity	<i>P</i> =0.38		-	<i>P</i> =0.21
	Second and third trimester	Single second or third trimester fetal loss	Case-control	OR	3.26	2.8		4.12
				95% CI	1.82-5.83	1.3-6.2		1.93-8.81
				heterogeneity	<i>p</i> =0.6	<i>p</i> =0.4		<i>p</i> =0.0003
			Cohort	OR		1.2		
				95%CI		0.6-2.5		
				heterogeneity		<i>p</i> =0.9		
			PGV	OR				8.60
				95% CI				2.18-95
				heterogeneity				<i>P</i> =0.59
	>1 fetal loss	fVL	Case control	OR		3.9	2.7	

including at least 1 second or third trimester fetal loss	PGV	Case-control	95% CI	1.9-8.2	2.0-3.7
			heterogeneity	$p=0.4$	-
			OR		2.2
			95% CI		1.1-4.4
			heterogeneity		-
≥ 2 second or third trimester fetal losses	fVL	Case-control	OR	10.7	
			95%CI	4-28.5	
			heterogeneity	$p=0.9$	

2.2.5.2 Pre-eclampsia

Table 3 lists and summarises the results of the four meta-analyses exploring an association between: 1) pre-eclampsia and maternal fVL; and 2) pre-eclampsia and maternal PGV.

With respect to fVL, Robinson et al (6) combined case-control and cohort studies to calculate a pooled OR, whereas Kosmas et al (3) and Lin et al (5) only examined case-control studies. Due to heterogeneity between the studies, Dudding et al (8) subdivided case-control and cohort studies.

Most studies examined the outcomes of pre-eclampsia and severe pre-eclampsia separately. All the meta-analyses that included case-control studies showed a significant association between maternal fVL and pre-eclampsia ranging from 1.81 to 2.52, but all the pooled groups were statistically heterogeneous. On the other hand, the pooled cohort studies described by Dudding et al (8) were statistically homogeneous, but failed to show a statistically significant association (OR 1.1 95% CI 0.4-2.9) between fVL and pre-eclampsia, which may be due to insufficient sample size. The fact the cohort studies do not support an association between fVL and pre-eclampsia casts doubt on the results of the previous case-control studies. Therefore, the effect of maternal fVL on the risk of pre-eclampsia still remains uncertain.

By combining case-control and cohorts studies Lin et al and Robertson et al (5, 6) showed an association between maternal PGV and pre-eclampsia that ranged from 1.23- 2.54, but the pooled groups were heterogeneous. Combining case-control and cohort studies with an outcome of severe pre-eclampsia, Lin et al (5) reported an OR of 1.98 (0.94-4.17), but the pooled studies were heterogeneous. Therefore, the effect of maternal PGV on the risk of pre-eclampsia still remains uncertain.

Table 3 Summary of meta-analyses exploring an association between: 1) pre-eclampsia and maternal fVL; and 2) pre-eclampsia and maternal PGV

Outcome	Gene variant	Study design included in meta-analysis	Results	Kosmas 2003 (3)	Lin 2005 (5)	Dudding 2004 (8)	Robertson 2006 (6)
Pre-eclampsia	fVL	Case-control	OR	2.25	1.81		
			95% CI	1.50-3.38	1.14-2.87		
			heterogeneity	$p=0.002$	$p=0.04$		
		Case-control and cohort combined	OR				2.19
			95% CI				1.46-3.27
			heterogeneity				$p=0.04$
		Cohort	OR			1.1	
			95%CI			0.4-2.9	
			heterogeneity			$p=0.5$	
	PGV	Case control and cohort combined	OR		1.37		2.54
			95% CI		0.72-2.57		1.52-4.23
			heterogeneity		$P=0.57$		$p=0.58$
Severe	fVL	Case-control	OR		2.24	3.0	2.04

pre-eclampsia			95% CI	1.28-3.94	2.0-4.7	1.23-3.36
			heterogeneity	$p=0.09$	$p=0.3$	$p=0.13$
PGV	Case control	OR	1.98			
	and cohort	95% CI	0.94-4.17			
	combined	heterogeneity	$p=0.55$			

2.2.5.3 Fetal growth restriction

Table 4 lists and summarises the results of the three meta-analyses that explored a possible association between: 1) maternal fVL and fetal growth restriction (FGR); and 2) maternal PGV and FGR.

When the outcome was defined as FGR < 10th centile or < 5th centile, all meta-analyses reported statistically significant heterogeneity between the pooled studies. In trying to identify the source of heterogeneity, Dudding et al 2004 noted two different populations of recruited patients. Five of the eight studies stated that known causes of FGR were excluded. The pooled OR in this group was 4.7 (95% CI 2.3-9.5), indicating an association with maternal fVL with no heterogeneity. However, it is worth noting that the details of how the authors excluded other causes of FGR were scanty, and the total number within this group was only 695.

Three studies used a relatively unscreened population and did not mention the exclusion of causes of FGR. The results of these three studies were also homogeneous with a pooled OR of 1.07 (95% CI of 0.67-1.75). These studies included a total of 2116 participants and included the most rigorous study from a methodological viewpoint (10). Therefore, it is not possible from these results to determine whether or not maternal fVL or paternal PGV are associated with an increased risk of FGR.

Table 4 Summary of meta-analyses exploring an association between: 1) maternal fVL and FGR; and 2) maternal PGV and FGR

Outcome	Gene variant	Study design included in meta-analysis	Results	Howley 2004 (4)	Dudding 2004 (8)	Robertson 2006(6)
Birth weight <10 th centile	fVL	Case-control	OR	1.97		2.68
			95%CI	0.84-4.62		0.59-12.3
			heterogeneity	Yes		<i>P</i> =0.02
		Cohort	OR	0.96		
			95% CI	0.5-1.8		
			heterogeneity	?		
	PGV	Case-control	OR	1.97		2.92
			95%CI	0.72-5.4		0.63-13.70
			heterogeneity	Yes		<i>P</i> =0.0006
<10 th centile (other causes of FGR excluded)	fVL	Case-control	OR		4.7	
			95%CI		2.3-9.5	
			heterogeneity		<i>P</i> =0.3	
Birth weight <5 th centile	fVL	Case-control	OR	4.68		
			95% CI	1.59-13.78		
			heterogeneity	Yes		

2.3 Fetal factor V Leiden genotype or prothrombin gene variant and adverse pregnancy outcome – a summary of the literature

2.3.1 Intrauterine fetal death

Dekker et al 2004 evaluated the effect of fetal thrombophilia on the risk of intrauterine fetal death in 139 consecutively recruited women with a late fetal death (>16 weeks gestation) delivered between 1994 and 1998. Fetal DNA was recovered from umbilical cord blood in 123 (88%), tested for fVL and PGV and compared to a historic control group from which DNA was extracted. Combining the results for fetal fVL and fetal PGV, a greater frequency of fetal genetic thrombophilia was found in cases (9.8%) of intrauterine fetal death compared to controls (2%) with an OR of 4.8 (95% CI 1.1-22). Of interest, fVL was detected in 8/64 fetuses with a second-trimester fetal death compared to 1/59 fetuses with a third-trimester loss. There was also a strong association between fetal fVL and placental abruption as the cause of late fetal death with an OR of 7.6, (95% CI 1.5–37).

2.3.2 Fetal growth restriction

Infante-Rivard et al (2002) compared maternal and infant thrombophilia in 493 consecutively recruited newborns (birth weight < 10th centile) with 466 controls and reported no significant association between maternal and or fetal thrombophilia and fetal growth restriction (FGR). 15/461 (3.3%) of control newborns were fVL+ compared to 18/466 (3.9%) of case newborns with an OR of 1.35 (0.76–2.88). 6/460 (1.3%) of control newborns were PGV positive compared to 11/468 (2.4%) of case newborns with an OR of 1.92 (0.70–5.82) (10). McCowan et al (2003) also reported no association between infant fVL/PGV and risk of birth weight <10th centile in a smaller study recruiting infants and 290 controls (11). Gibson et al 2006 investigated a possible association between fetal thrombophilia and small-for-gestational age <10th centile. The cases were selected from a pathology-enriched cohort of babies obtained from the Cerebral Palsy Register as part of a study investigating the role of

thrombophilia and cerebral palsy. This exploratory study, which undertook 642 separate analyses, reported that for babies born < 28 weeks of gestation, the PGV was associated with an increased risk of small-for-gestational-age with an OR of 6.40 (95%CI 1.66-24.71), and concluded that future studies were needed to explore the role of fetal thrombophilia and adverse pregnancy outcomes(12).

Anteby et al 2004 identified 70 babies born with adverse pregnancy outcomes delivered within a 12-month period in a university hospital. 61% (11/18) of the thrombophilia-positive fetuses had FGR<10th centile compared to 58% (30/52) of thrombophilia-negative fetuses, which was not a statistically significant difference(13).

2.3.3 Pre-eclampsia

Livingston et al 2001 evaluated a possible association between severe pre-eclampsia and maternal or fetal thrombophilia (fVL, MTHFR &PGV) in 110 case women (and 75 fetuses) compared to 97 control normotensive pregnant women (and 80 fetuses) of African-American descent. The ORs for maternal or fetal thrombophilia being present in a pregnancy complicated by severe pre-eclampsia were 1.15 (95%CI 0.5-2.67) and 3.21 (95% CI 0.76-6.38) respectively. Although they found no significant difference between the frequency of fetal or maternal thrombophilia in the case and control groups, the numbers were small and the possibility of Type II error exists. The main limitation of this study is that fVL is extremely rare in individuals of African descent (14).

Vefring et al 2004 recruited 92 mother-father-child triads of mild, moderate or severe pre-eclamptic pregnancies. From 129 women who fulfilled the criteria for pre-eclampsia between January 1994 and December 1995, 92 triads consented to participate. The DNA from the mother, father and fetus were genotyped for the MTHFR c677T and fVL mutations. The authors found no effect of the fetal genotype on risk of pre-eclampsia. The relative risk of pre-eclampsia in case-mothers who were homozygous for the MTHFR mutation was 2.0 (CI=1.0-4.1). Factor V Leiden heterozygosity in the mother was associated with a 2.5-fold risk (CI=1.1-5.7) of pre-

eclampsia. There was insufficient statistical power in the mothers to detect whether or not this effect was higher for severe pre-eclampsia. The combination of fVL and homozygosity for the MTHFR mutation was associated with a 4.6-fold (1.0-21) risk of pre-eclampsia (15).

2.4 Conclusion

This meta-analysis and literature review highlighted the need for further research in this area.

Review of the literature up to January 2007 exploring possible associations between: 1) maternal/fetal fVL and adverse pregnancy outcomes intrauterine fetal death, pre-eclampsia and fetal growth restriction; and 2) maternal/fetal PGV and adverse pregnancy outcomes intrauterine fetal death, pre-eclampsia and fetal growth restriction confirms unclear and conflicting results.

It is not possible to determine from the current literature whether or not maternal fVL or maternal PGV was associated with an increased risk of FGR. Although meta-analyses of homogeneous case control studies exploring the outcome of non-recurrent intrauterine fetal death after 19 weeks showed a statistically significant association, the pooled OR of homogeneous cohort studies with an outcome of non-recurrent second-/ third-trimester loss was not statistically significant. The difference in results between the case-control studies and cohort studies was also apparent for the pre-eclampsia and intrauterine fetal death meta-analyses. Unfortunately, if a disease occurrence is rare, a large number of people are required to generate enough power to show a small, but clinically significant, association.

A possible effect of fetal fVL or PGV had not been tested in a large cohort study.

To address the shortfalls observed in the large number of small and possibility underpowered case-control studies, a decision was made to undertake a large nested case-control study within the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort. The aim of this was to evaluate the association between: 1)

maternal/fetal fVL and intrauterine fetal death, fetal growth restriction and pre-eclampsia; and 2) maternal/fetal PGV genotype and risk of intrauterine fetal death, fetal growth restriction and pre-eclampsia.

2.5 Hypothesis

The survey of the literature and reported meta-analyses led us to formulate the following hypotheses.

PRIMARY HYPOTHESES

1. The presence of heterozygosity or homozygosity for the factor V Leiden mutation in the mother is associated with an increased risk of FGR, late fetal death or pre-eclampsia in a nested case-control study.
2. The presence of heterozygosity or homozygosity for the factor V Leiden mutation in the fetus is associated with an increased risk of FGR or pre-eclampsia in a nested case control study.
3. The presence of heterozygosity or homozygosity for the prothrombin G20210A mutation in the mother is associated with an increased risk of FGR, late fetal death or pre-eclampsia in a nested case-control study.
4. The presence of heterozygosity or homozygosity for the prothrombin G20210A mutation in the fetus is associated with an increased risk of FGR or pre-eclampsia in a nested case-control study.

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CHAPTER 3

FACTOR V LEIDEN IS ASSOCIATED WITH PRE-ECLAMPSIA BUT NOT WITH FETAL GROWTH RESTRICTION: A GENETIC ASSOCIATION STUDY AND META-ANALYSIS.

3.1 Introduction to publication III

In order to undertake this study, I successfully applied for a NHMRC grant (NHMRC grant number 209558).

Many meta-analyses had already been published in an attempt to clarify the contribution of inherited thrombophilias to the risk of fetal growth restriction (FGR), pre-eclampsia and fetal loss. But because the effect of fetal fVL or PGV had not been tested in a large cohort study, it was still unclear whether maternal factor V Leiden (fVL) or prothrombin gene variant G20210A (PGV) was associated with an increased risk of FGR, intrauterine fetal death or pre-eclampsia.

To overcome the shortfalls of the many, but small and possibly underpowered, studies, research was conducted within the large population based cohort, Avon Longitudinal Study of Parents and Children (ALSPAC), appendix 1,

6755 mother/infant pairs within the ALSPAC study were genotyped to determine whether maternal or fetal FVL or PGV, either alone or in combination, was associated with FGR or pre-eclampsia. (Late fetal death could not be included in the analysis because of the low incidence of late fetal death combined with incomplete data collection within the ALSPAC cohort).

Data from other published cohort studies relating to fVL and risk of pre-eclampsia was combined by meta-analysis to increase the power of detecting an association.

Overall, the results of this study within the large ALSPAC cohort show no statistically significant association between maternal or fetal fVL or PGV, either alone or in combination with birth weight <10th centile. Furthermore, the FGR meta-analysis which

pooled the results of this cohort study and other cohort studies found no evidence of an effect of maternal fVL on FGR. Given the size of the pooled sample, there was 80% power to detect an OR of 1.09, indicating that if an effect of fVL on FGR was missed by this meta-analysis, it would be quite small.

In light of this, it is clear that the previous estimates of fVL increasing the risk of FGR were driven largely by small case-control studies not supported by this cohort study or the meta-analysis with other cohort studies.

The results of this study within the large ALSPAC cohort show no statistically significant association between maternal or fetal fVL or PGV, either alone or in combination with pre-eclampsia. However, increasing the power by combining this study with other cohort studies by meta-analysis revealed a positive association between maternal fVL and pre-eclampsia with an OR of 1.49 (95% CI 1.13-1.96 $p=0.003$).

These results publication III are relevant to women in the general population with fVL or PGV mutations identified through cascade testing. They suggest these women are not at an increased risk of FGR, but that fVL positive women have an approximately 50% increased risk of pre-eclampsia.

Addendum: Rodger et al 2010 published a subsequent meta-analysis of cohort studies, in which the data from this publication was included and provided the greatest weight (appendix 2). The Roger et al 2010 meta-analysis casts doubt on a possible association between fVL and pre-eclampsia with a combined odds ratio of 1.23 (95% 0.89-1.70).

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Dudding T, Heron J, Thakkestian A, Nurk E, Golding J, Pembrey M, Ring SM, Attia J, Scott RJ. Factor V Leiden is associated with pre-eclampsia but not with fetal growth restriction: a genetic association study and meta-analysis. *J Thromb Haemost.* 2008 Nov; 6(11):1869-75.

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CHAPTER 4

MATERNAL FACTOR V LEIDEN AND ADVERSE PREGNANCY OUTCOME: DECIDING WHETHER OR NOT TO TEST

4.1 Introduction to publication IV

The aim of this narrative review was to examine the translation from statistical association to change in clinical practice with respect to factor V Leiden (fVL) and adverse pregnancy outcomes.

To explore how differences in the number of fetal losses and the gestation period at which they occur influences the relative importance of fVL, the results of five meta-analyses exploring an association between fVL and fetal loss were divided into six different subgroups based on the trimester at which the fetal loss occurred and on the number of prior fetal losses.

The results of the six meta-analyses exploring an association between fVL and pre-eclampsia were categorised according to severity of pre-eclampsia and the type of studies included in the meta-analyses.

The results of three meta-analyses exploring an association between fVL and fetal growth restriction (FGR) were categorised according to the degree of FGR and the type of studies included in the meta-analyses.

The review illustrates that previous history, as well as severity of an adverse pregnancy outcome, are strong candidates for explaining heterogeneity between the results of different meta-analysis; highlighting the relevance of different study populations to different clinical scenarios. The results of population-based cohort studies, which represent an unselected group of women within the population, are relevant for women without a previous history of adverse pregnancy outcome who are identified as fVL heterozygotes through cascade family testing. Conversely, meta-analyses of studies which include women with a previous history of at least one adverse pregnancy outcome are relevant to women with a history of adverse pregnancy outcome who are subsequently identified as fVL heterozygotes.

Although these meta-analyses show a clear trend towards a greater association with fVL as the severity and number of adverse pregnancy outcomes increases, statistical association is not sufficient to answer the question of whether or not to test for fVL in different clinical scenarios. Moving from statistical association to change in clinical practice also requires consideration of: 1) the yield of testing in different clinical scenarios; 2) the calculated post-test probability of a recurrence based on testing in different clinical scenarios; and 3) the clinical utility of a positive test. The review examines each of these issues with respect to fVL, and reports that the yield of fVL testing in women with previous adverse pregnancy outcomes is up to six times higher than in the general population. Calculated post-test probabilities illustrate that the combined effect of fVL and poor pregnancy history places these women at a high risk of recurrent events.

The clinical utility of genetic testing for fVL refers to the ability of this test to guide management decisions to significantly improve outcomes. The manuscript summarises the studies to date, which evaluate the safety and efficacy of low molecular weight heparin (LMWH) during a subsequent pregnancy for thrombophilia carriers with a history of previous pregnancy loss.

Despite some suggestion that preventative treatment with LMWH may be beneficial in the group of women with a very poor pregnancy history, adequately designed randomised controlled trials are needed to confirm whether or not anticoagulant therapy will improve the prognosis in this group of thrombophilic women. Ideally, thrombophilic women at high risk of recurrence should be enrolled in a well-designed adequately powered multicentre clinical trial. However, while awaiting the outcome of treatment trials, we propose that these post-test probabilities, in addition to the preliminary treatment data in high-risk women, justify consideration of screening for fVL in women with a strong past history of poor pregnancy outcome, as well as discussion of the current data concerning LMWH. There is no consensus threshold that merits fVL testing, and the threshold to prompt screening will depend on the patient's previous pregnancy history, patient's and physician's perception of risks and benefits, and the value they place on the outcome.

The results to date of LMWH treatment trials cannot be extrapolated to all women with thrombophilia; however, the results provide a rationale for randomised prophylactic anticoagulant treatment trials in thrombophilic women with severe adverse pregnancy outcomes. While we await the results of well designed, adequately powered treatment trials, we propose that post-test probabilities, in addition to the preliminary treatment data in high-risk women, justify consideration of screening for fVL in women with a strong past history of poor pregnancy outcome.

REVIEW ARTICLE

Maternal factor V Leiden and adverse pregnancy outcome: deciding whether or not to test

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This narrative review examines the translation from statistical association to change in clinical practice with respect to factor V Leiden and adverse pregnancy outcome. A collation of published meta-analyses illustrates a clear trend towards a greater association with factor V Leiden (fVL) as the severity of adverse pregnancy outcomes increases, and highlights that different study populations are relevant to different clinical scenarios. The yield of fVL testing in women with previous adverse pregnancy outcomes is up to six times higher than in the general population. Calculated post-test probabilities illustrate that the combined effect of fVL and poor pregnancy history places these women at a high-risk of recurrent events. The results to date of low molecular weight heparin (LMWH) treatment trials cannot be extrapolated to all women with thrombophilia; however, the results provide a rationale for randomized prophylactic anticoagulant treatment trials in thrombophilic women with severe adverse pregnancy outcomes. While we await the results of well-designed, adequately powered treatment trials, we propose that post-test probabilities, in addition to the preliminary treatment data in high-risk women, justify consideration of screening for fVL in women with a strong past history of poor pregnancy outcome.

Keywords: Fetal growth restriction, intrauterine fetal death, pre-eclampsia, recurrent miscarriage, thrombophilia

Introduction

Intrauterine fetal death, recurrent miscarriage, intrauterine growth restriction (IUGR), and pre-eclampsia continue to be major causes of fetal and maternal morbidity and mortality. Given the importance of establishing and maintaining an adequate placental circulation, hereditary thrombophilias have been postulated as a possible cause of placental insufficiency. The factor V Leiden mutation (fVL) is the most common form of inherited thrombophilia, being present in about 5% of Caucasians [1]. A point mutation in the factor V gene at nucleotide position 1691, resulting in an arginine to glutamine substitution, reduces the sensitivity of the factor V protein to inactivation by activated protein C (activated protein C resistance) resulting in a pro-coagulant state and an increased risk of thrombosis. However, despite numerous case-control and cohort studies, a possible association between fVL and adverse pregnancy outcome remains controversial [2].

The purpose of this narrative review is to explore the reasons for the heterogeneity between meta-analyses. The review illustrates that previous history as well as severity of an adverse

pregnancy outcome are strong candidates for explaining this heterogeneity; and highlights that different study populations are relevant to different clinical scenarios. The results of population-based cohort studies, which represent an unselected group of women within the population, are relevant for women without a previous history of adverse pregnancy outcome who are identified as fVL heterozygotes through cascade family testing. Conversely, meta-analyses of studies which include women with a previous history of at least one adverse pregnancy outcome are relevant to women with a history of adverse pregnancy outcome who are subsequently identified as fVL heterozygotes. Although these meta-analyses show a clear trend towards a greater association with fVL as the severity and number of adverse pregnancy outcomes increases, a statistical association is not sufficient to answer the question of whether or not to test for thrombophilia. Moving from statistical association to change in clinical practice also requires consideration of (1) The yield of testing in different clinical scenarios; (2) The calculated post-test probability of a recurrence based on testing in different clinical scenarios; and (3) The clinical utility of a positive test. We complete the review by examining each of these issues with respect to factor V Leiden.

Method

Electronic databases Embase and Medline were searched using the following search terms: (1) thrombophilia and meta-analysis and pregnancy or fetal loss; miscarriage; stillbirth; pre-eclampsia, fetal growth restriction; intrauterine growth retardation and (2) factor V Leiden and meta-analysis and pregnancy or fetal loss; miscarriage; stillbirth; pre-eclampsia, fetal growth restriction; intrauterine growth retardation. Data from 10 meta-analyses was extracted and collated based on: (1) The history of previous adverse outcomes and (2) The types of studies included in the meta-analysis i.e. case-control studies or cohort studies.

The fVL yield data was extracted from large population-based cohorts in whom women, identified with pre-eclampsia, severe pre-eclampsia, recurrent miscarriage or late fetal death/stillbirth, were tested for fVL. The electronic databases Embase and Medline were searched using the following search terms: factor V Leiden and pre-eclampsia; factor V Leiden and miscarriage; and factor V Leiden and fetal death or stillbirth. Four hundred and twenty four studies were identified and, based on the titles, 40 studies were examined in more detail. Data was extracted from 17 population-based cohort studies. The post-test probability was calculated using the recurrence risk of each of the adverse pregnancy outcomes adjusted according to the OR associated with fVL.

Results

The importance of the number of fetal losses and the gestation period at which they occur.

To explore how differences in the number of fetal losses and the gestation period at which they occur influences the relative importance of fVL, the results of five meta-analyses [3–7] exploring an association between fVL and fetal loss are summarized in Table I. Based on the trimester at which the

fetal loss occurred and the number of prior fetal losses, there were six different subgroups of women. Combining all women with one fetal loss across all trimesters by meta-analysis, Rodger et al. 2010 [7] showed a small increase in risk (OR 1.52; 95% CI 1.06–2.19) of fetal loss associated with fVL. Therefore, whereas the risk of fetal loss is eight percent for women in the general population [8], primigravida women known to be heterozygote for fVL have a 12% absolute risk of fetal loss during their first pregnancy. The association with recurrent or late fetal loss

Table I. Summary of factor V Leiden and fetal loss meta-analyses.

Trimester	Number of fetal losses	Gene variant	Study design included in meta-analysis	Results	Rey 2003 [3]	Dudding 2004 [4]	Kovalevsky 2004 [5]	Robertson 2006 [6]	Rodger 2010 [7]
Any trimester	Single fetal loss	fVL	Case-control	OR	1.73				
				95% CI	1.18–2.54				
			Cohort	Heterogeneity	$p=0.086$				
				OR					1.52
First trimester	Single fetal loss	fVL	Case-control	95% CI					1.06–2.19
				Heterogeneity					$p=0.06$
			Cohort	OR		1.8		1.68	
				95% CI		1.2–2.7		1.09–2.58	
	>1 fetal loss	fVL	Case-control	Heterogeneity		$p=0.11$		$p=0.08$	
				OR		1.1			
			Cohort	95% CI		0.5–2.6			
				Heterogeneity		One study			
Second and third trimester	Single second or third trimester fetal loss	fVL	Case-control	OR	2.01	2.6		1.91	
				95% CI	1.13–3.58	1.7–3.8		1.01–3.6	
				Heterogeneity	$p=0.11$	$p=0.7$		$p=0.001$	
			Cohort	OR	3.26	2.8		4.12	
				95% CI	1.82–5.83	1.3–6.2		1.93–8.81	
				Heterogeneity	$p=0.6$	$p=0.4$		$p=0.0003$	
	>1 fetal loss including at least 1 second or third trimester fetal loss	fVL	Case control	OR		3.9	2.7		
				95% CI		1.9–8.2	2.0–3.7		
				Heterogeneity		$p=0.4$	–		
	≥2 second or third trimester fetal losses	fVL	Case-control	OR		10.7			
				95%CI		4–28.5			
				heterogeneity		$p=0.9$			

Table II. Summary of factor V Leiden and pre-eclampsia meta-analyses.

Outcome	Gene variant	Study design included in meta-analysis	Results	Kosmas 2003 [9]	Lin 2005 [10]	Dudding 2004 [4]	Dudding 2008 [11]	Robertson 2006 [6]	Rodger 2010 [7]
Pre-eclampsia	fVL	Case-control	OR	2.25	1.81				
			95% CI	1.50–3.38	1.14–2.87				
			Heterogeneity	$p=0.002$	$p=0.04$				
		Case-control and cohort combined	OR					2.19	
			95% CI					1.46–3.27	
			Heterogeneity					$p=0.04$	
		Cohort	OR			1.1	1.49		1.23
			95%CI			0.4–2.9	1.13–1.96		0.89–1.70
			Heterogeneity			$p=0.5$	$p=0.93$		$p=0.99$
Severe pre-eclampsia	fVL	Case-control	OR		2.24	3.0		2.04	
			95% CI		1.28–3.94	2.0–4.7		1.23–3.36	
			Heterogeneity		$p=0.09$	$p=0.3$		$p=0.13$	

is evaluated largely by meta-analyses of case-control studies (Table I). These meta-analyses report that fVL is associated with recurrent first trimester fetal loss with ORs between 1.91 (95% CI 1.01–3.6) [6] and 2.6 (95% CI 1.7–3.8) [4]. Factor V Leiden is associated with non-recurrent loss after 19 weeks with ORs ranging from 1.2 (95% CI 0.6–2.5) [4] to 4.12 (95% CI 1.93–8.81) [6]. There is a consistent increase in OR with increasing trimester and number of fetal losses. Factor V Leiden associated with two or more second/third trimester fetal losses has an OR of 10.7 (4–28.5) [4].

The importance of the severity of pre-eclampsia

Table II summarises the results of the six meta-analyses [4,6,7,9–11] exploring an association between fVL and pre-eclampsia; these meta-analyses are categorized according to severity of pre-eclampsia and the type of studies included in the meta-analyses. A recent meta-analysis including published cohort studies casts doubt on a possible association between fVL and pre-eclampsia with a combined OR of 1.23 (95% CI 0.89–1.70) [7]. The association with severe pre-eclampsia, on the other hand, is evaluated by meta-analyses of case-control studies and generally indicates stronger association (Table II). These meta-analyses report that fVL is associated with severe pre-eclampsia with ORs ranging from 2.04 (95% CI 1.23–3.36) [6] to 3.0 (95% CI 2.0–4.7) [4].

The importance of severity of intrauterine growth restriction

Table III summarizes the results of three meta-analyses exploring an association between fVL and IUGR [7,12,13]. A meta-analysis of cohort studies in 2008 [7] reported no increase risk (OR 1.00; CI 0.80–1.25) of small for gestational age (<10th centile) associated with fVL. There have been no cohort studies evaluating a possible association between small for gestational age (<5th centile); and although meta-analyses of case controls report a 4.68 (1.59–13.78) fold increased risk of SGA (<5th centile) associated with fVL, the pooled studies were heterogeneous [12]. Therefore, it remains unclear whether or not fVL is associated with SGA (<5th centile).

Factor V Leiden and adverse pregnancy outcome: translation to clinical practice

The literature supports an association between fVL and recurrent miscarriage, late-trimester fetal loss and severe pre-eclampsia, but what does this mean for clinical practice? There are a number of factors to be considered when moving from a statistically significant association to a change in clinical practice. These include: (1) Who to test for fVL? (2) What is the absolute risk of a particular adverse pregnancy outcome given that the individual is positive for fVL? and (3) How does a positive fVL test change management?

Who to test? The yield of fVL testing in different clinical scenarios.

Possible clinical settings in which fVL genetic testing could be offered include: (1) All women planning a family; (2) Women with a family history of adverse pregnancy outcome; and (3) Women with a prior history of recurrent unexplained miscarriage, late fetal death, intrauterine fetal growth restriction or pre-eclampsia. The yield of fVL testing will differ according to the different clinical scenarios (Table IV). Within large cohort studies ($n > 1000$), the yield of fVL testing in women with a previous history of pre-eclampsia ranges from 3.4–13% [11,14–22]. The fVL yield in women with severe pre-eclampsia ranges from 5–11% [23,24]. Cohort studies including women with three trimester miscarriages report a 4.8–8% fVL yield [25,26]. Within the largest cohort of 100,000 consecutive pregnancies, there were 44 unexplained stillbirths at/or after 22 weeks with a 9 percent yield for fVL [27]. Data from another prospective cohort of women with a stillbirth after 20 weeks reported a 23% yield for fVL; however, 49% of his cohort had stillbirth accompanied by placental pathology. A subgroup analysis confined to stillbirths with placental pathology reported a fVL yield of 33% [28]. Within a case-control study, Foka et al. reported a 31.5% (6/19) fVL yield when testing women with a history of two second trimester miscarriages [29]. Hence, there is evidence to support that fVL testing has a higher yield in those with previous adverse pregnancy outcomes; these data are summarized in the first column of Table IV.

Table III. Summary of factor V Leiden and IUGR meta-analyses.

Outcome	Gene variant	Study design included in meta-analysis	Results	Howley 2004 [12]	Facco 2009 [13]	Rodger 2010 [7]
Birth weight <10th centile	fVL	Case-control	OR	1.97	1.91	
			95% CI	0.84–4.62	1.17–3.12	
			Heterogeneity	Yes		
		Cohort	OR		1.16	1.00
			95% CI		0.98–1.38	0.8–1.25
			Heterogeneity			$p = 0.95$
Birth weight <5th centile	fVL	Case-control	OR	4.68		
			95% CI	1.59–13.78		
			Heterogeneity	Yes		

Table IV. Yield of factor V Leiden testing and post-test probability of current event.

Outcome	fVL yield	Pre-test odds of having a recurrence	Likelihood ratio	Post test probability
Pre-eclampsia	3.4–13% [11,14–22]	14% [30–36]; 31% when PE in two previous pregnancies [30]	1.2–2 [6,7]	16–25%, 35–47%
Severe pre-eclampsia	5–11% [23,24]	6.8–40% ^a [37,38]	2–2.5 [6,10,11]	13%–15%, 57–62% ^a
Recurrent first trimester miscarriage (≥2 miscarriages)	4.8–8% [25,26]	26% [39–41]	2 [3,4,6]	41%
Late stillbirth >22 weeks	9–23% [27,28]	2% [42,43]	1.2–4 [4,6]	2.4–7.5%
Placental stillbirth	33% [28]			
≥2 second or third trimester losses	31.5% [29]		10 [4]	

^aStudy included women with onset of pre-eclampsia between 18–27 weeks.

What is the risk of a subsequent adverse pregnancy outcome given that a woman is fVL positive?

The recurrence risk of a particular adverse pregnancy outcome in the general population is required to calculate the post-test probability of recurrence in women identified as fVL positive (Table IV). Within a large population-based cohort of 763,795 women, Hernandez-Diaz et al. [30] report a 14.7% recurrence rate of pre-eclampsia. This is consistent with the results of other cohort studies, all reporting a pre-eclampsia recurrence rate between 13–15% [31–36]. The recurrence rate of severe pre-eclampsia is less consistent in the literature, with recurrence rates ranging for 6.8–40% [37,38]. One large population-based cohort of 188768 women identified 133 women (mean age 28.9 years) with *denovo* severe pre-eclampsia with a recurrence risk of 6.8% (95% CI 5.7–7.9%) [38]. This risk was lower than an earlier study with younger patients (mean age 23.6 years) with earlier onset pre-eclampsia (18–27 weeks) which reported a recurrence risk for severe pre-eclampsia of 40%. A lower use of Aspirin in the Sibai study [37] may be another explanation for the difference. Within the largest population-based cohort of 151,021 women, Bhattacharya [39] reported a 26% risk of subsequent miscarriage following two previous consecutive first trimester miscarriages, which is similar to two previous smaller studies [40,41]. As demonstrated by Brigham et al. 1999, the risk of subsequent first trimester miscarriage increases with increasing maternal age and the number of previous miscarriages [40]. Within the largest population-based cohort including 2677 women with a previous stillbirth >24 weeks gestation, the recurrence rate was 1.9% [42], which is similar to the 2.1 % recurrence rate within another large population cohort including 1050 women with previous stillbirth >20 weeks gestation [43].

Women with a past history of adverse pregnancy outcomes are at higher risk of future events, and this risk is further increased by the presence of fVL. The highest risk group is women with early onset severe pre-eclampsia and fVL, whose calculated absolute risk of recurrent severe eclampsia is higher than 50%. Other calculated post-test probabilities are as high as 25–41% (Table IV).

How does the positive fVL test change management? The clinical utility of genetic testing for fVL

The clinical utility of genetic testing for fVL refers to the ability of this test to guide management decisions to significantly improve outcomes. Based on a positive test for fVL, possible treatment options include: (1) Screening for other environmental or genetic risk factors; and (2) Treatment with low molecular weight heparin (LMWH). The presence of a second genetic or acquired form of thrombophilia, or the presence of other risk factors such as obesity or smoking may influence counseling of the women. A number of studies have attempted to evaluate the efficacy and safety of LMWH during a subsequent pregnancy for thrombophilia carriers with a history of previous pregnancy loss [44–50]. For ethical reasons, many of these studies did not have a no-treatment arm [44,45,47]. The Live-Enox study was a multicentre, prospective, randomized, open-labeled trial between 2000 and 2002. Women aged ≥ 18 years with thrombophilia and a history of recurrent pregnancy loss (RPL) were enrolled at 5–10 weeks of pregnancy. RPL was defined as ≥ 3 losses during the first trimester, ≥ 2 in the second trimester, or one intrauterine fetal death in the third trimester. The outcome of previous pregnancies for women who enrolled in the Live-Enox study was poor, with only 28% of previous pregnancies resulting in a live birth. In contrast, treatment with enoxaparin 40 mgs or enoxaparin 80 mgs resulted in a live birth of rate of 84.3% and 78.3% (both doses equally effective and safe) [45]. Carp et al. 2003 assessed the effect of enoxaparin

on the subsequent live birth rate in 85 women with three or more consecutive pregnancy losses and a hereditary thrombophilia. Thirty-seven patients were treated with enoxaparin 40 mgs, and 48 patients were not treated. The live birth rate was 70.2% in the treated patients compared to 43.8% in the untreated patients ($p < 0.02$, OR 3.03 95% CI 1.12–8.36). The main effect was in primary aborters with a 42% improvement in the live birth rate compared to eight percent in secondary aborters. Results were not statistically significant, possibly due to inadequate sample size [48]. Gris et al. 2004 compared aspirin 100 mgs with enoxaparin 40 mgs daily in 160 thrombophilic women (heterozygous fVL, PGV or protein S deficiency) with a previous history of one unexplained pregnancy loss from the 10th week of amenorrhea, and reported a live birth rate of 86% (69/80) in the enoxaparin treated group compared to 29% (23/80) in the aspirin treated group [47]. The outcome of previous pregnancies for women enrolled in this study was also low with a previous live birth rate of 29%. Notably, the previous live birth rate of 28% and 29% for women enrolled in the Live-Enox study and the subsequent study by Gris et al. 2004 is much lower than the estimated 75% live birth rate following three miscarriages which has been reported by previous cohort studies [40,41]. A retrospective observational study of 116 thrombophilic women with severe adverse pregnancy outcomes observed a subsequent adverse pregnancy outcome in 7% of the women treated with LMWH compared to 21% of the untreated group [50]. Although the results of the above studies cannot be extrapolated to all women identified with fVL, they provide some evidence that preventative treatment with LMWH may be beneficial in the group of women with a very poor pregnancy history.

The results of recently published SPIN and ALIFE trials failed to demonstrate a benefit of combination low molecular weight heparin and aspirin or aspirin alone compared to placebo in women with recurrent pregnancy loss, defined as at least two unexplained miscarriages [51,52]. The SPIN study excluded women with known thrombophilia. An argument can be made that recruiting women with a history of < 3 miscarriages will increase the number of women with repeat sporadic fetal chromosomal abnormalities. The percentage of recruited women who had ≥ 3 previous miscarriages was 42.9% and 59.8% for the SPIN and ALIFE studies respectively. These studies do not address the smaller group of patients presenting with pregnancy losses > 10 weeks gestation and a documented thrombophilia. We propose that severity of previous pregnancy outcome in thrombophilic women is important to consider when designing future treatment trials. The design of such studies should also include karyotyping fetal losses, where possible, to avoid embryos with chromosome abnormalities being included as a treatment failure. Unfortunately, the inclusion of low risk patients in ongoing randomized controlled trials evaluating the possible benefit of anticoagulant treatment will dramatically lower their power.

Sarig et al. 2009 published a risk stratification scoring system. Based on four major categories – obstetric history, previous thromboembolic events, family history and type of thrombophilia – the authors proposed that women be stratified into four levels of risk [53]; however, this study has methodological problems as the point score was assigned arbitrarily. The development and validation of a clinical prediction model based on logistic regression may facilitate the identification of fVL heterozygote women at high-risk of an adverse pregnancy outcome.

Conclusion

Analyzing the literature based on severity of adverse pregnancy outcome illustrates that the reported risk associated with fVL increases when there is a strong history of adverse pregnancy

outcomes (such as more or later pregnancy losses and more severe pre-eclampsia). This is consistent with a study by Kist et al. 2008, which concluded that severity of adverse pregnancy outcome influences the association between thrombophilia and adverse pregnancy outcome [54]. The yield of fVL testing in women with previous adverse pregnancy outcomes is up to six times higher than in the general population, i.e. up to 30%. Calculated post-test probabilities illustrate that the combined effect of fVL and poor pregnancy history places these women at a high-risk of recurrent events. Despite some suggestion that preventative treatment with LMWH may be beneficial in the group of women with a very poor pregnancy history, adequately designed randomized controlled trials are needed to confirm whether or not anticoagulant therapy will improve the prognosis in this group of thrombophilic women. Ideally, thrombophilic women at high-risk of recurrence should be enrolled in a well-designed adequately powered multicentre clinical trial. However, while awaiting the outcome of treatment trials, we propose that these post-test probabilities, in addition to the preliminary treatment data in high-risk women, justify consideration of screening for fVL in women with a strong past history of poor pregnancy outcome and discussion of the current data concerning low molecular weight heparin (LMWH). There is no consensus threshold that merits fVL testing, and the threshold to prompt screening will depend on the patient's previous pregnancy history, patient's and physician's perception of risks and benefits, and the value they place on the outcome.

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CHAPTER FIVE

CONCLUSION

5.1 Introduction

The factor V Leiden (fVL) mutation is present in one in 20 Caucasian women. Asymptomatic gene carriers are often identified through cascade family testing and seek genetic counseling for risk assessment. Conversely, women with adverse pregnancy events present the opposite dilemma of whether to instigate genetic testing. In the final chapter of this thesis, results relating to fVL and analysis of the concurrent research have been integrated to suggest some possible directions in both scenarios; concluding with the identification of future priority research areas.

5.2 Clinical Scenarios

5.2.1 Group 1: Factor V Leiden positive women identified through cascade family testing with no history of adverse pregnancy outcome

A recent meta-analysis (1) combined all cohort studies to show a small increase in risk (OR 1.52; 95% CI 1.06-2.19) of fetal loss across all trimesters associated with fVL. Therefore, whereas the risk of fetal loss is eight percent in the general population (2), primigravida women known to be heterozygote for fVL have a 12% absolute risk of fetal loss during their pregnancy.

Meta-analyses of homogeneous case-control studies (Rey 2003 & Dudding 2004) suggest around a three-fold increased risk of late fetal loss in primigravida women known to be heterozygote for fVL, with OR ranging from 3.26 (95%CI 1.82-5.83) to 2.8 (95% CI 1.3-6.2). This translates to a 1.5% risk of late fetal loss compared to a population risk of 0.5%. However, a meta-analysis of three homogenous cohort studies (3-5) (total pooled cohort of 3418) reported a combined OR of 1.2 (95% CI 0.6-2.5), casting doubt on a possible increased risk of late fetal loss in this group of women (Dudding 2004)(6). Although late fetal loss was

an outcome assessed in Publication III (7), the low rate of late fetal death meant there was still insufficient power within the cohort of 6755 mother-infant pairs to determine whether maternal or fetal fVL or PGV, either alone or in combination, were associated with fetal loss.

Combining all cohort studies, including data from Publication III (7), Rodger et al 2010 casts doubt on a possible association between fVL and pre-eclampsia with a combined OR of 1.23 (95% CI 0.89-1.7)(1). Therefore, there is no evidence to suggest that primigravida fVL + women within the general population have an increased risk of pre-eclampsia. The risk of severe pre-eclampsia in primigravida fVL + women remains unclear. A meta-analysis of homogenous case-control studies (Dudding 2004, Lin 2005, Robertson 2006) reports fVL is associated with a two-to-three-fold increase risk of severe pre-eclampsia (6, 8, 9). This translates into a 1-1.5% risk of severe pre-eclampsia compared to a 0.5% risk in the general population. This small absolute increased risk has not been confirmed in cohort studies.

Combining all cohort studies, including data from publication III(7), Rodger et al 2010 reports no increase risk of birth weight < 10th associated with fVL (OR 1.00; (95% CI 0.08-1.25)(1). There have been no cohort studies evaluating a possible association between fVL and birth weight < 5th centile, and although meta-analyses of case controls report a 4.68- (1.59-13.78) fold increased risk of birth weight < 5th centile associated with fVL, the pooled studies were heterogeneous(10). Therefore, it remains unclear whether or not fVL is associated with a birth weight < 5th centile.

Treatment

There have been no randomised controlled treatment trials to support benefit of anticoagulation treatment in primigravida women identified as fVL + on cascade genetic testing.

Conclusion

Therefore, although a small absolute increased risk of fetal loss or severe pre-eclampsia may warrant further surveillance, in the absence of other acquired or inherited forms of thrombophilia, anticoagulation prophylaxis is not indicated in women identified as fVL carriers through cascade genetic testing.

5.2.1 Group 2: Genetic testing for factor V Leiden in women with a history of first-trimester fetal loss

Recurrent first-trimester fetal loss has been evaluated by meta-analyses of homogeneous case-control studies (Rey 2003 & Dudding 2004). When women have had one first-trimester loss, the presence of maternal fVL is associated with around a two-fold increased risk of another first-trimester fetal loss, with OR ranging from 2.01 (95%CI 1.13-3.58) to 2.6 (95%CI 1.7-3.8) (6, 11). This translates into an absolute risk of 20-40% compared to a population risk of first-trimester miscarriage of 10-20% (12). The risk of a subsequent miscarriage in fVL + women with a previous history of miscarriage has not been evaluated within a population-based cohort study.

Post-test probability of recurrent miscarriage in fVL+ women with a previous history of \geq first-trimester miscarriages

Within Publication IV, (13) the post-test probability of a recurrent miscarriage in fVL + women with a previous history of ≥ 2 first-trimester miscarriages was calculated using: 1) pre-test probability of recurrent miscarriage following ≥ 2 previous first-trimester miscarriages in the general population; and 2) likelihood ratios (estimate of how much a fVL + test result will change the odds of having a disease). Based on large population cohorts, the recurrence risk of miscarriage following two miscarriages is 26% (14-16). Using a likelihood ratio of two (6, 8, 11), the calculated post-test probability of recurrent first-trimester miscarriage (publication IV) in this group of fVL+ women is 41% (13) .

Treatment

Despite some suggestion that preventative treatment with low molecular weight heparin (LMWH) may be beneficial in the group of women with a very poor pregnancy history (17-23), adequately designed randomised controlled trials are needed to confirm whether or not anticoagulant therapy will improve the prognosis in this group of thrombophilic women. The results of the published SPIN and ALIFE trials failed to demonstrate a benefit of combination LMWH and aspirin or aspirin alone compared to placebo in women with recurrent pregnancy loss (defined as at least two unexplained miscarriages); however, the ALIFE authors concluded that women with thrombophilia warranted further study in an adequately powered control trial (24, 25).

Current recommendations

Although a number of societies have published different guidelines on thrombophilia screening women with a history of fetal loss (table 1), the overall consensus of more recent guidelines is similar to the recommendations published by the British Committee for Standards in Hematology guidelines (Baglin et al 2010): “with respect to recurrent pregnancy loss, screening for thrombophilia (except APLS) is not recommended pending the outcome of randomised controlled trials with a no-treatment or placebo arm evaluating the antithrombotic therapy in women with heritable thrombophilia and a history of pregnancy complications”.⁶

Table 1 Published guidelines on thrombophilia and adverse pregnancy outcomes

	Recurrent pregnancy loss	Previous history of pre-eclampsia	Previous history of fetal growth restriction
Canadian Society of Obstetricians and Gynecologists (SOGC) 2000 (26)	Reasonable to screen for thrombophilia	Reasonable to screen for thrombophilia	Reasonable to screen for thrombophilia
Scottish Intercollegiate Guideline Network (SIGN) 2002 (27)	Screen selected patients with recurrent fetal loss	No recommendation	No recommendation
American College of Chest Physicians (ACCP) 2008 (28)	Screening for thrombophilia not recommended	Screening for thrombophilia not recommended	Screening for thrombophilia not recommended
The Italian Society for Haemostasis and Thrombosis (SISCT) 2009 (29)	Recommend screening for thrombophilia (grade C evidence)	Recommend screening for thrombophilia (grade D evidence)	Recommend screening for thrombophilia (grade C evidence)
British Committee	Screening for	Screening for	Screening for

for Standards in Hematology (BCSH) 2010 (30)	thrombophilia except APLS not recommended	thrombophilia not recommended	thrombophilia not recommended
Royal College of Obstetrics and Gynecologists (RCOG) 2011 (31)	No recommendation	Screening for thrombophilia not recommended	No recommendation
American College of Obstetricians and Gynecologists 2011 (32)	Screening for thrombophilia not recommended	Screening for thrombophilia not recommended	Screening for thrombophilia not recommended

Identified area of future research

A randomised controlled trial evaluating the benefit of aspirin verses LMWH and aspirin to prevent adverse pregnancy outcomes in fVL + women with a history of ≥ 2 first-trimester fetal loss.

5.2.3 Group 3: Genetic testing for factor V Leiden in women with a history of late (>20 weeks) fetal loss

Recurrent late fetal loss is evaluated by meta-analysis of homogeneous case-control studies in Publication I. When cases had >1 previous fetal losses, including at least one second- or third-trimester fetal loss, the OR of a subsequent fetal loss was 3.9 (95% CI 1.9-8.2). When cases had ≥ 2 second- or third-trimester fetal loss, the OR was 10.7 (4-28.5) (6). The risk of subsequent late fetal loss in fVL+ women with a previous late fetal loss has not been evaluated within a population-based cohort study.

Post-test probability of recurrent late fetal loss in fVL+ women with a late fetal loss (> 22 weeks)

Within Publication IV, the post-test probability of recurrent late-fetal loss in women with a previous late-fetal loss (>22 weeks) was calculated using: 1) pre-test probability of late-fetal loss in the general population; and 2) likelihood ratios. Based on large cohort studies, the recurrence risk of late-fetal loss is 2% (33, 34). Using a likelihood ratio of 1.2-4 (6, 8), the calculated post-test probability of recurrent late-fetal loss in this group of fVL+ women is 2.4-7.5%(13).

Current recommendations

Although a number of societies have published different guidelines on thrombophilia screening women with a history of fetal loss (table 1), the overall consensus of more recent guidelines is similar to the recommendations published by the British Committee for Standards in Hematology guidelines (Baglin et al 2010): “with respect to recurrent pregnancy loss, screening for thrombophilia (except APLS) is not recommended pending the outcome of randomised controlled trials with a no-treatment or placebo arm evaluating the antithrombotic therapy in women with heritable thrombophilia and a history of pregnancy complications”⁶.

Identified area of future research

A randomised controlled trial evaluating the benefit of aspirin verses LMWH and aspirin to prevent adverse pregnancy outcomes in fVL+ women with a history of previous late fetal loss (>20 weeks).

5.2.4 Group 4: Genetic testing for factor V Leiden in women with a history of pre-eclampsia or severe pre-eclampsia

There has been one small study evaluating the recurrence risk of pre-eclampsia in fVL+ women with a previous history of pre-eclampsia. In a multi-centre observational cohort study, Facchinetti et al 2009 observed 172 Caucasian patients with a previous history of pre-eclampsia, 90 of who had a diagnosis of severe pre-eclampsia. All the patients were tested for thrombophilia and 17/172 (10%) were positive for fVL. However, the authors do not comment on what proportion of these 17 fVL+ women had pre-eclampsia as opposed to severe pre-eclampsia. Within the group of fVL+ women, the recurrence rate of pre-eclampsia was 59% (10/17). For statistical analysis, the authors pooled all the different forms of thrombophilia together and reported that

thrombophilia was associated with a 2.5-fold increase risk in the recurrence of pre-eclampsia (OR 2.5; 95% CI 1.2-5.1). The authors further clarify this result by stating “whereas mild forms of pre-eclampsia occurred independently of the thrombophilic trait, this was not the case for severe pre-eclampsia (OR, 6.5; 95%CI, 2.7-15.9; $P=0.001$). In the subset of patients with previous severe pre-eclampsia (90/172 women), the rate of recurrence of severe pre-eclampsia was significantly higher in thrombophilic women compared to women without thrombophilia (44.4% vs. 9.3% OR, 7.35; 95% CI, 2.1-27.1; $P=0.0005$)”. Although the numbers are small, this study suggests that the presence of fVL may be particularly important in the subgroup of women with a history of severe pre-eclampsia. The risk of subsequent pre-eclampsia in fVL+ women with a previous history of previous pre-eclampsia or severe pre-eclampsia has not been evaluated within a large population-based cohort study.

Post-test probability of recurrent pre-eclampsia in fVL+ women

Within Publication IV, the post-test probability of recurrent pre-eclampsia for fVL+ women was calculated using: 1) pre-test probability of pre-eclampsia recurrence in the general population; and 2) likelihood ratio. Based on large population cohorts, the recurrence risk of pre-eclampsia in women with a previous history of pre-eclampsia is around 14% (35-41). Using a likelihood ratio of 1.2-2, the calculated post-test probability of recurrent pre-eclampsia in fVL + women is 16-25% (13).

Post-test probability of recurrent severe pre-eclampsia in fVL+ women

The recurrence rate of severe pre-eclampsia is less consistent in the literature, with recurrence rates ranging for 6.8- 40% (42, 43). One large population-based cohort of 188,768 women identified 133 women (mean age 28.9 years) with de novo severe pre-eclampsia with a recurrence risk of 6.8% (95% CI 5.7–7.9%) (43). This risk was lower than an earlier study with younger patients (mean age 23.6 years) with earlier onset

pre-eclampsia (18-27 weeks) which reported a recurrence risk for severe pre-eclampsia of 40%. A lower use of aspirin in the Sibai study (42) may be another explanation for the difference. Based on a pre-test probability of 6.8%, and a likelihood ratio of 2-2.5 (7-9), the calculated post-test probability of recurrent severe pre-eclampsia in fVL+ women with a previous history of severe pre-eclampsia is 13-15%. Based on the higher pre-test probability of 40%, the calculated post-test probability of recurrent severe pre-eclampsia in fVL+ women with a previous history of severe pre-eclampsia is 57-62%(13).

Current recommendations

Although a number of societies have published different guidelines on thrombophilia screening women with a history of pre-eclampsia (table 1), the overall consensus of more recent guidelines is similar to the recommendations published by the British Committee for Standards in Hematology guidelines (Baglin et al 2010): “with respect to pre-eclampsia, screening for thrombophilia is not recommended pending the outcome of randomised controlled trials with a no-treatment or placebo arm evaluating the antithrombotic therapy in women with heritable thrombophilia and a history of pregnancy complications”⁶.

Identified areas of future research

- 1) A prospective study to determine the recurrence risk of adverse pregnancy outcome in fVL+ women with a previous history of pre-eclampsia.
- 2) A prospective study to determine the recurrence risk of adverse pregnancy outcome in fVL+ women with a previous history of severe pre-eclampsia.
- 3) A prospective study to determine the recurrence risk of adverse pregnancy outcome in fVL+ women with a previous history of early onset pre-eclampsia

- 4) A randomised controlled trial evaluating the benefit of aspirin verses LMWH and aspirin to prevent adverse pregnancy outcome in fVL+ women with a history of pre-eclampsia.
- 5) A randomised controlled trial evaluating the benefit of aspirin verses LMWH and aspirin to prevent adverse pregnancy outcome in fVL+ women with a history of severe pre-eclampsia.
- 6) A randomised controlled trial evaluating the benefit of aspirin verses LMWH and aspirin to prevent adverse pregnancy outcome in fVL+ women with a history of early-set pre-eclampsia.

5.2.5 Group 5: Genetic testing for fVL in women with a previous history of fetal growth restriction

There have been no studies evaluating the risk of recurrent fetal growth restriction <10th in fVL+ women with a previous history of FGR.

There have been no studies evaluating the risk of recurrent fetal growth restriction <10th in fVL+ women with a previous history of early onset FGR.

Current recommendations

Although a number of societies have published different guidelines on thrombophilia screening women with a previous history of fetal growth restriction (table 1), the overall consensus of more recent guidelines is similar to the recommendations published by the British Committee for Standards in Hematology guidelines (Baglin et al 2010): “with respect to fetal growth restriction, screening for thrombophilia is not recommended pending the outcome of randomised controlled trials with a no-

treatment or placebo arm evaluating the antithrombotic therapy in women with heritable thrombophilia and a history of pregnancy complications".⁶

Identified area of future research

- 1) A prospective study to determine the recurrence risk of FGR in fVL+ women with a previous history of FGR.
- 2) A prospective study to determine the recurrence risk of FGR in fVL+ women with a previous history of early-onset FGR.
- 3) A randomised controlled trial evaluating the benefit of aspirin verses LMWH and aspirin to prevent adverse pregnancy outcome in fVL+ women with a history of previous early-onset FGR.
- 4) A randomised controlled trial evaluating the benefit of aspirin verses LMWH and aspirin to prevent adverse pregnancy outcome in fVL+ women with a history of previous FGR.

5.3 Design for proposed future research trial

Analysing the literature based on severity of adverse pregnancy outcome illustrates that the reported risk associated with fVL increases when there is a strong history of adverse pregnancy outcomes (such as more or later pregnancy losses and more severe pre-eclampsia).

Despite some suggestion that preventative treatment with LMWH may be beneficial in the group of women with a very poor pregnancy history (17-21, 23), adequately designed randomised controlled trials are needed to access the efficacy and safety of anticoagulant therapy in this group of thrombophilic women.

Study objective for proposed future research trial: To determine whether low molecular weight heparin enoxaparin decreases the rate of pre-eclampsia, fetal death,

placental abruption, FGR or prematurity in fVL+ women or PGV + women with a previous history of severe pre-eclampsia or late fetal loss (definitions in appendix 1).

Null Hypothesis: Treatment with low molecular weight heparin combined with aspirin compared to aspirin alone or placebo does not reduce the rate of pre-eclampsia, fetal death, placental abruption, IUGR or prematurity in fVL+ or PGV+ pregnant women with a history of severe pre-eclampsia or late fetal losses.

Study design: We propose a multi-centre, prospective, randomised-controlled trial. Clinicians can offer patients participation in either a two- or three-arm trial, which will enable clinicians the option of enrolling their patients in a trial without a placebo arm. Within the two-arm trial, the first group would be treated with a daily dose of aspirin 100 mg orally and a subcutaneous injection of placebo commencing at 12 weeks and continuing until delivery; and the second group would be treated with aspirin 100mg/day and enoxaparin commencing at 12 weeks and continuing until delivery. Within the three-arm trial, the first group would receive a daily dose of aspirin 100 mg orally/day and a placebo subcutaneous injection commencing at 12 weeks and continuing until delivery; the second group would receive aspirin 100mg/day and enoxaparin commencing at 12 weeks and continuing until delivery; and the third group would receive a daily dose of placebo orally and a subcutaneous injection of placebo commencing at 12-weeks gestation and continuing until pregnancy.

Women would be offered participation in the study at their first-trimester screening test (nuchal translucency) between 11 and 14 weeks. Inclusion and exclusion criteria (appendix 1) would be searched, and suitable women would be consecutively recruited. The recruitment process will be a two-part process. Women who reach the inclusion criteria will initially be screened for fVL, PGV and other forms of inherited thrombophilia if this has not already been done. For women who test positive for fVL or PGV, the treating clinician will decide whether to offer participation in a two-arm or a three-arm trial. Participating women would have a blood sample taken monthly to test platelet count. Post-delivery management will proceed according to current guidelines for thrombophilic women. Primary outcome measures will include pre-eclampsia, FGR, placental abruption and intrauterine fetal death (with normal fetal

karyotype). Secondary outcome measures will include gestational age, enoxaparin toxicity, post-partum hemorrhage and the need for blood transfusion.

Calculation of sample size

1) To detect a 25% reduction in risk of adverse pregnancy outcomes in study group

A conservative risk of pre-eclampsia in the general population is two percent and the literature suggests that this risk doubles in women with fVL to around four percent. The risk of stillbirth in the general population is 0.5%, and the literature suggests this risk doubles in women with fVL to around one percent. The risk of placental abruption in the general population is 0.5% and the estimated risk in fVL+ women is estimated as one percent. The risk of having an infant with a customised birth weight < 5th centile in the general population is five percent. The estimated risk of fVL+ or PVG+ women having an infant with a customised birth weight < 5th centile in weight is estimated as 10%. Therefore the estimated event rate in the control group is 16% (4% + 1% + 1% + 10%) or 0.16.

Using a P value of 0.5 ($\alpha = 0.05$); power of 0.80 ($1 - \beta$), an event rate in the control population of 16% (0.06) with m (ratio of cases to controls) set as 1, and a RR of 0.75 (25% reduction in the outcome in the study group), a sample of 1229 is required for each arm. For a three-arm study, 3687 women would need to be recruited.

As the fVL yield is five to 10% in the women with severe pre-eclampsia or an unexplained fetal loss after 20-weeks gestation, 36,870 women with a history of severe pre-eclampsia or fetal death would need to be screened for fVL or PGV. To find 36,870 women with a history of severe pre-eclampsia (one in 200) or stillbirth > 20 weeks (one in 200), the study would need to be an international multi-centre trial including centres with a combined birth rate of 3,687,000 over three years (1,229,000 a year). Because only 80% may have a nuchal translucency scan, a recruiting source of 1,474,800 babies is needed per year. Estimating a 60% participation rate in an international multi-centre

trial, the recruiting source would need to have a combined birth rate of 2,064,720 Caucasian mothers over three years or 688,240 per year. This would require a multi-national, multi-centre collaboration.

2) Pilot study: to detect a 75% reduction in risk of adverse pregnancy outcomes in the study group

In an attempt to estimate the level of risk reduction, a pilot study would be undertaken with the risk reduction set as 0.25 (75% reduction in the outcome in the study group). The pilot study would require a sample size of 113 in each arm. Therefore, 339 women would need to be recruited for a three-arm pilot study. Because the fVL yield is five to 10% in the women with severe pre-eclampsia or an unexplained fetal loss after 20-weeks gestation, 3390 women with a history of severe pre-eclampsia or fetal death would need to be screened for fVL or PGV. To find 3390 women with a history of severe pre-eclampsia (one in 200) or stillbirth > 20 weeks (one in 200), the study would need to recruit from centres with a combined birth rate of 339,000 over three years (113,000 yearly). Because only 80% may have a nuchal translucency scan, a recruiting source of 135,600 per year would be needed. Estimating a 60% participation rate in the pilot study, this requires a recruiting source of 189,840 annually.

The population of Australia is 22,000,000. The Caucasian population is 19,800,000 (90%). The yearly birth rate is 12/1000, yielding an estimated 237,000 infants born to Caucasian mothers in Australia each year. Therefore, the pilot study would need to include all the major high-risk clinics around Australia for a number of years. To put this in perspective, we can look at an Australian city like Newcastle. There are approximately 4000 births a year in Newcastle and most women with previous adverse events are seen in the high-risk clinic. At this rate 16 centres the size of Newcastle would be needed to reach the target sample required over a three-year period. When designing such a trial, the feasibility of recruiting the required number of patients needs to be carefully considered. For example, the ongoing TIPPS trial, with a sample size of ~ 385 patients is assessing antepartum LMWH verses placebo in

pregnant women with prior DVT or thrombophilia. This study has been ongoing for 12 years despite, over 20 participating sites (clinicaltrials.gov).

The recently published FRUIT-RCT (44) provides long awaited evidence to support the treatment of thrombophilic women with a previous history of early-onset (<34 weeks gestation) hypertensive disease (HD) and/or SGA with LMWH and aspirin commencing < 12 weeks gestation. Although the overall recurrence risk of HD was not reduced, the addition of LMWH to aspirin reduces recurrent HD onset <34 weeks gestation (risk difference [RD] 8.7%: confidence interval [CI] of RD 1.9–15.5%; $P = 0.012$; number needed to treat [NNT] 12). The trial recruited 139 women from multiple sites over a 10 year period, further illustrating that it is important to consider the feasibility of recruiting patients into such trials. Therefore, a future adequately powered RCT recruiting thrombophilic women with a previous history of severe pre-eclampsia or late fetal death will need to be undertaken within an international multicentre collaboration.

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APPENDICES

Chapter 3 Appendix 1.

The Avon Longitudinal Study of Parents and Children Study: methodology

References:

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Summary

1) Demographics

The Avon Longitudinal Study of Pregnancy and Childhood (ALSPAC) is a longitudinal study of 14000 children from the county of Avon, which has a population of one million and includes the city of Bristol (population 0.5 million). The study population is 120 miles west of London and situated on the Severn estuary. The population is a mixture of moderate-sized towns, inner-city deprivation, suburbs and rural area. Industries include petro-chemical industries on the Severn estuary and a British aerospace factory in Bristol; however most of the industry is light rather than heavy. Data from this region was compared to other areas in Britain through the Child Health and Educational Study (which followed up all children born in Great Britain in one week of 1970) and it was concluded that the Avon area was fairly similar to the rest of Britain.

2) Aims of the ALSPAC study

‘To determine which biological, environmental, social, psychological and psychosocial factors are associated with the survival and optimal health and development of the fetus, infant and child, and the ways in which causal relationships might vary with the genetic composition of mother and/or child.’

‘To identify the complex ways in which environmental features may be associated with the optimal development, health and well-being of the child. ALSPAC was specifically designed to analyse the interplay between genes and environment with respect to important relatively common health outcomes. ALSPAC has the long-term aim of following the children into adulthood and thus will be set to answer questions related to prenatal and postnatal factors associated, for example, with schizophrenia, delinquency, reproductive failure on the one hand, and realisation of full educational potential, health and happiness on the other.’

3) Recruitment

The recruitment aim was to recruit all pregnant mothers in the defined geographical region with an expected date of delivery between 1st April 1991 and 31st December, 1992. Prior to the enrolment period, health professionals including obstetricians, midwives and general practitioners were educated about the study. The study was also promoted by posters and media coverage. Mothers were approached by ALSPAC staff at the time of their routine antenatal scan, and community midwives were also involved in the recruitment process. Mothers who were happy to receive further information about the study were asked to complete a card providing their contact details and expected date of delivery. Once the card was received by study staff, the mother was sent further information about participation in the study.

4) Methods of data collection and management

Data collection relevant to this study was collected by: 1) self-completion questionnaires during pregnancy and post-delivery completed by the mother and her partner; 2) medical records; and 3) biological samples from the mother, her partner and child.

5) Self-completion questionnaires

Four questionnaires were sent to participating mothers during the pregnancy and questionnaires were also sent post-delivery. Questionnaire B - 'Having a Baby' - was sent at 18 weeks and Questionnaire C - 'Your Pregnancy' - at 32 weeks. Questionnaire A - 'Your Environment' - was sent as soon as possible after the mother's enrolment into the study. Questionnaire D - 'About Yourself' covered the mother's past medical, social and environmental history. Although mothers who enrolled late were not sent the 18-week Questionnaire B, they were sent Questionnaire E - 'Your Home and Lifestyle' because it was felt that there was still some important environment and lifestyle information that could be validly collected. The women could make a decision whether or not she wished to invite her current partner to participate in the study, and the two partner questionnaires were sent accompanying Questionnaires B and D. All births in the region were routinely reported to the health authorities. Systems were in place to ensure that information about miscarriage, perinatal or postnatal death and seriously ill babies was conveyed to the study centre. Post-delivery questionnaires were sent to all mothers, but the timing and content differed in the situation of an adverse pregnancy outcome.

For all fetal deaths, a letter of condolence was sent, which included an offer to continue in the study with further investigations concerning the miscarriage or death. Most of the possible responses to the self-completion questionnaires were in the form of a coded tick-box, and the coding process was double-checked by a second person.

6) Medical records

With the mother's consent, information relating to the pregnancy and the child could be obtained from the medical records. Prior to commencing the study, a sample of

computer records were checked against the medical records and were found to be missing data and frequently inaccurate. As a result, medical information was extracted from the paper medical records, which could take up to four hours per file.

7) Collection of biological samples

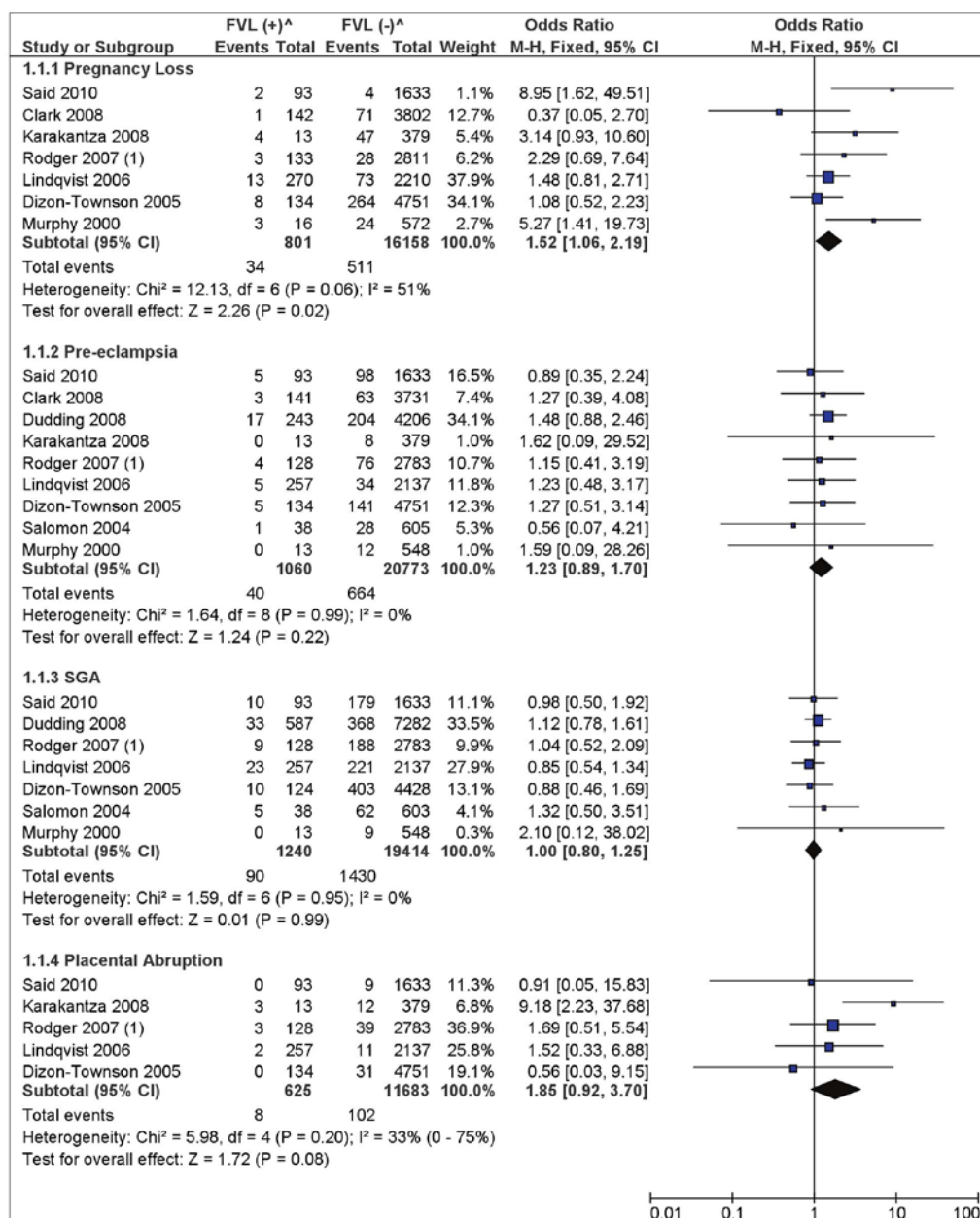
Maternal blood was collected from mothers during their antenatal care. It was either collected in EDTA and stored at -20 degrees C or collected in heparin, and the separated serum stored at -70 degrees C. Cord blood was collected at birth and separated serum was stored at -70 degrees C. These samples were stored for five to seven years before DNA was extracted. Overall DNA is available for approximately 85% of the cohort children. Information about the source, storage and processing of each sample from the mother and baby is recorded on the computer database. Initially the blood sample was given a sample identity number which links to the individual's confidential records in a database with limited access. Long-term storage samples often contained clot despite anti-coagulation and the phenol-chloroform method of DNA extraction was superior to the salting-out extraction method in this group of samples. The less toxic salting-out method of DNA extraction was used for samples stored at -20 degrees C for less than a month. Samples of extracted DNA were then given an accession number linked to the sample number, and transferred manually to deep 96-well arrays. Two laboratory workers were involved in all transfers and a masking system was used to prevent samples being put in the incorrect well.

These plates were then sealed and stored at -50 degrees C. From this stage, robotic processing using a Biomek 2000 was used to process the stock DNA to produce replica plates with 250ng DNA/well for use by collaborating groups. Robotic precision was tested by evaluating the ability of the robotic dilution techniques to generate replica plates containing DNA samples of the same concentration. As part of evaluating the reliability of the robotic processing of DNA samples, duplicated samples were included among samples prior to PCR. Presumed duplicate samples were tested using primers at the HLA-DBR locus to check for identification and sampling errors.

Chapter 3 Appendix 2

RODGER MA, BETANCOURT MT, CLARK P, et al. The association of factor V leiden and prothrombin gene mutation and placenta-mediated pregnancy complications: a systematic review and meta-analysis of prospective cohort studies. PLoS Med;7:e1000292.

Figure 2. Odds of placenta-mediated pregnancy complications in FVL + women



Chapter 5 Appendix 1.

Inclusion and exclusion criteria for proposed research trial

Inclusion Criteria:

- fVL+ OR PGV+ pregnant women
- AND
- Previous history of severe pre-eclampsia or pre-eclampsia with onset < 33 weeks gestation
- OR
- Previous unexplained fetal loss > 20 weeks

Exclusion Criteria:

- Multiple pregnancy
- Alcohol or illicit drug use
- Severe fetal malformations or chromosomal abnormalities
- Known thrombophilia in the father
- An additional form of inherited or acquired thrombophilia or homozygosity for fVL in the mother
- Allergy to low molecular weight heparin (LMWH)
- Allergy to Aspirin
- Thrombocytopenia related to Heparin use
- Thrombocytopenia < 100,000/u/l at first prenatal visit
- Known osteoporosis
- Inability to do subcutaneous injection of heparin
- Anticoagulant therapy in the last 3 months
- Severe liver disease (INR >1.8)
- Signs of thrombosis or a history of thromboembolism in previous pregnancy

- Need for anticoagulation during pregnancy e.g. cardiac valvular prosthesis
- Previous hematologic disease
- Severe hypertension (systolic BP > 200mmHg and/or diastolic BP >120Hg)
- A contraindication to anticoagulants e.g. previous hemorrhagic disease or gastric ulcer/ cerebral hemorrhage or cerebral aneurysm.
- Weight >120kgs
- Patient positive for HIV, hepatitis C virus or hepatitis B virus.
- Involvement in another intervention trial
- Unable or unwilling to provide informed consent.
- An absolute indication for anticoagulant therapy: venous deep thrombosis or pulmonary embolism.
- Metabolic disorders which increase the risk for development of pre-eclampsia or fetal growth restriction e.g. Type I diabetes, hyperthyroidism or chronic renal insufficiency.

Definitions

Fetal growth restriction < 5%: Defined as customized fetal growth restriction birthweight < 5% percentile.

Abruptio placentae: Defined as the association of bleeding and one of the following criteria:

- Abnormal fetal heart rate,
- Abdominal pain

Late intrauterine fetal death: Defined as fetal death of unknown etiology occurring after 20 weeks gestation.

Preeclampsia with onset < 33 weeks: Defined as the presence of hypertension (BP $\geq 140/90$ mm Hg) on 2 occasions, at least 6 hours apart, but without evidence of end-organ damage in the patient, with an onset < 33 weeks gestation

Severe preeclampsia: Defined as the presence of 1 of the following symptoms or signs in the presence of preeclampsia:

- Systolic BP ≥ 160 mm Hg or diastolic BP ≥ 110 mm Hg on two occasions at least 6 hours apart
- Proteinuria > 5 g in a 24-hour collection or $> 3+$ on 2 random urine samples collected at least 4 hours apart
- Pulmonary edema or cyanosis
- Oliguria (< 400 mL in 24 h)
- Persistent headaches
- Epigastric pain and/or impaired liver function
- Thrombocytopenia
- Oligohydramnios, decreased fetal growth or placental abruption

The End